OKLAHOMA TRANSPORTATION CENTER

ECONOMIC ENHANCEMENT THROUGH INFRASTRUCTURE STEWARDSHIP

CHARACTERIZATION AND MEDIATION OF MICROBIAL DETERIORATION OF CONCRETE BRIDGE STRUCTURES

SRAVANTHI VUPPUTURI
BABU FATHEPURE, PH.D.
GREGORY G. WILBER, PH.D., P.E.
SEIFOLLAH NASRAZADANI, PH.D.
TYLER LEY, PH.D., P.E.
JOSHUA D. RAMSEY, PH.D.

OTCREOS9.1-39-F

Oklahoma Transportation Center
2601 Liberty Parkway, Suite 110
Midwest City, Oklahoma 73110

Phone: 405.732.6580
Fax: 405.732.6586
www.oktc.org

DISCLAIMER

The contents of this report reflect the views of the authors, who are responsible for the facts and accuracy of the information presented herein. This document is disseminated under the sponsorship of the Department of Transportation University Transportation Centers Program, in the interest of information exchange. The U.S. Government assumes no liability for the contents or use thereof.

TECHNICAL REPORT DOCUMENTATION PAGE

1. REPORT NO.	2. GOVERNMENT ACCESSION NO.	3. RECIPIENTS CATALOG NO.
OTCREOS9.1-39-F		

4. TITLE AND SUBTITLE	5. REPORT DATE
Characterization and Mediation of Microbial Deterioration of Concrete Bridge Structures	April 2013
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT
Sravanthi Vupputuri, Babu Fathepure, Gregory Wilber, Seifollah Nasrazadani, Tyler Ley, and Joshua D. Ramsey	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. WORK UNIT NO.
Oklahoma State University School of Chemical Engineering 423 Engineering North Stillwater, OK 74078	11. CONTRACT OR GRANT NO. DTRT06-G-0016
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Oklahoma Transportation Center (Fiscal) 201 ATRC Stillwater, OK 74078 (Technical) 2601 Liberty Parkway, Suite 110 Midwest City, Oklahoma 73110	Final Report: June 2009 - April 2013 14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES

Project performed in cooperation with the Oklahoma Transportation Center and the University Transportation Center.

16. ABSTRACT

Samples obtained from deteriorated bridge structures in Texas were cultured in growth medium containing thiosulfate as an energy source and investigated for acid production, type of acid produced by microbes and the bio-deterioration of concrete cylinders. Enriched cultures decreased the pH to 3.5. Whereas in culture containing concrete the pH remained stable, although there was 97 % oxidation of thiosulfate to sulfate. Bio-deterioration was also evident from the 2-fold higher amount of calcium leached out from concrete compared to controls . Stereo microscope and scanning electron microscopy (SEM) images of the concrete cylinders attacked by acid revealed cracks, which could be due to the formation of expandable products gypsum and ettringite.

Microbe responsible for acid production was isolated. The16S rRNA-gene of microbe was genetically sequenced, analyzed with the use of basic local alignment search tool (BLAST) and was identified as *Streptomyces sp*. This strain was capable of reducing the culture pH to 3.4 in absence of concrete. Whereas in presence of concrete a drop in pH was observed , when there was sufficient amount of thiosulfate for oxidation reaction. There was 98 % oxidation of thiosulfate to sulfate and the amount of calcium leached out from concrete was 6 fold higher than control. SEM images revealed cracks on concrete exposed to *Streptomtces sp.* culture.

Approximately, 400 to 600 16S rRNA-gene sequences representing microbial communities on concrete surfaces from three different bridge structures were analyzed. Highly diverse bacterial and archael communities with only a few known acid producers existed at the time of sampling at all three sites. However, our laboratory studies revealed that the community composition dramatically shifted from a highly diverse to a highly rich dominated by mainly sulfur oxidizing acid-producers such as *Thiobacillus thioparus*, *Alicyclobacillus ferrooxydans*, *Alicyclobacillus pomorum*, *Alicyclobacillus acidocaldarius*, *Alicyclobacillus* sp. and *Bacillus* sp., when thiosulfate was provided. The acid producers were able to oxidize 40 mM thiosulfate to roughly 75 mM sulfate within 5 weeks suggesting almost stoichiometric conversion (94 %) of the added energy source. The pH of the culture decreased from 6.7 to 2.8. These results clearly demonstrate the role of sulfur oxidizing microorganisms in concrete corrosion and the availability of reduced sulfur compounds in the environment is important for corrosion to occur.

17. KEY WORDS: Bridge structures, Acid production, Concrete, Sulfur oxidizing microorganisms, 16S rRNA gene sequence.	18. DISTRIBUTION STATEMENT No restrictions. This publication is available at www.oktc.org and from the NTIS.		
19. SECURITY CLASSIF. (OF THIS REPORT) Unclassified	20. SECURITY CLASSIF. (OF THIS PAGE) Unclassified	21. NO. OF PAGES 78 + covers	22. PRICE

SI (METRIC) CONVERSION FACTORS

Approximate Conversions to SI Units

Symbol	When you know	Multiply by	To Find	Symbol
	LENGTH			
in	inches	25.40	millimeters	mm
ft	feet	0.3048	meters	m
yd	yards	0.9144	meters	m
mi	miles	1.609	kilometers	km
	AREA			
in^2	square inches	645.2	square millimeters	mm
ft^2	square feet	0.0929	square meters	m^2
yd^2	square yards	0.8361	square meters	m^2
ac	acres	0.4047	hectares	ha
mi^2	square miles	2.590	square kilometers	km^2
	VOLUME			
fl oz	fluid ounces	29.57	milliliters	mL
gal	gallons	3.785	liters	L
ft^3	cubic feet	0.0283	cubic meters	m^3
yd^3	cubic yards	0.7645	cubic meters	m^3
	MASS			
oz	ounces	28.35	grams	g
lb	pounds	0.4536	kilograms	kg
T	short tons (2000 lb)	0.907	megagrams	Mg
	TEMPERATURE (exact)			
°F	degrees Fahrenheit	(°F-32)/1.8	degrees Celsius	°C
	FORCE and PRESSURE or STRESS			
lbf	poundforce	4.448	Newtons	N
lbf/in^2	poundforce per square inch	6.895	kilopascals	kPa

Approximate Conversions from SI Units

Symbol	When you know	Multiply by	To Find	Symbol
	LENGTH			
mm	millimeters	0.0394	inches	in
m	meters	3.281	feet	ft
m	meters	1.094	yards	yd
km	kilometers	0.6214	miles	mi
	AREA			
mm^2	square millimeters	0.00155	square inches	in^2
m^2	square meters	10.764	square feet	ft^2
m^2	square meters	1.196	square yards	yd^2
ha	hectares	2.471	acres	ac
km^2	square kilometers	0.3861	square miles	mi^2
	VOLUME			
mL	milliliters	0.0338	fluid ounces	fl oz
L	liters	0.2642	gallons	gal
m^3	cubic meters	35.315	cubic feet	ft^3
m^3	cubic meters	1.308	cubic yards	yd^3
	MASS			
g	grams	0.0353	ounces	oz
kg	kilograms	2.205	pounds	lb
Mg	megagrams	1.1023	short tons (2000 lb)	T
	TEMPERATURE (exact)			
°C	degrees Celsius	9/5+32	degrees Fahrenheit	°F
	FORCE and PRESSURE or STRESS			
N	Newtons	0.2248	poundforce	lbf
kPa	kilopascals	0.1450	poundforce per square inch	lbf/in^2

ACKNOWLEDGEMENTS

The authors are very grateful for financial support from the Oklahoma Transportation Center (OkTC) and the RITA University Transportation Center program. Special thanks should be given to OkTC leadership team, Tony Dark, OkTC Executive Director, Michelle McFarland, OkTC Assistant Director, and Dr. Arnulf Hagen, OkTC Technical Director, for their support in making this research successful. A special thank you goes to TxDOT for providing us with the initial samples.

A heartfelt thanks goes out to Dr. Nasrazadani Seifollah, and Elias Sudoi of University of North Texas for helping us with analyzing the changes in the concrete chemistry and structure due to deterioration. We would like to thank Sonal Dalvi, Graduate Research Assistant of Department of Microbiology and Molecular Genetics, and Mohammed Aboustait, Graduate Research Assistant of School of Civil and Environmental Engineering, for their assistance in collecting some of the data. We also like to thank Robbie Fraizer for his assistance while collecting the field samples in Texas.

CHARECTERIZATION AND MEDIATION OF MICROBIAL DETERIORATION OF CONCRETE BRIDGE STRUCTURES

Final Report
April 2013

Sravanthi Vupputuri
Graduate Research Assistant, School of Chemical Engineering

Babu Fathepure Ph.D.
Co- Principal Investigator, Department of Microbiology and Molecular Genetics

Gregory G. Wilber P.E., Ph.D.
Co- Principal Investigator, School of Civil and Environmental Engineering

Tyler Ley P.E., Ph.D.
Co- Principal Investigator, School of Civil and Environmental Engineering

Joshua D. Ramsey Ph.D.
Principal Investigator, School of Chemical Engineering

423 Engineering North
Oklahoma State University
Stillwater, OK 74078

Nasrazadani Seifollah Ph.D.
Co- Principal Investigator, Department of Engineering Technology
University of North Texas

Oklahoma Transportation Center
Tinker Business & Industrial Park
2601 Liberty Parkway, Suite 110
Midwest City, Oklahoma 73110

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

EXECUTIVE SUMMARY

Based on comments from the Texas Department of Transportation (TxDOT), the research team came to suspect that microbes may be involved in the deterioration of more than 20 concrete bridge structures in the state of Texas. While the deterioration has not been found to be widespread it has impacted several critical structures including the some in major metropolitan areas. Similar deterioration has also been reported in other southern states including Alabama, Georgia, and Mississippi. While microbial attack is known to occur in concrete sewer pipes, it is not a common occurrence at bridge structures. With funding from the Oklahoma Transportation Center (OkTC), a multidisciplinary research team from Oklahoma State University (OSU) studied several bridge sites in Texas where microorganisms were suspected of having a role in the corrosion of concrete bridge columns. The research team identified acid-producing microbes at bridge sites in Texas where bridge columns were being eroded in a quite unusual manner. Identification of microbes using genetic analysis tools revealed that these microbes were closely related to microbes found in sewer systems and appeared to work in a similar manner.

Some of the important findings of this research include:

- Samples provided by TxDOT were cultured in the laboratory, and the mixed cultures decreased the pH of a thiosulfate-growth medium below 4.0, indicating the presence of acid producing microbes. An acid producing microbe (*Streptomyces* sp.) was isolated from the mixed cultures, and the mechanism of acid production was studied. Ion chromatography on both the mixed and isolated cultures indicated the oxidation of thiosulfate present in the growth medium into sulfate. Through inductively coupled plasma emission spectrometry (ICP-ES) the research team also observed that the acid produced by the microbes attacking the concrete and solubilizing the calcium thereby causing degradation.

- Scanning electron microscopy (SEM), stereo microscopy, Fourier transform infrared (FTIR) spectroscopy and energy dispersive spectrometer (EDS) were used to measure the changes in the concrete chemistry and structure due to deterioration. Deep cracks were observed on the cylinders exposed to culture containing acid producing microbes.
- Samples collected during the field study have been analyzed using molecular techniques to determine microbial diversity. Analysis revealed the presence of diverse organisms belonging to both domain bacteria and archaea. Among bacteria, members of the phyla actinobacteria (14-29 %), alpha-proteobacteria (13-21 %), cyanobacteria (9-21 %), chloroflexi (5-19 %) and acidobacter (4-11 %) dominated the community at all sites. Among archaea, a large number of crenarchaeota comprising 20 % and 11 % of clones were retrieved from Madisonville and Tarkington samples, respectively. The analysis showed the presence of only a few putative acid-producing organisms in the concrete collected at the site. However, when we incubated concrete samples from different bridge sites with thiosulfate, the population changed and more >60 % of the microbial community was comprised of sulfur oxidizing acid producers and the pH decreased from 6.7 to 3.0.
- The research team enriched the microorganisms responsible for corrosion to check the acid production and the mechanism of acid production. Similar to the earlier work with the samples provided by TxDOT, the cultures showed a significant decrease in pH and oxidation of thiosulfate present in the medium and the release of calcium from concrete.

INTRODUCTION

Microorganisms may be small, but they have a big impact on the life span of some bridges in the southern United States. In fact, there is growing concern that microbes present on the surface of concrete may be responsible for deterioration of more than 150,000 bridges in the United States, especially in southern states such as Alabama, Georgia, Mississippi, and Texas. While this problem has not been studied extensively, the effect of bio-deterioration of concrete-reinforced sewer pipes and its effects on the integrity and maintenance of our sewer systems have been well characterized [1-4]. The microbes in sewer pipes act as small chemical factories, taking in nutrients from their surroundings and producing chemical products useful to the organism, as well as byproducts and wastes. One byproduct produced by certain species of microbes is a harsh acid that reacts to weaken or even dissolve concrete. In this case, the microbes convert toxic hydrogen sulfide sewer gas into sulfuric acid that corrodes the concrete sewer pipe [4-6]. As analytical and molecular techniques improve, however, we are beginning to understand the much broader effects microbes are having on our concrete infrastructure, including many bridges.

Recent observations in Texas, Alabama, Georgia, and Mississippi have identified several sites where microorganisms have caused deterioration of the columns of concrete bridges (Figure 1). Before beginning the current study, we had data from bridges in East Texas that also supported this finding. While these cases are not yet widespread, they do involve critical infrastructure in some major metropolitan areas. Studying both the microbes and the environmental factors that lead to these problems will better enable us to care for the structures that have been affected and prevent future microbial bio-deterioration. Part of the goal of our study was to answer questions such as, “Is the cause of deterioration something unique about one particular microorganism or a combination of

microorganisms? Is it something unique about the environment? Is it a combination of the right microorganism in the right environment?"

Figure 1: Images of microbial bio-deterioration of concrete bridge columns. The images above show microbial bio-deterioration of the concrete bridge columns at several sites in Texas.

BACKGROUND AND SIGNIFICANCE

Microorganisms have a profound impact on our daily lives. These small, unseen organisms not only recycle our wastes and degrade toxic chemicals we produce but also give yogurt its characteristic taste; give cake and bread its light, airy texture; help us recover from disease by antibiotics and also help us grow our plants; they not only impact our climate, but also help us relax over a glass of wine or beer. What you may not realize is that microorganisms also impact our roads, bridges, miles of sewer, water and other public infrastructures. When we consider microorganisms we should think of them as small but powerful, powerful enough to deteriorate the surface of concrete structures such as bridges, buildings and sewer pipes. Few people will consider the role that microbes play in the challenges that we face with maintaining our roads and public infrastructure. As our analytical and molecular techniques improve, however, we are learning that microbes are capable of causing significant amounts of damage to concrete. Microbes utilize a variety of substrates as energy sources and produce a number of reactive metabolic products. This process often involves degradation of materials that most would consider unsuitable to sustain microbial growth. For example, microbial degradation of prehistoric and renaissance paintings [7] and historic buildings such as cathedrals and monuments [8, 9] are becoming issues of concern for many countries, and in the United States, bio-deterioration of concrete reinforced sewer pipes is a significant challenge to maintaining the public sewer systems [10, 11]. Over the last couple of years there has been growing concern over the impact that microbes are having on other sections of the public infrastructure in the US. Recently, several bridges in Texas, Alabama, Mississippi and Georgia have shown signs of bio-deterioration by microbes [12, 13].

How are small single cell organisms capable of such degradation? In order to

answer this question it is important to understand the nature of the microbes and their interactions with the environment.

Microbes work synergistically

Microbes have been detected in every conceivable environment on the Earth. The widespread presence of microbes is a result of microbes evolving to utilize the unique physical and chemical properties of their environment. Therefore, a microbial community at a specific site is determined by the physical and chemical characteristics of the surrounding environment, and as a result, a habitat that is favorable for one organism may be harmful or unsuitable for another. The environment, however, is also affected by the presence of microbial life. Because of this impact on the environment, a setting that may not be initially suitable for certain microbes may become a viable habitat to newly introduced organisms. This occurs because the microbes that flourish carry out metabolic processes that remove some nutrients and excrete others (as waste products) into their surroundings. The environment and its ability to support additional microbes can change through this process. It is no wonder that populations of microbes rarely live alone. They typically live in association with other populations of microbes called communities. It is important to consider this issue as we study the impact microbes are having on the concrete bridge columns. Though one microbe may be primarily responsible for deterioration it is possible the microbe may behave differently without the microbial community.

Microbial degradation of concrete

Research in the area of microbial impact on concrete has revealed that the microbes most frequently involved in biodegradation of concrete include bacteria, cyanobacteria, algae fungi, and lichens. These microbes require water, and their growth is dependent on the properties of the underlying material such as surface conditions, composition, porosity

and permeability as well as environmental conditions [14-17].

The first study investigating microbial bio-deterioration of concrete was reported in 1900 by Olmstead and Halmin [18]. Later, in the year 1945 C.D. Parker identified *Thiobacillus* species as a cause of concrete bio-deterioration in sewer systems [19]. The *Thiobacillus* microbes acted by oxidizing elemental sulfur, thiosulfate and hydrogen sulfate to produce sulfide, sulfuric acid and tetrathionates [20, 21]. The same microorganisms may also use hydrogen sulfide from the atmosphere to produce sulfuric acid, which in turn solubilizes calcium carbonate in concrete [11, 22, 23]. There are two types of sulfur-oxidizing bacteria (SOB) in *Thiobacillus* species: neutrophilic sulfur oxidizing bacteria (NSOB) and acidophilic sulfur oxidizing bacteria (ASOB). NSOB colonize on surface of concrete when pH is slightly alkaline and could have a great impact on the establishment of an environment which is suitable to ASOB (by further reducing the pH). Then ASOB start to colonize and further reduce the pH by oxidizing thiosulfate, elemental sulfur and polythionates present in the environment to sulfuric acid thereby causing concrete deterioration. Table 1 shows the main characteristics of *Thiobacillus* species. When studied in environmental chambers meant to simulate conditions similar to a sewer pipe, *Thiobacilli* produced corrosion rates of 4.3 – 4.7 mm/year [11, 24].

Table 1: Characteristics of five main acid producing *Thiobacillus* species.

Organism	Mechanism of degradation	pH range	Life style	References
T. thiooxidans	Production of sulfuric acid	0.5 – 4.0	Autotrophic	[2-4, 19, 25-27]
T. intermedius	Production of polythionates and sulfuric acid	1.7 – 9.0	Mixotrophic	[2-4, 19, 25-27]
T. novellus	Production of elemental sulfur	5.0 – 9.2	Mixotrophic	[2-4, 19, 25-27]
T. neopolitanus	Production of polythionates and sulfuric acid	4.5 – 8.5	Autotrophic	[2-4, 19, 25-27]
Thiomonas sp.	Production of sulfuric acid	5.0 – 7.5	Mixotrophic	[2-4, 19, 25-27]

Nitrifying bacteria, *Nitrosomanas* and *Nitrobacteria* species are also able to breakdown concrete by producing acid. These organisms were found on the inner walls of cooling towers and buildings, and they have been shown to reduce ammonia to nitric acid. Chemical attack results from the production of nitric acid which solubilizes calcium nitrate [11, 23, 28]. In stimulation chambers these bacteria were able to produce 220 millimoles of nitric acid in 1 year and 3 % of the original weight of the material was lost due to degradation [28]. A summary of these results and the literature in the field is provided in Table 2.

Table 2: Microbial effects on building materials.

Organism	Mechanism of Degradation	Analytical Techniques	References
Cyanobacteria Gloeothece PPC 6909 **Fungi** Synechococcus elongates Microcoleus vaginatus Phormidium tenue Phormidium fragile	Due to desiccation may remove stone as they detach. Sheath formation produces 1 mm colonization into stone. Filamentous, resistant to fragmentation. Contraction upon dryness causes mechanical alternation.	Biomass detection was done through chlorophyll estimations, SEM, and ESEM.	[29]
Cyanobacteria and algae Chlorophyta Cyanophyta Oscillatoriates Ulotrichates **Lichens and mosses** Hyperphysia agglutinate	Mechanical and physical stress caused by crusts and patina formation. Penetration of rhizoids causes mech. damage. Secretion of carbonic and oxalic acids and biogenic compounds acting as chelating agents.	Organism detected with SEM. Imaged by SEM. $CaCO_3$ solubilization at site of acid production determined by EDS, XRD, and FTIR.	[30, 31] [32]
Fungus Cladasporium Sphaerospermum	Organic acid production.	$CaCO_3$ solubilization at site of acid production determined by EDS, XRD, CA, and MC.	[30, 33]
Fungus C.Cladosporoides A. alternata Phoma glomerata Penicillium ferguentans	Biodegradation occurs through Fe and Mn oxidation and resulting metal ion transfer.	Fe and Mn concentrations measured by enzymatic assay.	[34]
Acidophilic bacteria	Secretion of metabolic acids. (Produces chemical and mechanical damage.)	High concentrations of soluble silicon and calcium was detected by EDS.	[32]
Nitrifying bacteria Nitobactor Nitrosomanas	Production of nitric acid.	Degradation characterized by sample weight and pH change.	[23, 28, 35-38]

Mechanism of bio-deterioration of concrete

Microbes are expected to present everywhere, such as on the surface of the material, interstitial space of the material or with in the material. Sometimes, just the presence of microbes on surface of the material is sufficient for microbial damage. Generally, their metabolic end products are responsible for concrete deterioration [4, 19, 27]. The two main inorganic acids produced by microbes as their metabolic end products are 1) sulfuric acid produced by sulfur oxidizing bacteria and 2) nitric acid produced by nitrifying bacteria.

The exact mechanism used by microbes during oxidation of various forms of sulfur present in the environment to sulfuric acid depends on the type of microbes abundantly present in that environment. The production of sulfuric acid and attack of sulfuric acid on concrete is described by the following pathways (Figure 2) [19, 39] :

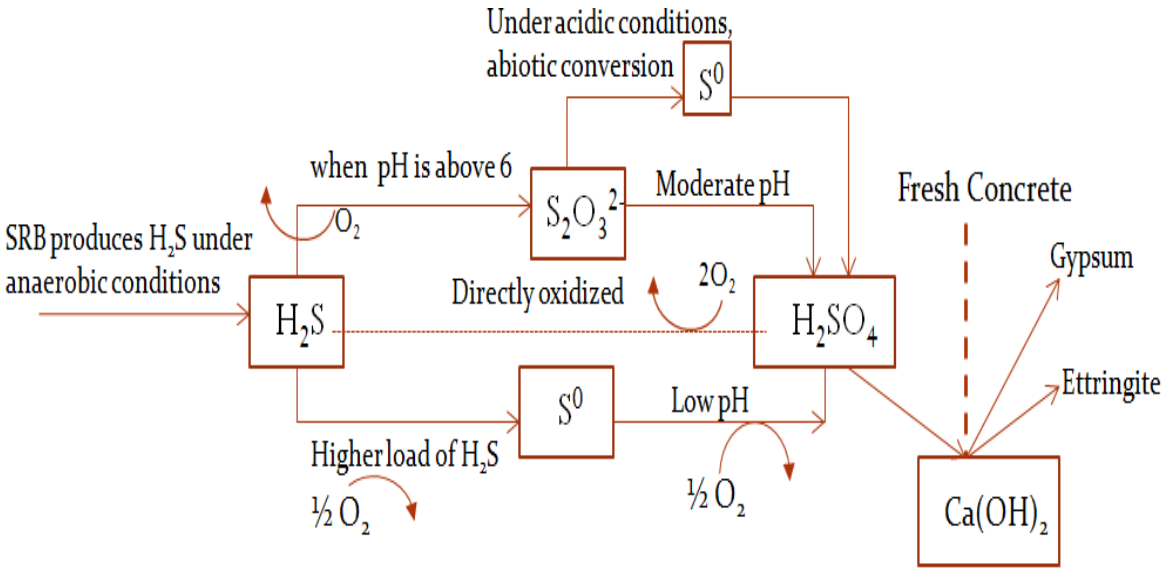


Figure 2: Possible pathways for hydrogen sulfide oxidation in deteriorated concrete.

Conditions for microbial activity

Microbial growth depends on suitable environmental conditions such as nutrients (carbon and energy sources), pH, temperature, osmotic pressure, humidity etc. From the above literature review it is clear that microbes involved in degradation of concrete thrive only when special environmental conditions are met and a community of microbes (or a single microbe) suitable for that environment is present. The role of microorganisms involved in degradation of concrete and the identification of major mechanisms of degradation are important in order to identify the specific effects of different organisms in regards to their environmental conditions.

Preventive techniques of concrete bio-deterioration

Preventive techniques of concrete bio-deterioration depend on type of microbe present, type of environment present at the site and the degree of damage occurred. Combination of the following measures can be taken to prevent deterioration:

- Coating concrete surfaces using paints, epoxies and polymers [40-42].
- By varying the concrete composition and also by using corrosion-inhibiting admixtures [40, 41, 43, 44].
- Surface cleaning using chemical or biological means (biocides) [42, 45, 46].

De Muynck et al. studied the effectiveness of the use of various surface coatings, ad mixtures and antimicrobial polymer fibers and zeolites towards biogenic sulfuric acid concrete corrosion. Effectiveness of the treatments was measured by means of accelerated chemical and biological tests. They found out that epoxy coatings and polyurethane linings offered best protective performance towards biogenic sulfuric acid concrete corrosion, while the use of cementetious coating showed greatest degradation. The addition of hydrous silicate ad mixtures, antimicrobial polymer fibers or zeolites failed to give improved performance [41].

The effectiveness of the addition of anti-fungal microcapsules to the motor and concrete during the casting stage for the prevention of fungal attack on concrete was studied by Park et al. In order to protect the microcapsule membrane from being damaged during mixing and casting of motor, Zeolite and Zeocarbon was added to the microcapsules. The specimens were observed after 45 days following a 30 day fungus application. Less amount of fungus was observed on the surface of motors with 5 % and 15 % anti-fungal microcapsules compared to surface of plain motors where huge amount of fungi was observed [44].

Recently, Brendt, M.L. investigated the effect of supplementary cementitious materials, epoxy coatings, latex modified mortars and calcium aluminate cement mortar in protecting concrete from microbial deterioration. Laboratory exposure tests to sulfur oxidizing bacteria as well as field tests were performed to select best working materials. Results showed that partial replacement of cement with 40 % slag or 5 to 10 % silica fume improved resistance to microbial deterioration. Whereas replacement of cement with 60 % slag did not show any resistance to deterioration, suggesting optimized amount of slag is required for better performance. Epoxy coatings and calcium aluminum silicate mortars showed excellent performance. In order to get best protection from epoxies, the thickness of the coatings should be within the suggested range. Calcium aluminum silicate mortar showed excelled acid resistance and potential bacteriostatic effect [40].

Muynck et al. investigated both traditional and innovative water repellents and biocides for prevention of algal fouling on two different types of concrete. Results of their study showed that performance of the treatments was dependent on the type of concrete. They identified that the combinations of water repellents and biocides were the most effective treatments [46].

Recently, Sobecky et al. showed that photocatalytic cement exposed to artificial sunlight can strongly inhibit the fungal colonization and fouling compared to normal cement

of same composition**.** The results of their study showed direct relationship between water-to-cement ratio and amount of fouling [43].

PART 1: ISOLATION AND CHARACTERIZATION OF A STREPTOMYCES SP. FROM DETERIORATING CONCRETE BRIDGE STRUCTURES

1.1- INTRODUCTION

Concrete deterioration is one of the most challenging problems that has been facing by sewage and transportation authorities, due to its major impact on maintenance costs, public health and safety [47, 48]. A better understanding of causative agents and mechanisms of concrete deterioration is essential for efficient prevention or control of deterioration process. Microbial deterioration is known to occur in concrete sewer systems and this issue has been well characterized and understood [47-51]. However, microbial deterioration of concrete bridge structures is recently attracting attention as a serious problem relating to the structural integrity and lifespan of a number of bridge structures [52]. In addition microbes are known to attack concrete and stone buildings, prehistoric and renaissance paintings and monuments over long time periods [16, 53, 54]. In some cases fungal species are also identified as a cause for bio-deterioration of concrete infrastructure [43, 55, 56].

In concrete sewer systems, first sulfate-reducing bacteria (SRB) present in water and sediments under anaerobic conditions produce hydrogen sulfide (H_2S), carbon dioxide and other acidic gases. These gases react with fresh alkaline concrete and gradually reduce its pH from initial value. H_2S is chemically oxidized to elemental sulfur (S^o) and thiosulfate ($S_2O_3^{2-}$). When alkaline pH reaches neutral pH, SOB start colonizing on the surface of concrete and further reduces the pH to acidic pH by oxidizing thiosulfate and elemental sulfur to sulfuric acid (H_2SO_4) [47-50, 57, 58]. Sulfuric acid reacts with calcium hydroxide, ($Ca(OH)_2$) which is present in concrete and forms gypsum and ettringite [59].

Both ettringite and gypsum are expandable products which increase the internal pressure of concrete that results in cracking and pitting [60]. Cracking provides larger surface area for the sulfuric acid reaction to occur and also provide more space for acid to penetrate into deeper layers of concrete causing structural failure of the facility [61].

The succession of different species of bacteria was observed at corroded parts of concrete [50]. Type of species depends on the pH of concrete, trophic conditions and ability to utilize different sulfur compounds such as hydrogen sulfide, elemental sulfur and thiosulfate [50]. Previous studies identified *Thiobacillus* species as the major source of bio-deterioration in concrete sewer systems [27, 49-52, 62]. Nitrosomanas and Nitrobacteria species are also able to breakdown concrete by producing nitric acid which solubilizes calcium compounds [63, 64]. The majority of microbial activity is known to occur on the surface of concrete. Okabe et al. (2007) reported logarithmically decrease in microbial populations with depth of concrete because of hydrogen sulfide and oxygen limitations [50].

Many modern analytical procedures are able to provide new capabilities for observation and characterization of microbes. Electron microscopy techniques such as scanning electron microscopy are capable of visualizing the presence and activities of microbes on concrete surfaces with no sample preparation or alteration [54, 65, 66]. Modern micro-analytical methods can be used to detect the changes induced by the microbes. The microstructure of the concrete can also be determined through the use of chemical analysis (CA), thermal analysis (differential and themogravimetric), FTIR, X-ray diffraction (XDR), and scanning electron microscopy with EDS [37, 67]. Chemical analysis through IC and ICP-ES will provide details concerning the types of acids that are being produced by the microbes and the structural and chemical effect acid production has on the concrete samples [50, 65].

The study presented here was intended to isolate and identify the acid- producing

microbe that is responsible for deterioration of bridge structures and to develop a better understanding of the biological and chemical processes associated with microbial deterioration. Additional experiments were also designed to identify the extent of deterioration in order to develop effective techniques to prevent microbial bio-deterioration of concrete.

The specific objectives are as follows: (1) to determine if acid producing microbes are present in samples collected from bridge sites exhibiting surface deterioration. (2) to isolate and identify the acid producing organisms and (3) to determine if these organisms, whether in pure or mixed culture, can produce significant deterioration of concrete surfaces.

1.2- MATERIALS AND METHODS

1.2.1- Sample collection and growth medium preparation

The TxDOT personnel collected microbial samples from deteriorated bridge structures in Texas during routine inspection. Samples were collected by chipping or scraping the concrete surface into plastic bags which were shipped to OSU where they were stored at -80 °C immediately upon arrival. The microbial cultures produced from the concrete were enriched in growth medium which was composed of 1 g of sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) as an energy source, 1 g ammonium chloride (NH_4Cl), 0.5 g magnesium chloride hexahydrate ($MgCl_2.6H_2O$), 0.6 g potassium phosphate (K_2HPO_4), 0.4 g potassium dihydrogen phosphate (KH_2PO_4), 0.02 g ferric chloride anhydrous ($FeCl_3$) per liter of distilled water. Growth medium was supplemented with yeast extract at a concentration of 0.01% (w/v) to provide a source of carbon and nitrogen. Prior to introducing the concrete samples collected in the field, the medium was sterilized by autoclaving at 121 °C for 30 minutes. All chemicals used for producing growth medium

were purchased from *Fisher Scientific* Inc.

1.2.2- Culture enrichment

Enrichment cultures were initiated in 1 L bottles containing 500 ml of thiosulfate-growth medium and 2.5 grams of TxDOT concrete particles (2 – 5 cm size) and incubated at 30 ^{0}C. Concrete particles of same weight were autoclaved and inoculated in thiosulfate-growth medium and used as control. An aliquot (2 ml) of the culture medium was collected on weekly basis and pH was measured. Sulfate ion (SO_4^{2-}) concentration in the cultures was measured at the end of the experiment.

1.2.3- Bio-deterioration of concrete samples

High strength and low permeability concrete cylinders, approximately 1-inch by 0.5-inch diameter, were produced using a 0.45 ratio of water to cement. The cylinders were cured in water for three weeks before being transferred to bottles containing either (i) 250 ml of enriched culture, (ii) 250 ml of growth medium, or (iii) 250 ml of autoclaved water. Bottles were stored at 30 °C for 6 months, and triplicate concrete samples at each condition were removed at 2 month intervals and either stored at -80 °C or analyzed immediately for signs of deterioration. Aliquots (2 ml) of the culture medium were collected on monthly basis and pH was measured. Aliquots (25 ml) were collected every two months to analyze the thiosulfate, sulfate and calcium ion concentration.

1.2.4- Structural deterioration

At the end of the experiment (after 6 months) the 1" concrete cylinders in bioreactors were analyzed under Zeiss Stemi 2000-C stereomicroscope with AxioCam MRc5 to observe the signs of physical degradation.

SEM imaging provides detailed morphological features of fractured concrete samples. The SEM scans a focused beam of electrons across the specimen and collects

secondary and backscattered electron signals. Even though specimens intended for secondary imaging benefit from a conductive coating of a heavy metal such as gold or gold/palladium to increase secondary electron flux and therefore improving the imaging signal, the samples were not coated in order to retain samples original conditions and preserve the bio-deterioration of the concrete samples. They were cut into sizeable dimensions of (1.5 x 1.5 x1.5) cubic inches so as to fit into the SEM chamber. A beam of electrons with an accelerating voltage of 15 kV was used in such in this study. The electron micrographs were obtained using low-energy secondary electrons and X-ray radiation allowed for the identification of elemental compositions. The semi-qualitative elemental analysis and elemental mapping was done using Jeol 840 scanning electron microscope retrofitted with EDS Kevex detector. The same procedure was followed to obtain SEM images of concrete samples stored in pure cultures for 12 months.

1.2.5- Isolation of pure cultures

In order to isolate the acid producing microbes, enriched samples were serially diluted (10^{-1} to 10^{-6} serial dilutions) and 100 µl from the 10^{-4} to 10^{-6} dilutions were spread on agar plates. Agar plates were prepared with same recipe of growth medium with the addition of 15 g/L agar. The plates were incubated for 10-15 days at 30 ^{0}C. Single colonies with different morphologies were streaked on the agar plates amended with 0.02 g/L of chlorophenol red (pH indicator dye) and plates were incubated for 7-10 days at 30 ^{0}C. Acid-producing microbes were identified by their ability to change agar color of indicator plate. Representative colonies were further used to extract the genomic DNA and also to enrich the pure cultures. The colonies of acid producing microbe was transferred to 200 ml of the growth medium and incubated at 30 ^{0}C on shaker for 2 months before using these pure cultures for other experiments.

1.2.6- DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using GeneQuik Bacteria DNA extraction kit (Orochem Technologies Inc., USA). The 16S rDNA genes were amplified from genomic DNA using bacterial primers 27F: AGAGTTTGATCMTGGCTCAG and 1492F: TACGGYTACCTTGTTACGACTT. The PCR program used was as follows: 95 °C for 5 minutes, 30 cycles of 60 seconds at 74 °C, 45 seconds at 54 °C, and 1.5 minutes at 72 °C, followed by a final extension for 8 minutes at 72 °C. The PCR products were purified with ExoSAP-IT (Affymetrix, inc., USA) using PCR (15 minutes at 37 °C and 15 minutes at 80 °C) and the purified PCR products were sequenced at the recombinant DNA/Protein Core Facility (Oklahoma State University, Stillwater, Oklahoma). The sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) and were checked from the reference sequences obtained from the GeneBank database (**http://www.ncbi.nlm.nih.gov/genbank/**) provided by the National Center for Biotechnology Information (NCBI).

1.2.7- Oxidation of thiosulfate to sulfate

10 % (v/v) of the pure culture was added to 150 ml of thiosulfate-growth medium containing 0.01 % (w/v) yeast extract and were incubated at 30 ^{0}C while shaking at 150 rpm. Enriched culture in growth medium without thiosulfate was used as control. Aliquots (6 ml) were collected on weekly basis and analyzed for pH, thiosulfate and sulfate ion concentration.

1.2.8- Effect of pure culture on concrete

10 % (V/V) of the pure culture and 2.5 grams of concrete chips were inoculated in 250 ml of the thiosulfate-growth medium with 0.01 % (w/v) yeast extract and were incubated at 30 °C while shaking at 150 rpm. Un-inoculated flasks prepared similarly were

used as control. Aliquots (8 ml) were collected on weekly basis and analyzed for pH, thiosulfate, sulfate and calcium ion concentration. Before inoculation, all the concrete samples which were used to start the cultures were carbonated for few weeks to neutralize the pH of concrete and autoclaved thrice keeping 24 hours gap between each autoclave.

1.2.9- pH measurements

The pH was measured at room temperature (25 °C) using an Accumet AB15 Basic and Biobasic pH/mV/°C meter calibrated at room temperature using three different pH buffers (pH 4 , 7 and 10).

1.2.10- Detection of sulfate and thiosulfate ion concentration

Dionex DX-120 Ion chromatograph equipped with a Dionex IonPac® AS14 anion exchange column was used to measure the concentration of sulfate and thiosulfate ions in the growth medium. The samples were filtered with 0.45 µm filters before injection and were injected automatically by the autosampler (Dionex AS40) in to the 25 µl sample loop. The sample flow rate was maintained at 1.14 ml/min. Sodium carbonate/bicarbonate buffer 2.1/0.8 mM/l was used as eluent for the detection of anions. The eluent was prepared with deionized water. The retention time for sulfate ions and thiosulfate ions was 6 min and 11 min. Sodium sulfate (Na_2SO_4) and sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) of concentration range 10, 50, 100, 200, 300, 400 mg/l were used as standards.

1.2.11- Analysis of calcium ion concentration

Analysis of calcium ion concentration that had been leached from concrete into the cultures was done at OSU, Soil Laboratories, Stillwater, Oklahoma. This analysis qualitatively determines analyte concentrations in sample filtrate using an ICP-ES. The samples were filtered using 0.45 µm filter before analysis to remove bacterial biomass.

1.3- RESULTS

1.3.1- Enrichment of mixed cultures

Since some microbes from the environment cannot be cultured, the initial interest was to investigate if the acid-producing microbes from the bridge structures could be cultured in laboratory. In order to make sure that, samples provided by TXDOT were cultured in laboratory and their growth rate and pH was monitored over time. The control samples, which comprise the respective growth medium and small autoclaved sample of concrete showed an increase in solution pH approximately from 7 to 9.5 over the duration of the experiment (Figure 3). This observation is consistent with our expectation that calcium hydroxide in hydrated concrete will dissolve in the broth and increase the pH of the solution. The samples, which comprise the microbes and concrete in growth medium showed a drop in pH from 7 to 4.3 (Figure 3). Approximately 7.8 mM sulfate was accumulated in the medium by the end of the experiment (Figure 4), suggesting some of the microbes present in the samples are sulfur oxidizers. At the end of the experiment cultures were serially diluted and streaked on agar plates to confirm the growth of microbes. It indicates that microbes are present in the samples and that the decrease of the pH and oxidation of thiosulfate is likely due to the acid production by these microbes (data not shown).

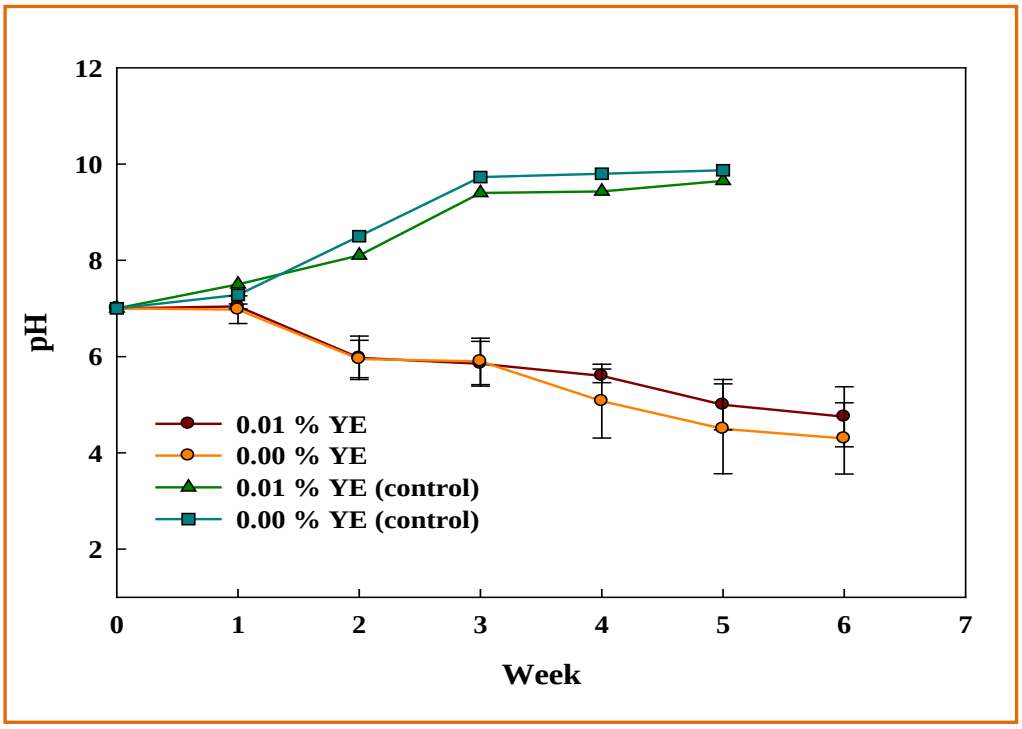


Figure 3: The effect of microbial growth on solution pH. YE in figure represent yeast

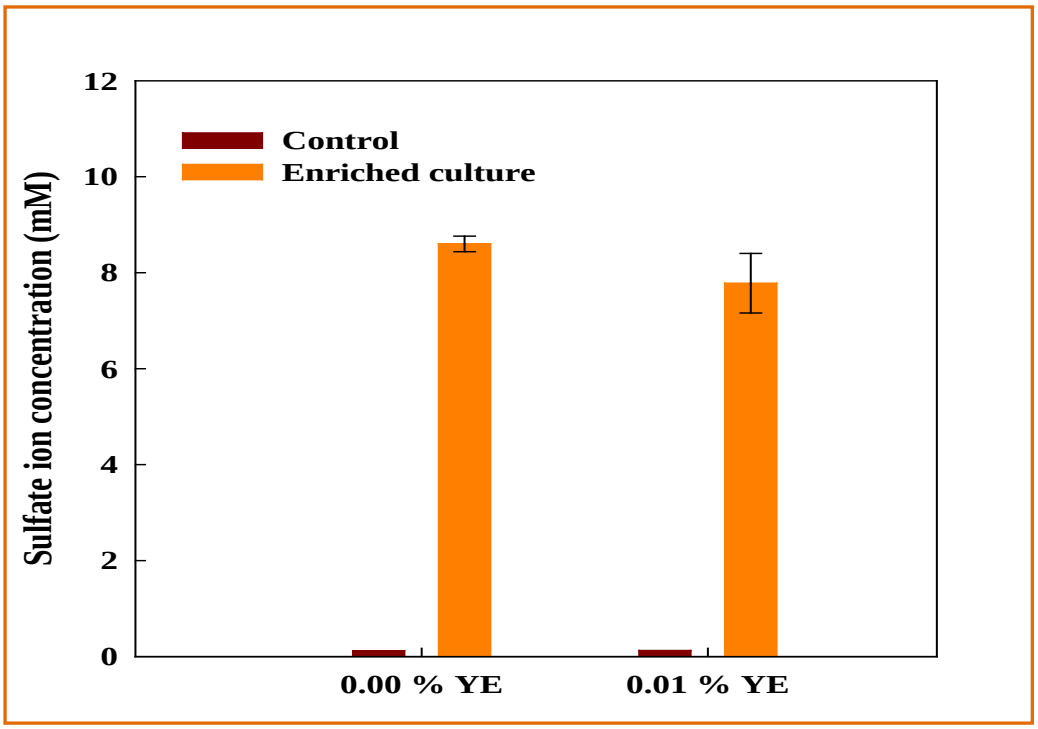


Figure 4: Sulfate ion concentration in enriched culture and in control at the end of the experiment (6 months). YE in figure represent yeast extract.

1.3.2- Deterioration of concrete by TxDOT cultures

After making sure that acid-producing microbes can be cultured in laboratory, enriched cultures from the TxDOT samples were used to develop a standardized laboratory assay intended to replicate degradation of concrete as observed in the field (Figure 5). The change in pH during the growth of microbes was shown in Figure 6. Results in Figure 6 showed decrease in culture pH from 6.3 to 3.4 in 4 weeks only in bottles lacking concrete cylinders. We surmise the decrease in pH was due to the oxidation of thiosulfate to sulfuric acid by sulfur oxidizing microbes. The pH remained almost neutral in culture bottles containing concrete cylinders and this could be due the neutralization capacity of the fresh concrete. The pH increased from 7.6 to 8.5 in un-inoculated control bottles containing concrete cylinders due to the alkalinity of fresh concrete.

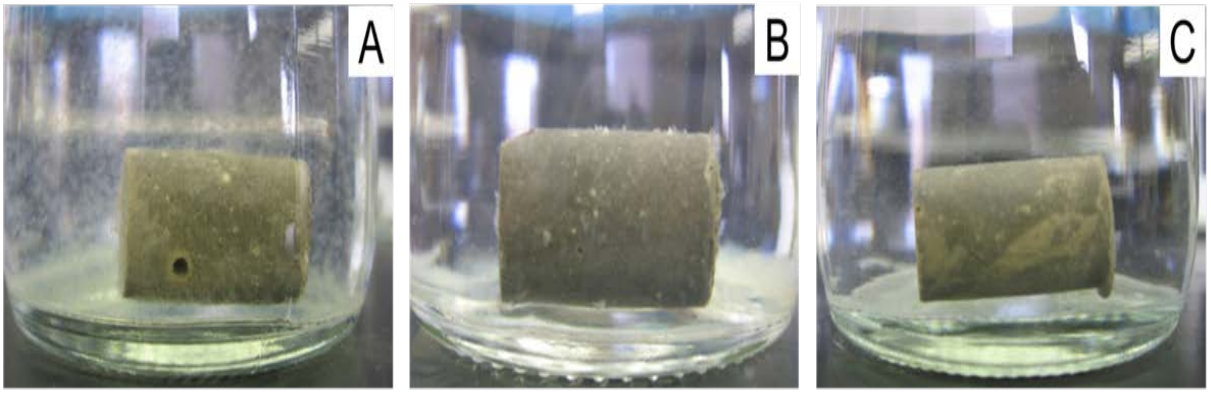


Figure 5: Assay for measuring effect of microbes on concrete. Concrete cylinders were stored in (A) Enriched culture, (B) Growth medium, and (C) Autoclaved water.

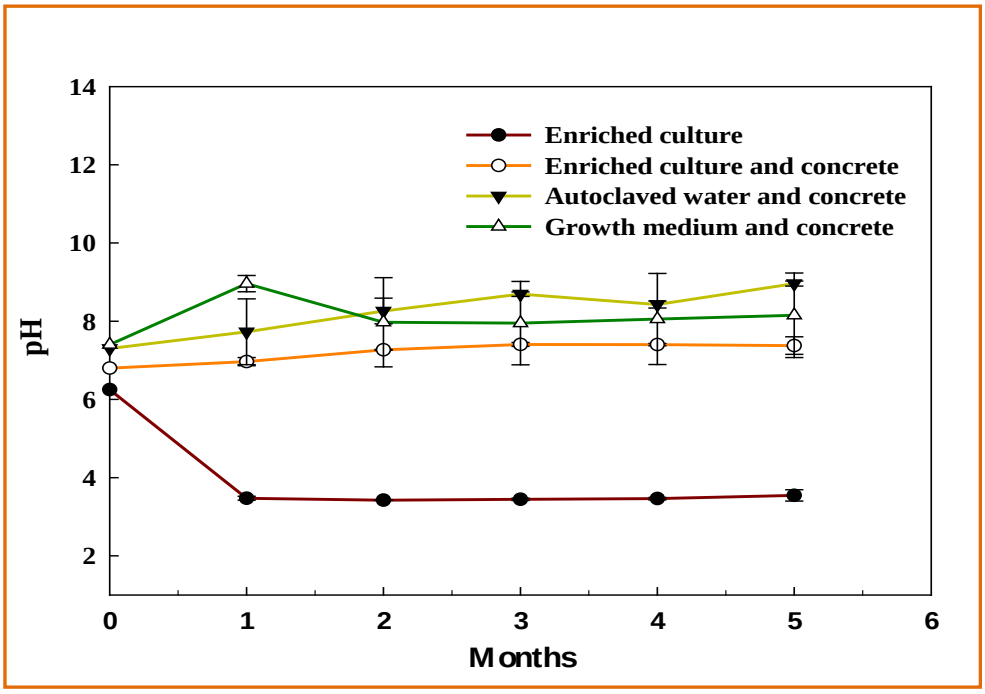


Figure 6: Solution pH in presence and absence of concrete cylinders and microbes.

In order to test for the oxidation of thiosulfate to sulfate by TxDOT culture, samples were withdrawn periodically and analyzed for the accumulation of sulfate using IC. Figure 7 shows the concentrations of thiosulfate and sulfate in inoculated and un-inoculated bottles with and without concrete cylinder. Results (Figure 7) clearly showed the oxidation of thiosulfate (as energy source) by the culture both in the presence or absence of concrete. Thiosulfate was not oxidized in un-inoculated control bottles containing concrete cylinder. These results show that microbes are responsible for the oxidation of thiosulfate. Analysis shows complete oxidation of thiosulfate occurred in 2 months with a corresponding accumulation of sulfate. Interestingly, sulfate continues to accumulate even after the added thiosulfate was completely removed. Approximately, 5 to 6.7 mM sulfate was accumulated accounting for only 62 to 84 % of the added thiosulfate suggesting incomplete oxidation of thiosulfate to sulfate and accumulation of intermediates (e.g., elemental sulfur, tetrathionates) that we did not detect using current methods. In the control bottles with concrete we did not observe either the oxidation of thiosulfate or formation of sulfate.

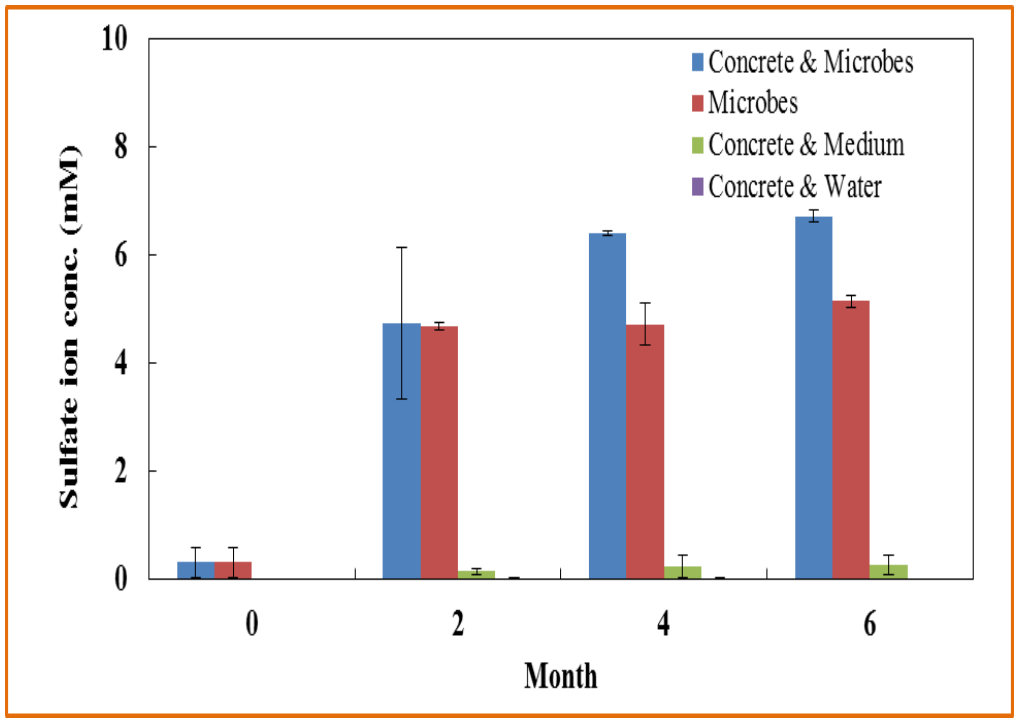


Figure 7: Sulfate ion concentration in cultures with and without concrete cylinders and microbes.

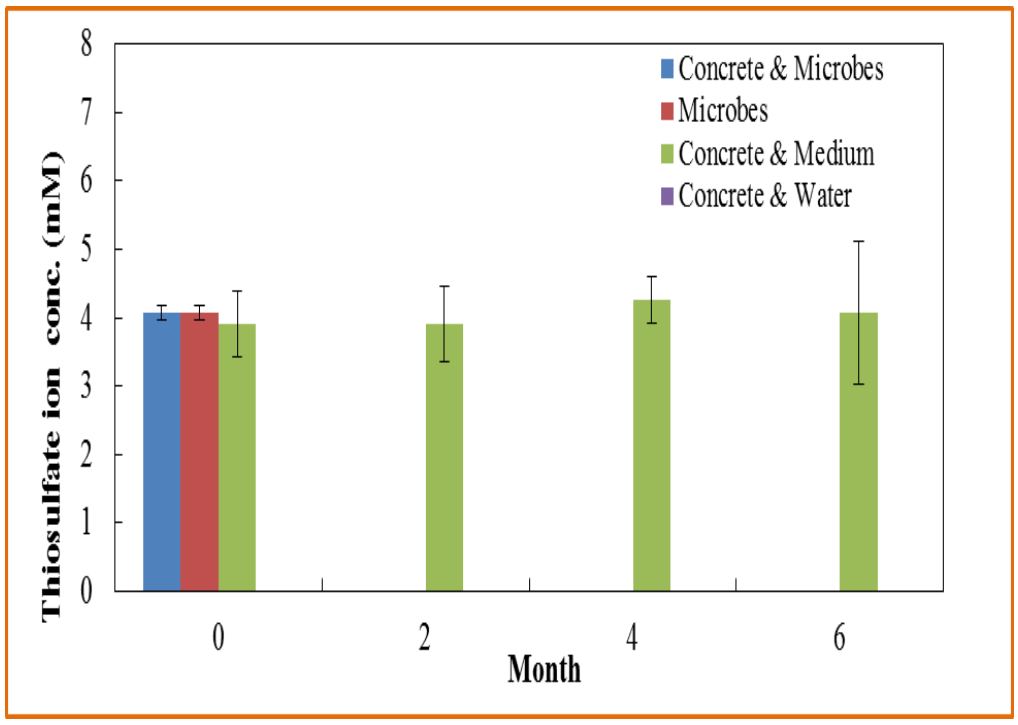


Figure 8: Thiosulfate ion concentration in cultures with and without concrete cylinders and microbes.

Figure 9 shows the concentration of calcium leached from the concrete due to acid attack over the duration of the six month study. Previous studies have demonstrated that sulfuric acid can react with calcium hydroxide present in the concrete to form gypsum and ettringite. The calcium ion concentration in bottles with microbes was 2 fold higher than that in the control (Figure 9).

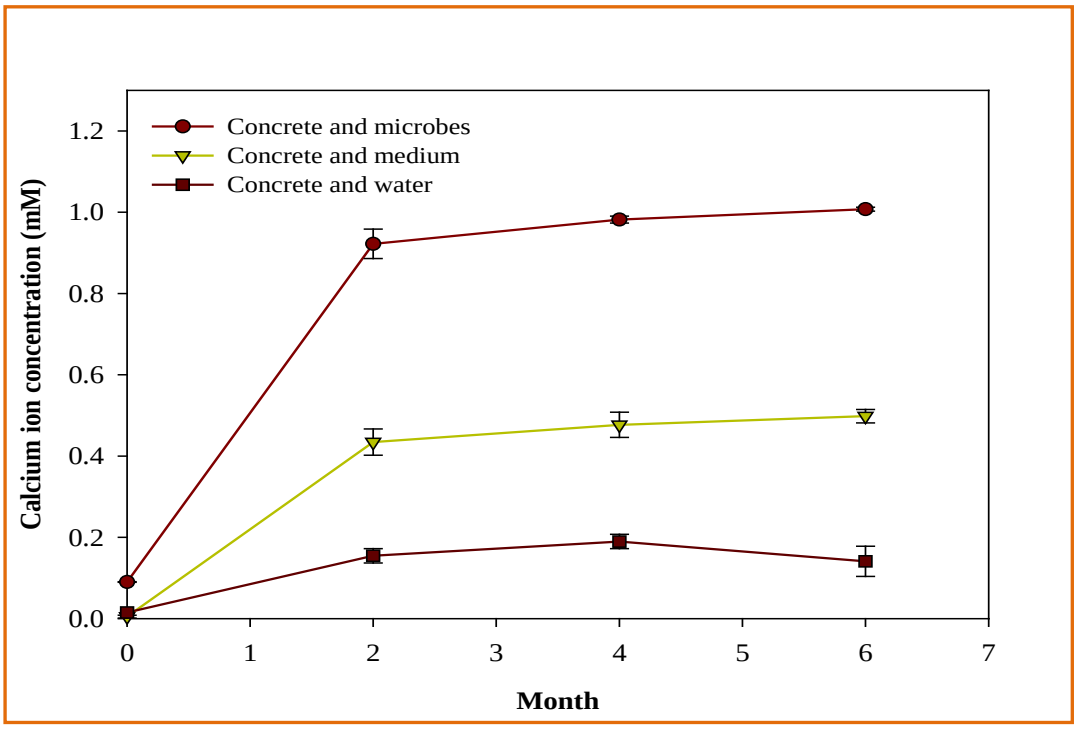


Figure 9: Leaching of calcium from concrete cylinders exposed to mixed cultures.

1.3.3- Stereo microscope and SEM of concrete cylinders

Stereomicroscopy was performed on the concrete cylinders to determine the impact of acid production on morphology of concrete cylinders. When the cylinder was exposed to sulfuric acid produced by enrichment culture, deep cracks and holes were observed on concrete cylinder as shown in Figure 10. The microbes present in the growth medium were involved in acid production and concrete deterioration where the oxidation of thiosulfate to sulfate led to the formation of deep cracks on concrete cylinder. In contrast to the concrete cylinders exposed to acid produced by microbes, the surface of the control cylinders were

smooth without any damage. Although there was no damage in the case of concrete and medium, concrete and water we observed white yellowish powder like material covering the surface of the concrete. Whenever moisture vapor is present, the moisture will dissolve the alkali salts and typically rise to the "green" concrete surface with the moisture vapor then remain as a white residue when the water evaporates (Figure 11, 12).

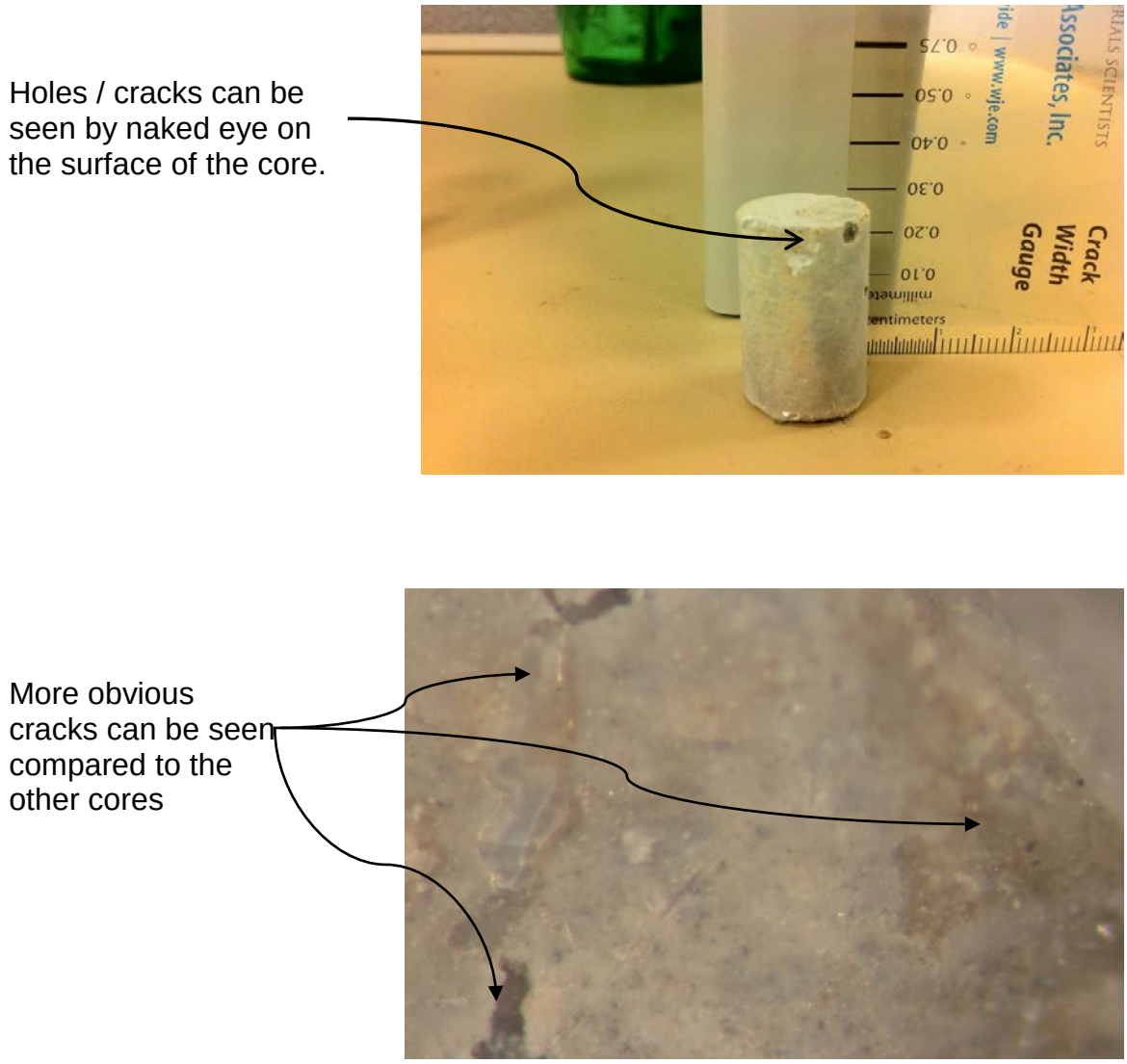


Figure 10: Stereomicroscope images of concrete surfaces after 6 months of exposure to microbes.

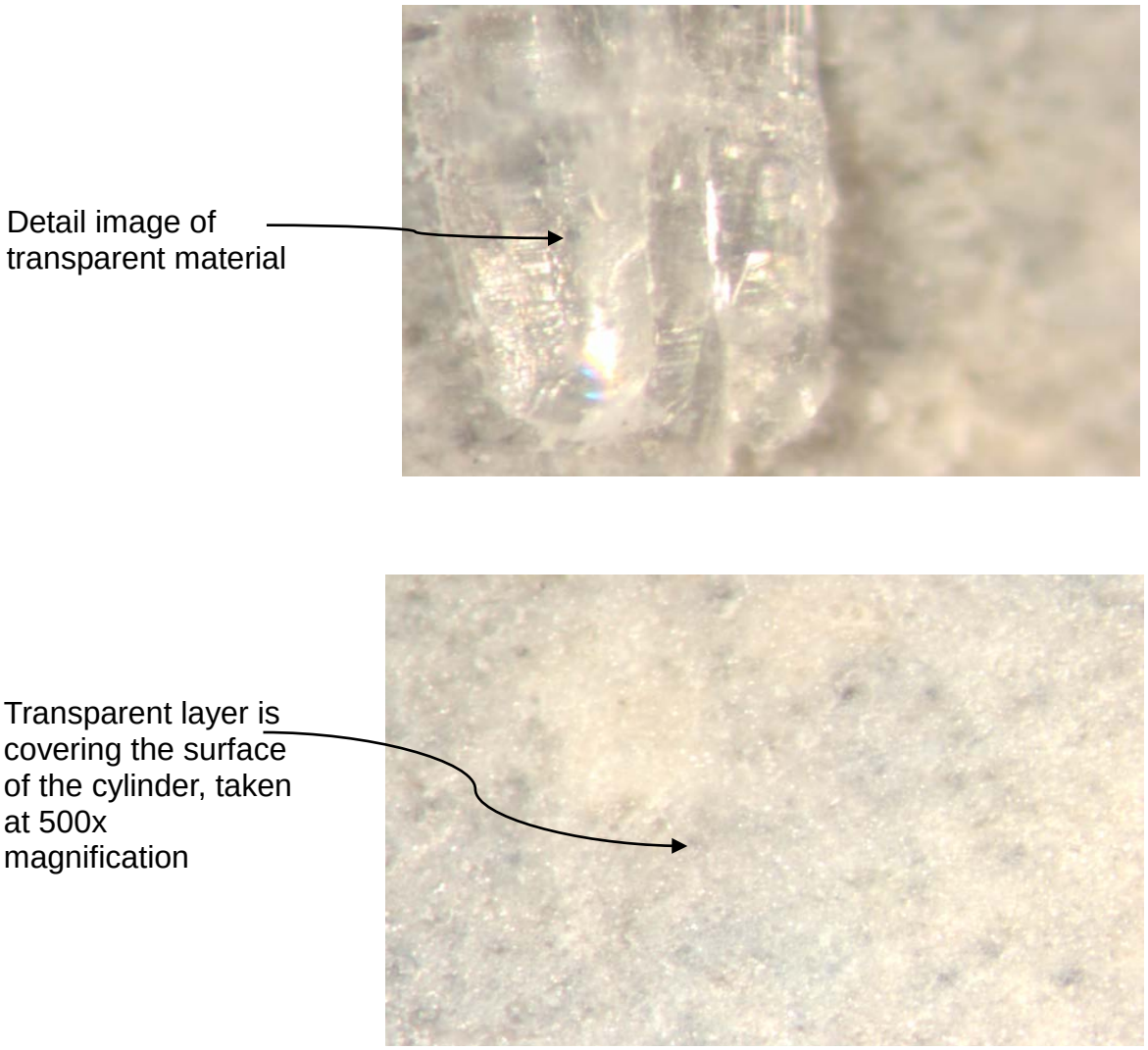


Figure 11: Stereomicroscope images of concrete surfaces after 6 months of exposure to growth medium.

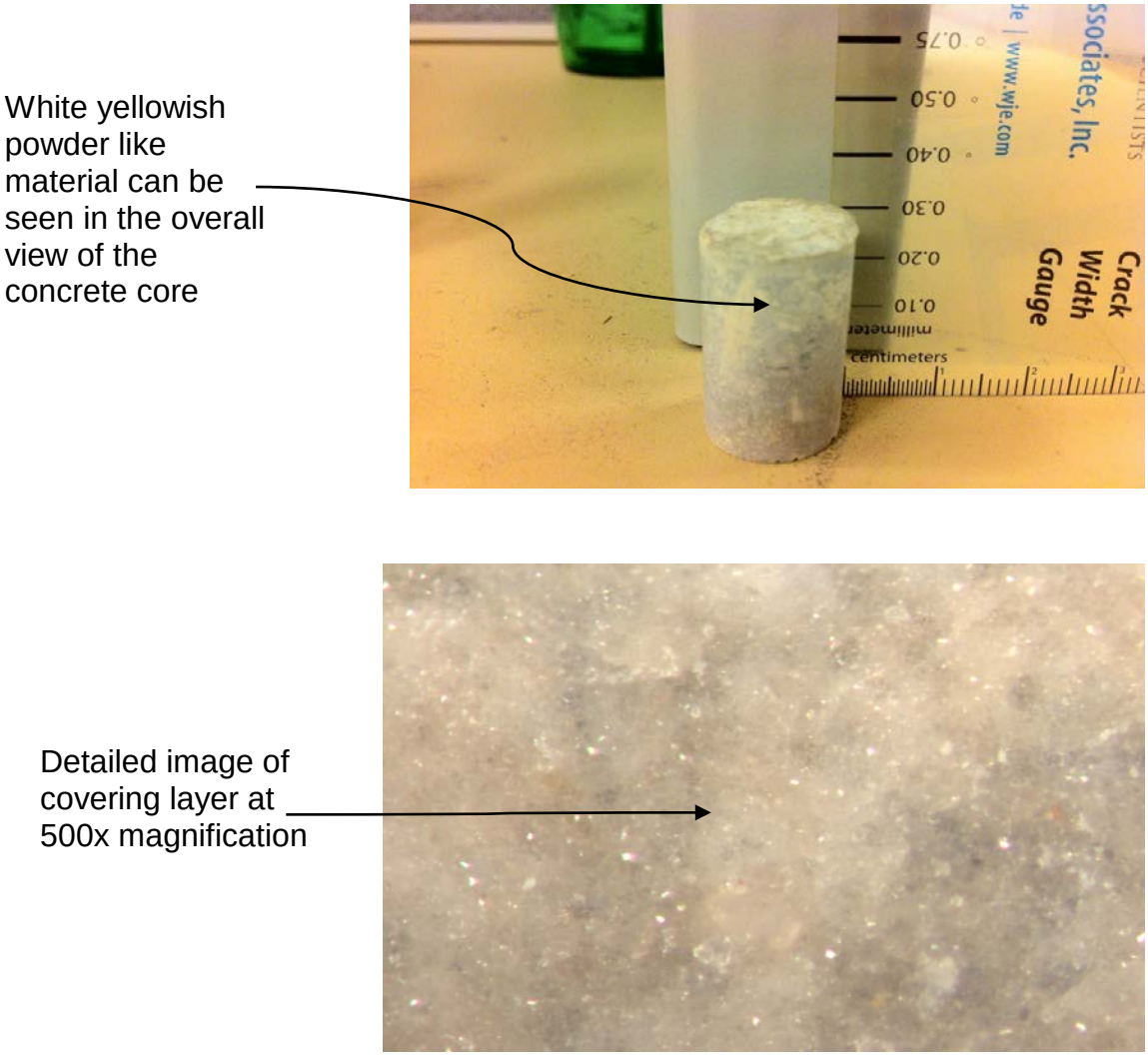


Figure 12: Stereomicroscope images of concrete surfaces after 6 months of exposure to autoclaved water.

SEM was performed on another set of concrete cylinders from the above experiment to verify the observations from stereomicroscopy. Consistent with the stereomicroscopy results, SEM also showed deep cracks on the cylinders exposed to enrichment cultures (Figure 13A), and that no cracks were observed on control cylinders (concrete cylinder in growth medium) (Figure 13B).

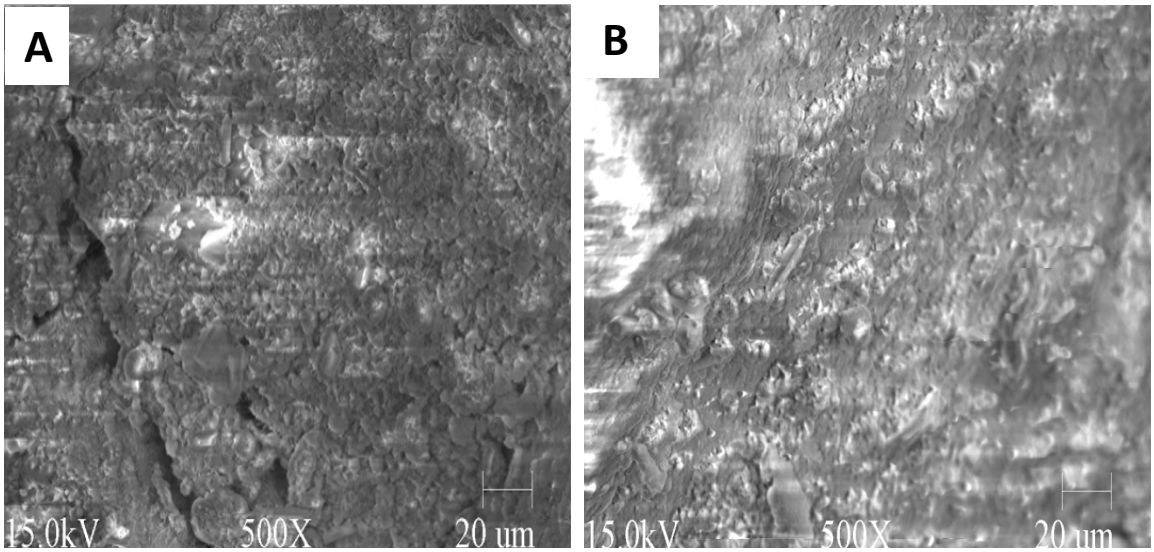


Figure 13: SEM images of cement surfaces after 6 months (end of experiment) of exposure to a growth medium (B) and to a growth medium with microbes (A).

1.3.4- EDS Analysis of Concrete Samples

EDS was first carried out on a concrete sample that was stored in 10 % sulfuric acid to give us an idea of what to expect from EDS analysis of a sample that had been attacked by sulfuric acid, regardless of the source of that acid. Figure 14 shows the EDS spectrum of the sulfuric acid attacked sample.

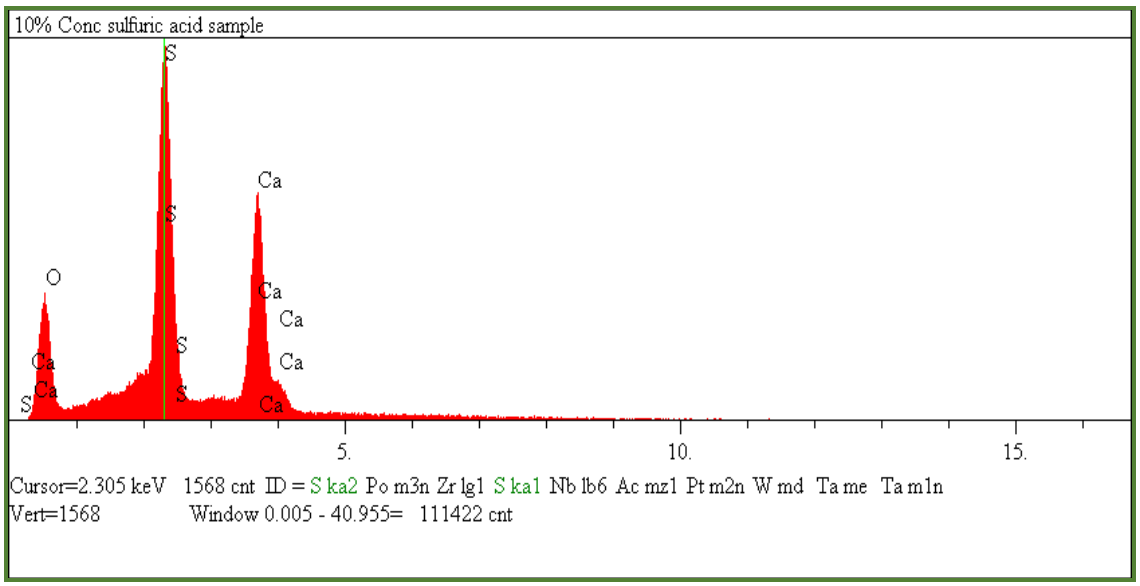
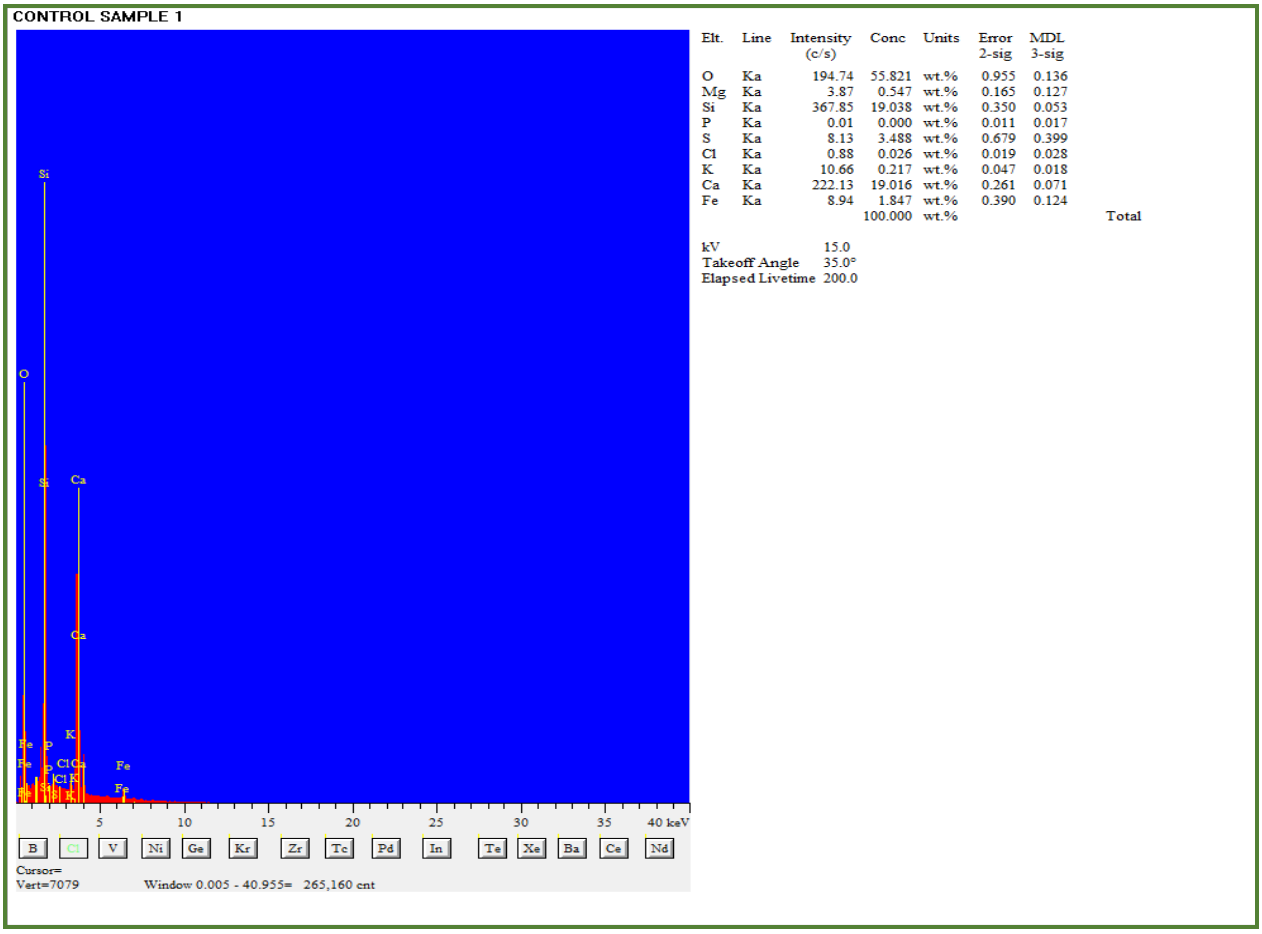


Figure 14: EDS spectrum of the concrete sample exposed to 10 % concentrated sulfuric acid.

The spectrum indicates high levels of calcium, as one would expect from the concrete alone, and high levels of sulfur. After observing calcium and sulfur peaks for the control sample that was stored in sulfuric acid, EDS was carried out on a control sample that was never exposed to the microorganism that produces acid. Figure 15 shows the EDS spectrum of the control sample. As we expected the concentration of calcium and silicon was high on the control concrete, and the amount of sulfur is low.



Elt.	Line	Intensity (c/s)	Conc	Units	Error 2-sig	MDL 3-sig	
O	Ka	194.74	55.821	wt.%	0.955	0.136	
Mg	Ka	3.87	0.547	wt.%	0.165	0.127	
Si	Ka	367.85	19.038	wt.%	0.350	0.053	
P	Ka	0.01	0.000	wt.%	0.011	0.017	
S	Ka	8.13	3.488	wt.%	0.679	0.399	
Cl	Ka	0.88	0.026	wt.%	0.019	0.028	
K	Ka	10.66	0.217	wt.%	0.047	0.018	
Ca	Ka	222.13	19.016	wt.%	0.261	0.071	
Fe	Ka	8.94	1.847	wt.%	0.390	0.124	
			100.000	wt.%			Total

kV	15.0
Takeoff Angle	35.0°
Elapsed Livetime	200.0

Figure 15: EDS spectrum of the concrete control sample.

When control spectrum is compared to the EDS spectrum of concrete that was stored in the medium lacking microbes, and medium containing microbes, we see a similar signal for sulfur (Figure 15, 16, 17). The low level of sulfur is not too surprising since the acid produced by the microorganism remains ionized in the liquid phase (Figure 7). In addition, two months is likely too short a time period to see insoluble sulfur compounds on the concrete cylinder.

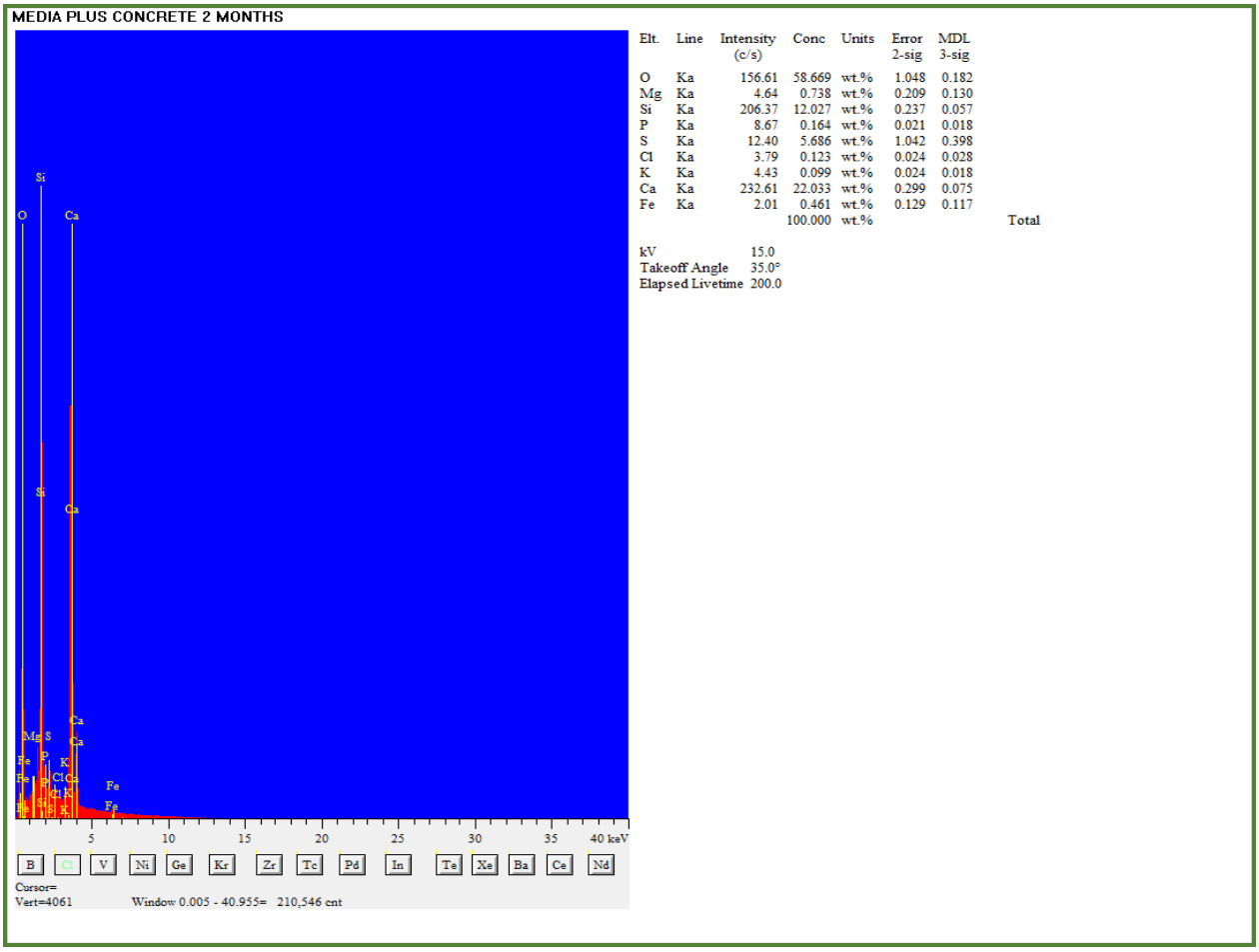


Figure 16: EDS spectrum of the concrete sample stored in growth medium for 2 months.

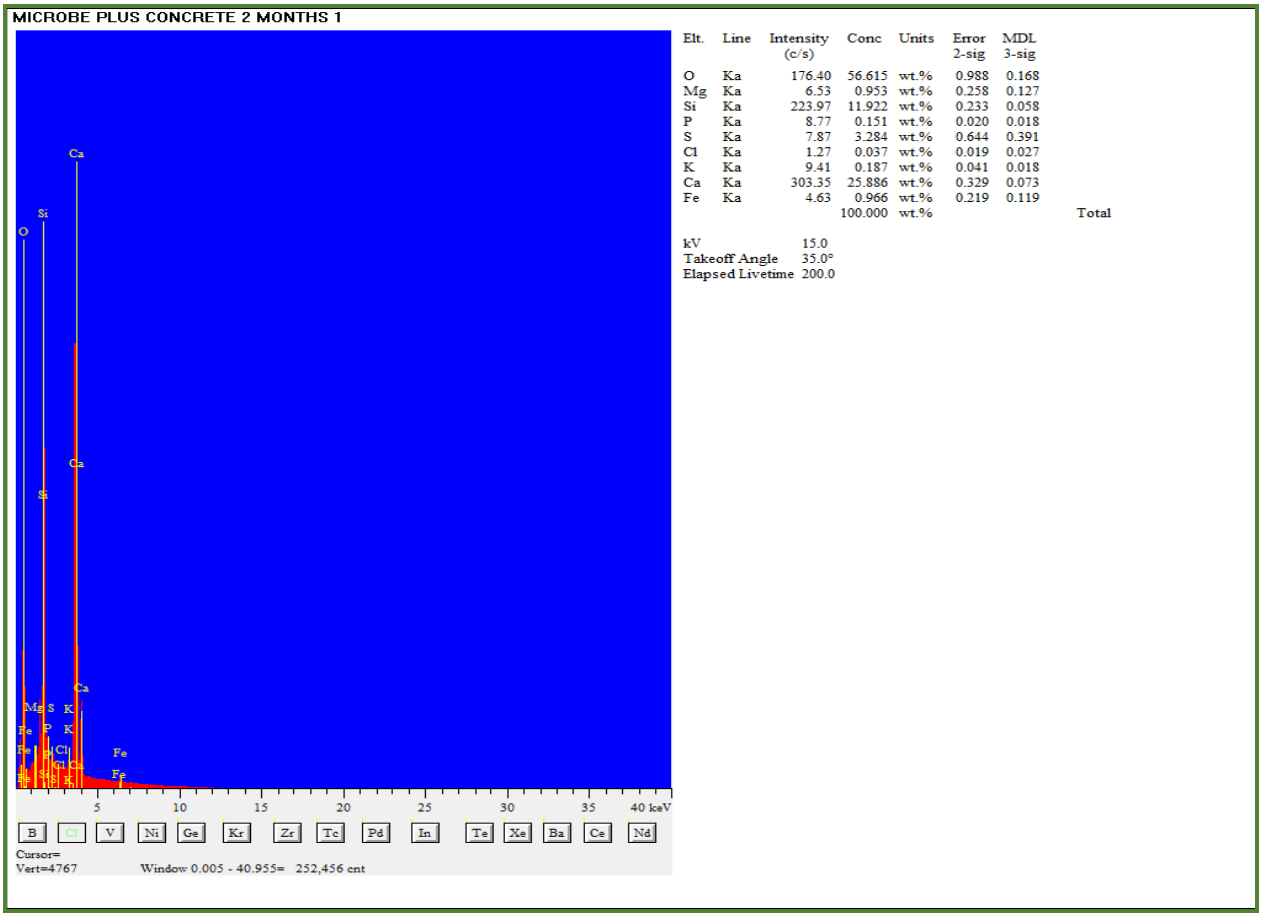


Figure 17: EDS spectrum of the concrete sample stored in medium containing microbes for 2 months.

1.3.5- FTIR Analysis of Concrete Samples

Similar to EDS, FTIR was used to look for the presence of sulfur on the samples. The control sample was stored in 10 % concentrated sulfuric acid for 24 hours and then left to dry at room temperature for 24 hours. As seen from the FTIR spectrum, there is a strong peak for sulfur at 2341 cm^{-1} and 2509 cm^{-1} (Figure 18). Since sulfur compounds typically produce signals between 2600 and 2200 cm^{-1} [68], the peaks within this range for the control sample were used as a reference in the analysis of the samples stored in enriched cultures or sterile growth medium for 6 months.

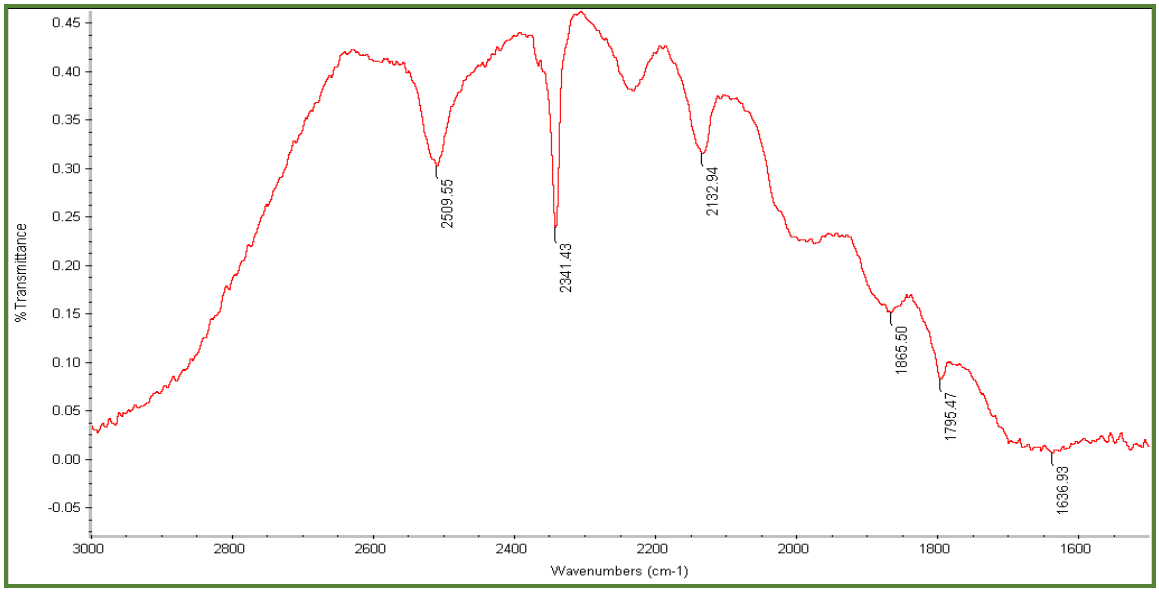


Figure 18: FTIR spectrum of the concrete sample exposed to 10 % concentrated sulfuric acid.

Figure 19 shows the FTIR spectrum for three control samples that were stored in medium lacking the microbes. While Figure 18 serves as a control showing an upper limit of what we might expect, in terms of sulfur content, Figure 19 serves as a control showing a lower limit (i.e., medium with thiosulfate). As expected, Figure 19 shows that the control samples stored in medium lacking microorganism have very little sulfur.

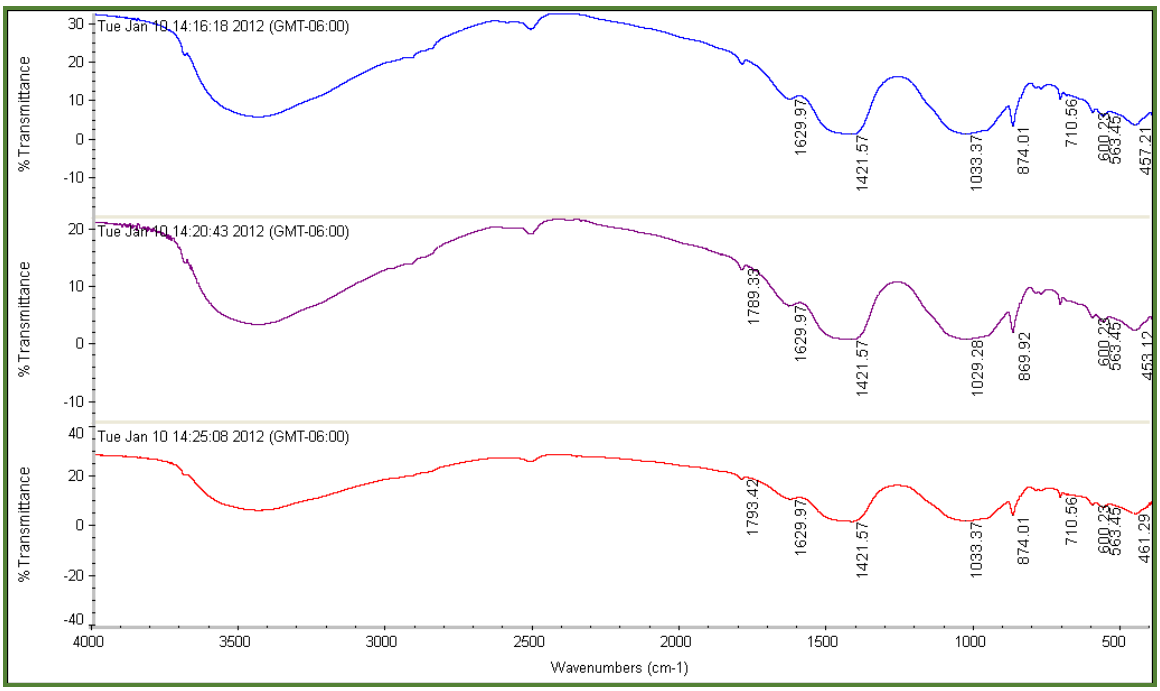


Figure 19: FTIR spectra of concrete cylinders stored in growth medium lacking microbes for 6 months.

There is some trouble with the signal to noise ratio for the concrete samples that were stored in the microbial culture for 6 months (data not shown). This may be due to the formation of a biofilm on the surface of concrete.

While not the main topic of Part 1, we have also used FTIR to analyze samples that were collected in the field. Figure 20 shows FTIR spectra for a concrete sample stored in 10 % concentrated sulfuric acid and samples from bridges on Bayou Creek, Madisonville, and State Highway 6. From these spectra, we see a clear indication (i.e., peak near 2511 cm^{-1}) that sulfur is present in the control sample and at the bridge sites for Madisonville and State Highway 6.

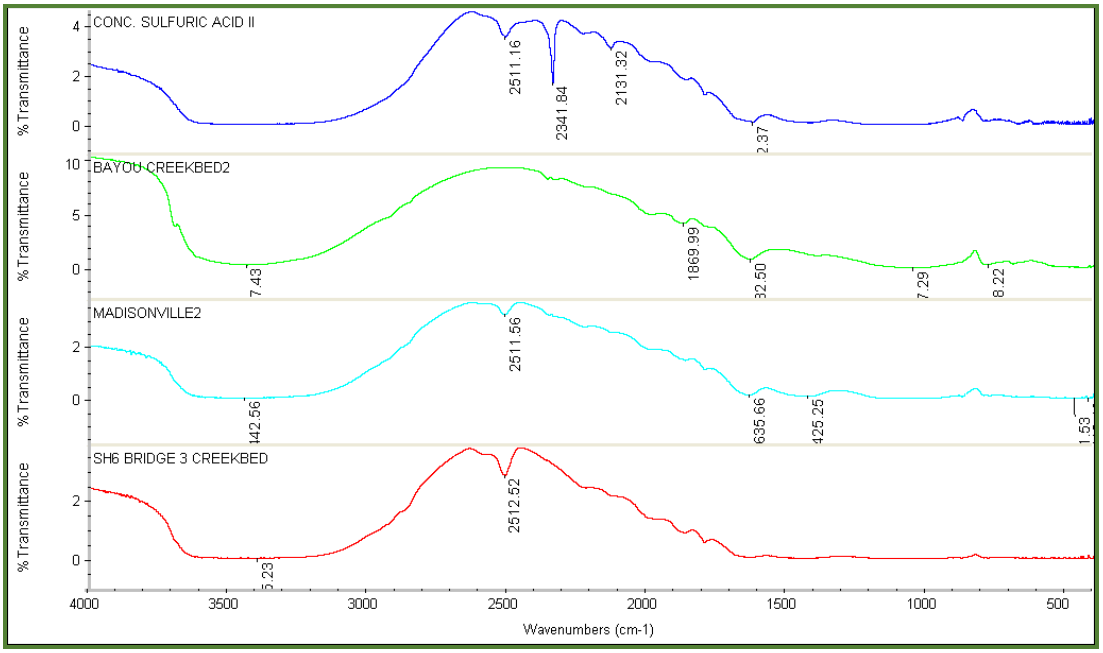


Figure 20: FTIR spectra overlay of the control sample and samples collected from bridges on Bayou Creek, Madisonville and State Highway.

1.3.6- Isolation and identification of acid-producing microbes

In order to isolate the microbe responsible for acid production, the mixed cultures were streaked on agar plates and then isolated colonies are transferred to agar plates containing chlorophenol red, a pH indicator dye. The acid producing microbes changed the color of the indicator from purple to yellow while the microbes not producing acid leave the

color unchanged (Figure 21). Using this method, seven acid-producing microbes were isolated and identified by sequencing the 16S rRNA gene. The BLASTN program was used to BLAST searched the sequences against the GenBank database and all seven isolates belonged to the genus Streptomyces with homologies of 98 % or greater. Among the seven Streptomyces isolates, *Streptomyces sp.* which showed 99 % similarity was used for further experiments The results of BLAST analyses are summarised in Table 3.

Table 3: Matching identities of Streptomyces species obtained from corroded concrete specimens.

Accession	Description	Max Identity
EF012136.1	Streptomyces sp. S6-212 16S ribosomal RNA gene, partial sequence	99 %
AB184583.1	Streptomyces thermocyaneomaculatus gene for 16S rRNA, partial sequence, strain: NBRC 14272	98 %
AB184582.1	Streptomyces thermocyaneoviolaceus gene for 16S rRNA, partial sequence, strain: NBRC 14271	98 %
AB184545.1	Streptomyces thermoviolaceus subsp. thermoviolaceus gene for 16S rRNA, partial sequence, strain: NBRC 13905	98 %
NR_027616.1	Streptomyces thermoviolaceus subsp. thermoviolaceus strain DSM 40443, 16S rRNA, partial sequence >emb|Z68096.1| S.thermoviolaceus 16S rRNA gene	98 %
AB184489.1	Streptomyces tosaensis gene for 16S rRNA, partial sequence, strain: NBRC 13798	98 %
AB184685.1	Streptomyces thermoviolaceus subsp. apingens gene for 16S rRNA, partial sequence, strain: NBRC 15459	98 %

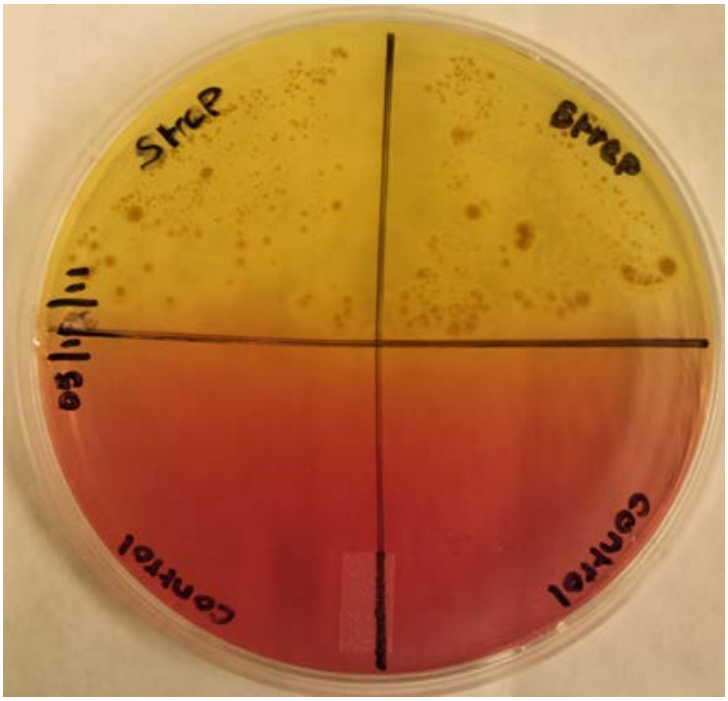


Figure 21: Phenol red pH indicator plate showing color change in the segments represented as 'Strep'. *Streptomyces sp.* that produce acid changed the color of agar plate with indicator dye from purple to yellow.

1.3.7- Oxidation of thiosulfate to sulfate by *Streptomyces sp.*

In order to verify the isolation of acid producers from mixed cultures and to identify the mechanism of acid production, the *Streptomyces sp.* strain was cultured in the growth medium with and without thiosulfate. The pH of these cultures was measured weekly over a time period of 14 weeks (Figure 22). As expected, *Streptomyces sp.* grown in medium with no thiosulfate failed to lower the pH while *Streptomyces sp.* grown in thiosulfate growth medium lower the pH (up to 3.5) to levels slightly below those observed with the mixed cultures. This observation confirms the isolation of acid producers from the initial mixed culture. In order to verify the oxidation of thiosulfate to sulfate, ion concentration in the cultures was measured (Figure 23). Consistent with the results presented in Figure 22, in the culture containing thiosulfate there is 96.8 % oxidation thiosulfate to sulfate and thus facilitate the production of sulfuric acid. The sulfate ion concentration remained low for the controls which lack *Streptomyces sp.*

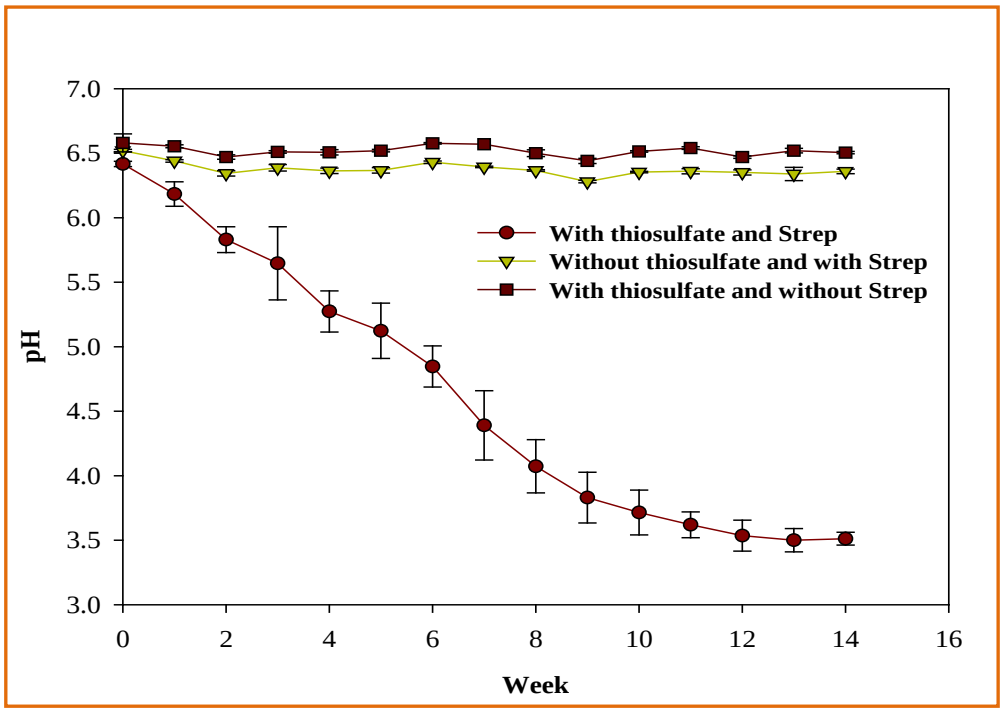


Figure 22: Acid production by *Streptomyces sp. Streptomyces* was cultured with and without thiosulfate in medium supplemented with 0.01 % yeast extract.

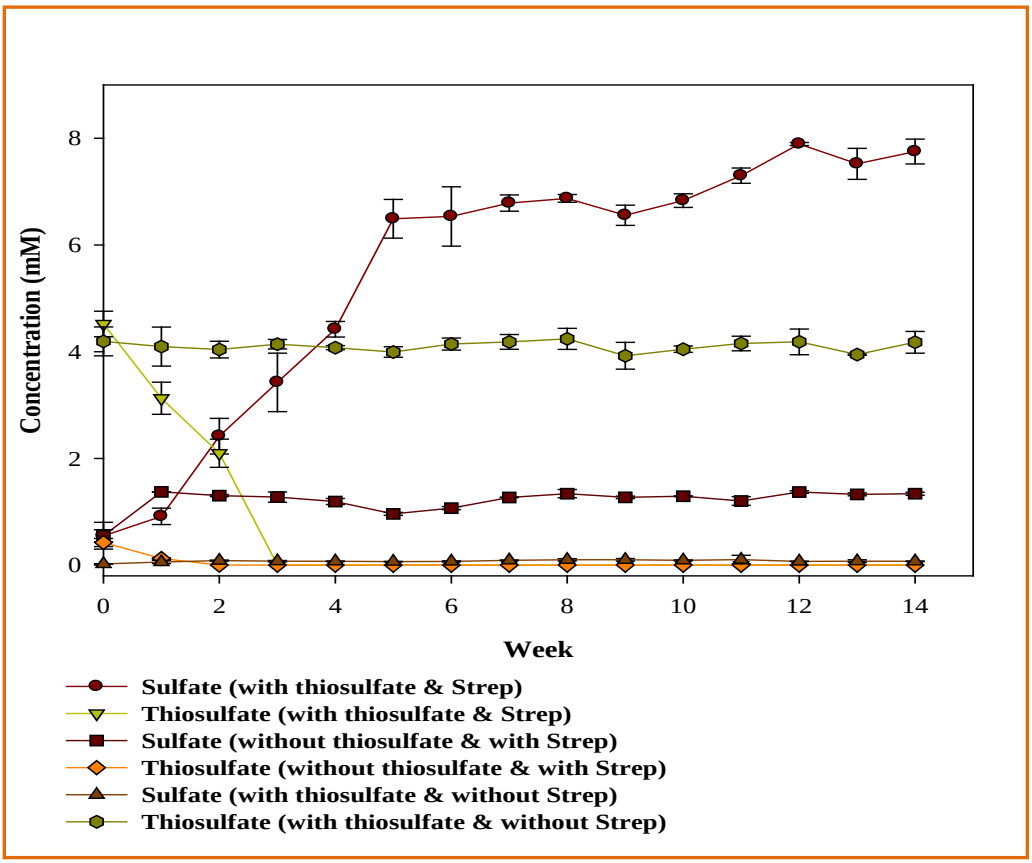


Figure 23: Oxidation of thiosulfate to sulfate by *Streptomyces sp.*

1.3.8- Biodegradation of concrete by *Streptomyces sp.* in semicontinous supply of thiosulfate

To assess the ability of the *Streptomyces* sp. to deteriorate concrete, it was inoculated in growth medium with concrete chips. The change in pH during the growth of microbe, was recorded on weekly basis (Figure 24). Measurement showed that pH dropped to 4.5 in the first week and increased back to pH 6.5 in bottles containing concrete. We believe this increase in pH was due to alkalinity of the concrete. As anticipated the pH increased from 6.5 to 7.5 in un-inoculated bottles containing concrete.

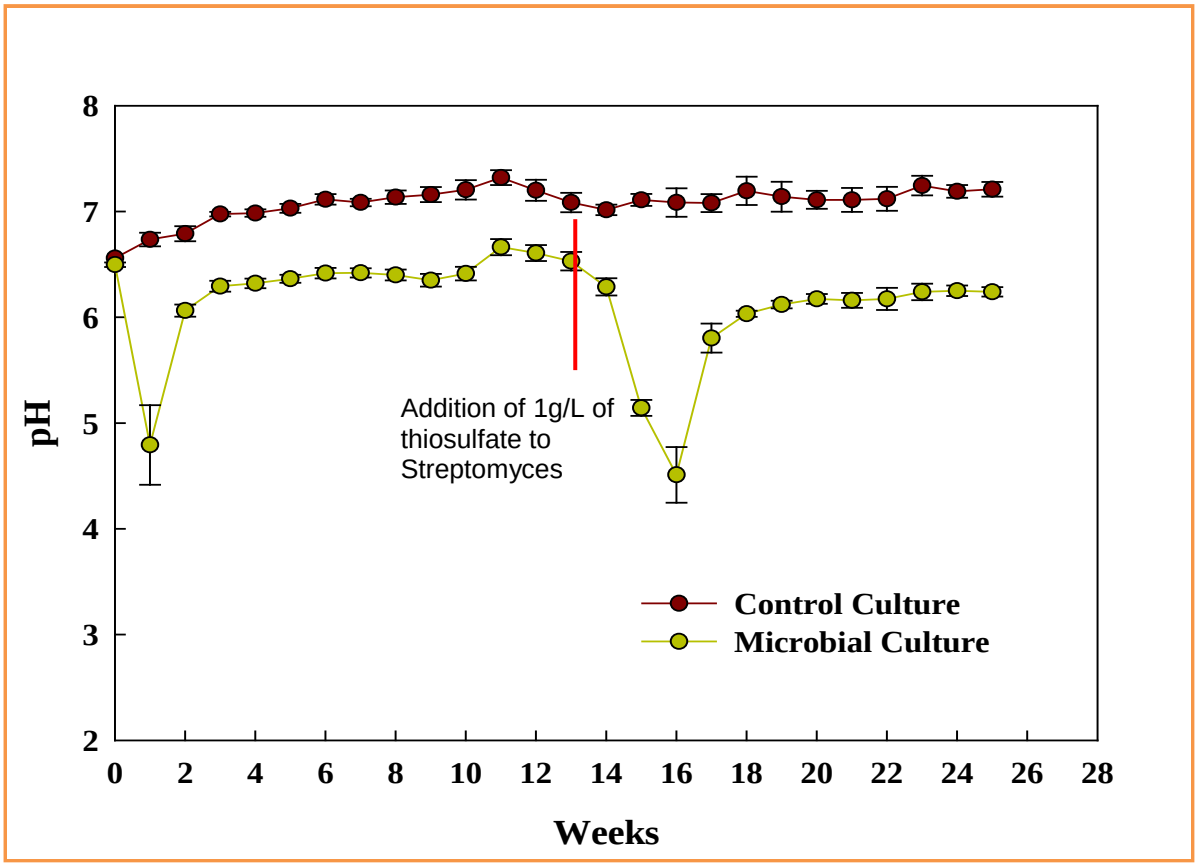


Figure 24: The pH changes of pure culture (*Streptomyces sp.*) over a time of 24 weeks. At 13th week the *Streptomyces sp.* culture was supplied with 1 g/L of thiosulfate, assuming supply of thiosulfate will produce more sulfate and further reduce the pH.

The ability of *Streptomyces sp.* to oxidize thiosulfate present in the medium to sulfate was studied. The accumulation of sulfate ions and hydrogen ions in the medium during oxidation results in the formation of sulfuric acid as indicated by this stoichiometric reaction ($S_2O_3^{-2} + H_2O + 2O_2 \rightarrow 2H^+ + 2SO_4^{2-}$). Complete oxidation of thiosulfate (4 mM) was observed within 3 weeks in cultures with a nd without concrete. According to the predicted mechanism, for every mole thiosulfate oxidized two moles of sulfate is produced. Although there was complete oxidation of thiosulfate with in 3 weeks, we observed only 31 % production of sulfate. This indicated the presence of intermediates in the reaction, which later oxidize to sulfate. There was 98.6 % oxidation of thiosulfate to sulfate within 9 weeks (Figure 25). At this point our working hypothesis was that maximum deterioration is observed when there is a maximal production of sulfuric acid as well as maximal exposure sulfuric acid. Thus, it is important that the amount of thiosulfate was not limiting. We therefore added 1 g/L of thiosulfate to culture containing microbes and concrete at 13th week, assuming supply of thiosulfate will produce more sulfate through oxidation and this sulfate further reduce the pH. As assumed addition of thiosulfate reduced the culture pH until 4.5 and then started increasing as there is nomore thiosulfate remained in the culture broth. Due to the addition of thiosulfate to culture containing microbes at 13th week, IC results indicated thiosulfate peak again at 13th week. Consistent with previous results there was a complete oxidation of thiosulfate within 3 weeks and 99 % oxidation of thiosulfate to sulfate was observed in 24 weeks.

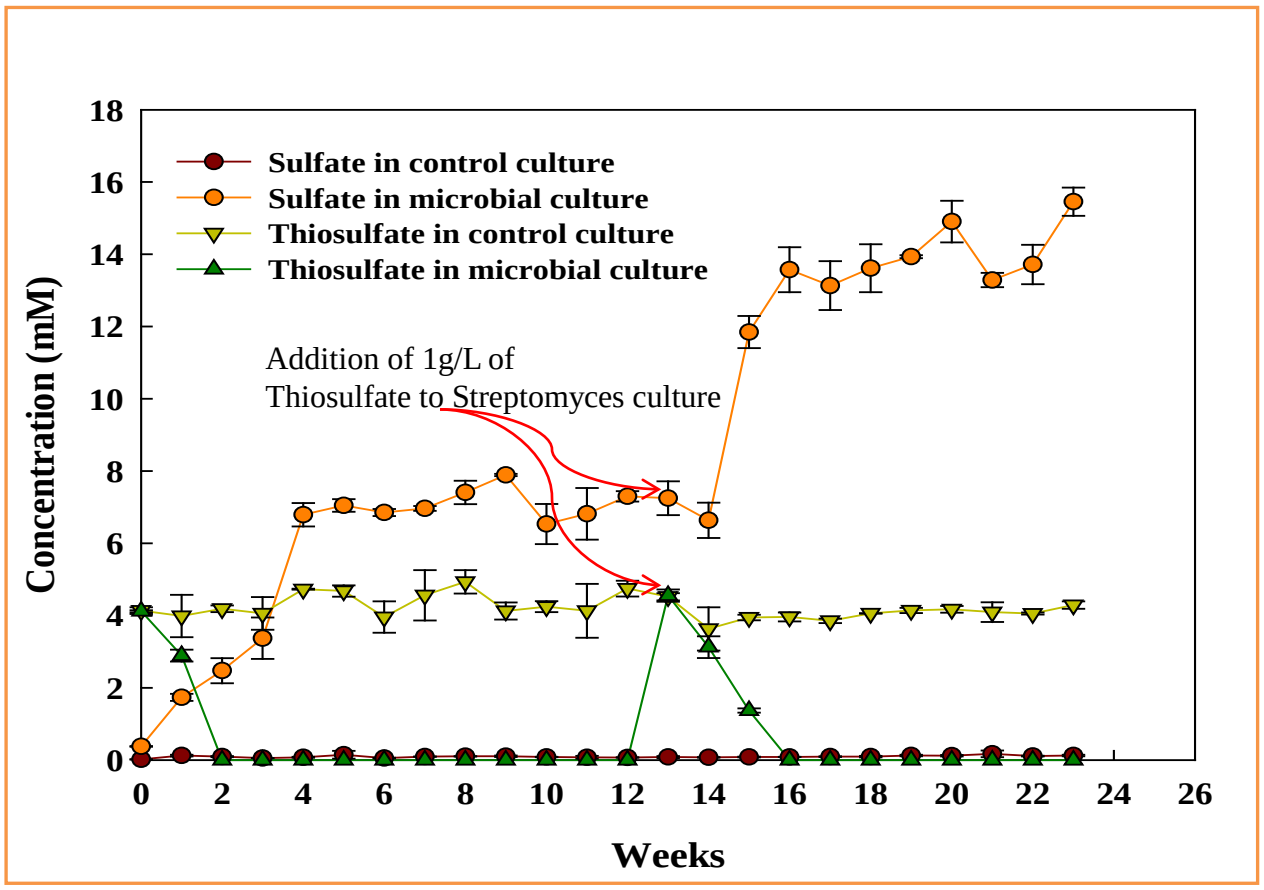


Figure 25: Oxidation of thiosulfate to sulfate by *Streptomyces sp.* in presence of concrete. At 13th week the *Streptomyces sp.* culture was supplied with 1 g/L of thiosulfate, assuming supply of thiosulfate will produce more sulfate thereby reduce pH.

Figure 26 shows the concentration of calcium leached from concrete over the duration of the study. The calcium ion concentration in control remains low, whereas the calcium ion concentration in culture containing microbes increased with time (Figure 26). The increase in soluble calcium is a result of the acid produced by the microbes attacking the concrete and solubilizing the calcium. These calcium readings are in good agreement with pH values. As pH increases, amount of calcium in medium slightly decreases. It may be due to precipitation of calcium. After addition of 1 g/L of thiosulfate to medium at 13th week, the pH dropped, sulfate ion concentration increased and correspondingly the amount of calcium ion concentration increased. The leaching rate of calcium was up to 6- fold higher than that of control.

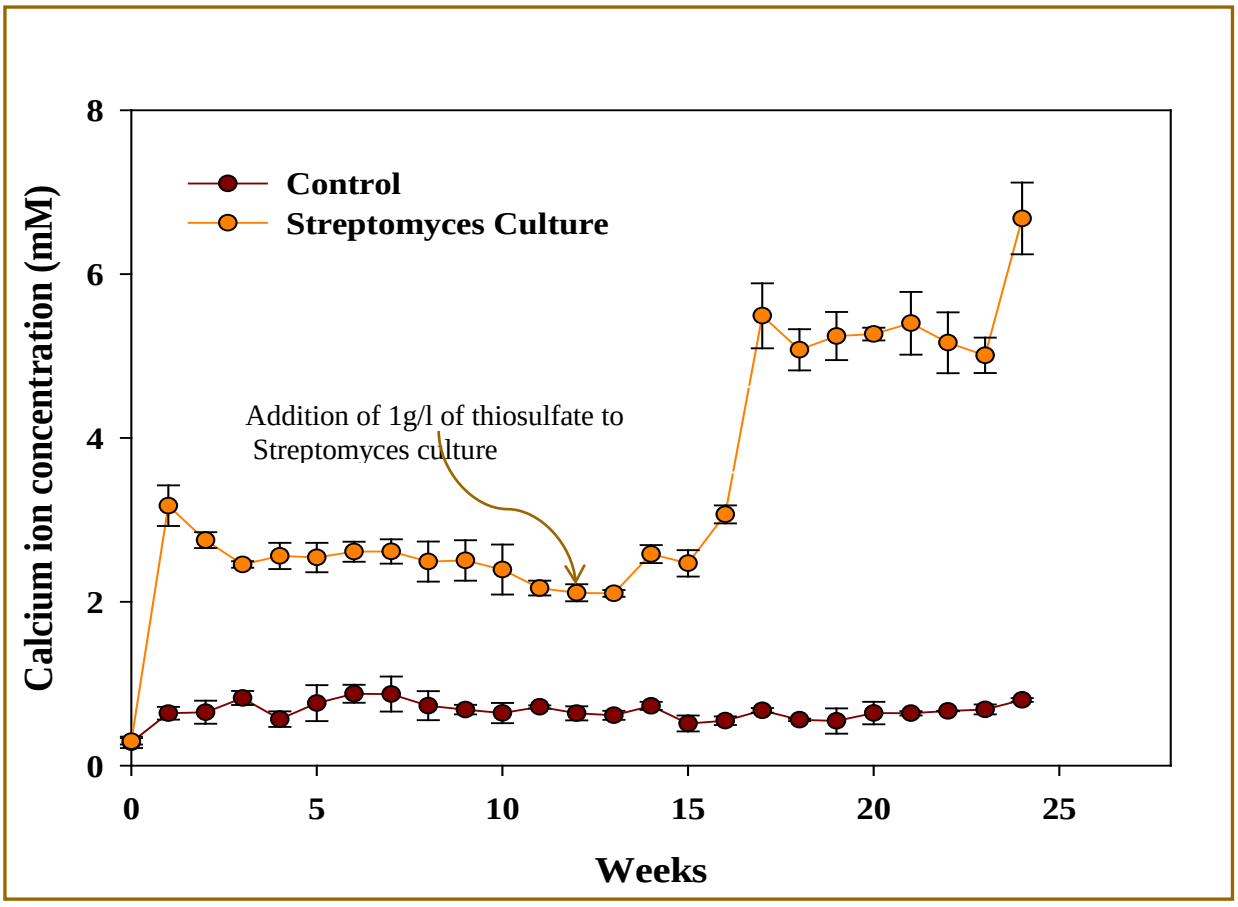


Figure 26: Leaching of calcium from concrete cylinders exposed to *Streptomyces sp.* At 13th week the *Streptomyces sp.* culture was supplied with 1 g/L of thiosulfate, assuming supply of thiosulfate will produce more sulfate which leaches out more calcium from concrete.

1.3.9- SEM images of concrete exposed to *Streptomyces* culture

SEM was performed on concrete exposed to *Streptomyces sp*. culture and to control culture. Consistent with the mixed culture results, deep cracks were observed on the cylinders exposed to *Streptomyces sp.* culture (Figure 27b), and that no cracks were observed on control cylinders (concrete cylinder in growth medium) (Figure 27a).

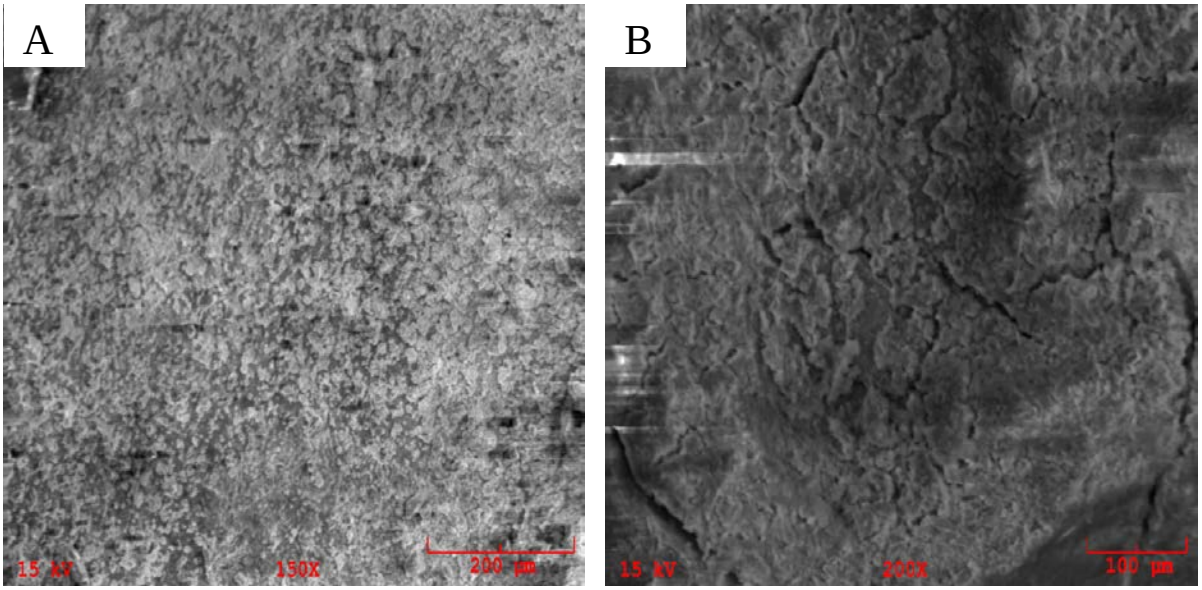


Figure 27: SEM Images of concrete exposed to control culture (A) and Streptomyces Culture (B) for 12 months.

1.4- DISCUSSION

Research in the area of bio-deterioration of concrete has revealed that microbes are frequently involved in concrete deterioration. Earlier studies demonstrated that concrete corrosion in sewer systems is caused mainly by microbial oxidation of various forms of sulfur into sulfuric acid, which reacts with the concrete and is known as microbially induced concrete corrosion (MICC) [50, 52, 61]. Our results showed that MICC also occurs on the surface of some concrete bridge structures.

Since many microorganisms are difficult to culture, the initial goal was to investigate if the acid-producing microorganisms from the bridge structures could be cultured and their growth and effect on pH measured. Low nutrient growth medium was used to grow the

samples, where most of the non-acid producing microorganisms present on the concrete surface were expected to not survive in the low nutrient growth medium while the acid-producing microorganisms were expected to grow in the low nutrient environment similar to that found at the bridge site [56, 61]. Enrichment of the acid-producing microbes in the low nutrient growth medium would enable the microbes to produce acid without interference from other microbes. The results confirmed production of acid, growth of acid-producing microbes and also indicated that some of these acid producing microorganisms are sulfur oxidizers (Figure 3, 4). These results are consistent with previous studies, in which a variety of acid-producing microorganisms have been enriched in a laboratory setting [52, 69, 70]. Further, the microcosm studies with TxDOT enrichment cultures grown in the presence of thiosulfate (no concrete) decreased the pH due to the oxidation of thiosulfate by the microbes. In contrast, the pH remained neutral when concrete was present due to the alkalinity of the concrete (Figure 6). These results agree with earlier findings that the alkalinity of concrete interferes with the pH of the culture [65].

Having confirmed that acid producing microorganisms were growing in the cultures, our aim was to i) determine the type and amount of acid produced, ii) quantify the amount of soluble calcium in the growth medium, and iii) visualize physical degradation of the concrete cylinders. Regarding the type of acid, in general the two main inorganic acids produced by microbes as an end product of their metabolism are sulfuric and nitric acid [62-64, 71]. The thiosulfate present in the growth medium acts an energy source and is oxidized to elemental sulfur, polythionates, and sulfate. The sulfate ion concentration in enriched cultures, with and without concrete, increased with time and 62 - 84 % of the thiosulfate was oxidized to sulfate (Figure 7 and Figure 8). Production of nitric acid in the enriched cultures was not observed (data not shown).

Acid produced by the microbes reacts with calcium hydroxide on the surface of the concrete and lowers the pH [52, 65]. As a result, calcium and silica, which are the main

constituents of concrete, start to leach out of the concrete. The leaching rates of calcium and silica ions are directly proportional to concrete bio-deterioration since they indicate chemical changes in the concrete that will eventually lead to surface erosion [65]. Consequently, the leaching rates of calcium and silica serve as a means of assessing concrete bio-deterioration. The results of our study (Figure 9), showed that in the presence of the acid-producing microbes the amount of calcium in solution (i.e., leached from the concrete) was 2-fold higher than the control. The high concentration of calcium in the microbial culture indicates the high dissolution rate of calcium hydroxide in sulfuric acid compared to calcium silicate.

Further evidence demonstrating the physical degradation of concrete was observed by stereomicroscope and SEM. Micrographs showed the formation of deep cracks on the concrete cylinders incubated in enriched cultures (Figure 10 and Figure 13a). Reaction of sulfuric acid with calcium hydroxide and calcium carbonate in the concrete produce expandable products such as gypsum and ettringite. These products increase the internal pressure of concrete thereby generating cracks. The transparent layer observed on the control cylinders was possibly due to the precipitation of calcium phosphate on the surface of cylinders (Figure 11, 12, 13b), which had no negative effect on the structural integrity of concrete as no cracks were observed on the control cylinder (Figure 11, 12, 13b). Some minor improvement in the resistance of the cement to acid because of precipitate filling the pores and preventing penetration of acid into the concrete cylinder cannot be ruled out. In case of concrete cylinders in the enriched culture, however, our results demonstrated that although there was some precipitation there was also significant bio-deterioration (Figure 11, 12, 13b).

The EDS and FTIR results of the concrete samples inoculated in enriched cultures did not show peaks corresponding to sulfur (Figure 17 and Figure 19), which was thought to be a result of the short-term exposure (6 months) of the concrete to enriched cultures.

To verify that sulfur was likely present in samples exposed to the microbes for an extended time, FTIR was performed on the deteriorated concrete samples collected during the field study. These samples from the Madisonville and State Highway 6 bridge sites have been undergoing biogenic degradation for presumably long periods of time. Madisonville and State Highway 6 bridge sites showed sulfur peaks comparable to control samples that were exposed to 10 % sulfuric acid (Figure 20).

Various microbes such as bacteria and fungi have been previously isolated from deteriorated concrete sewer systems, monuments, and buildings [16, 54]. Our enriched cultures contained various strains of microorganisms. Analysis of the pure culture, however, revealed that *Streptomyces sp.* was capable of significantly reducing the pH compared to other isolated strains. While *Streptomyces sp.* have not been commonly associated with biogenic acid attack of concrete, Actinobacteria, which belong to the genus *Streptomyces*, has been identified at deteriorated monuments, paintings and sewer pipes [53, 54, 72]. These strains were able to grow in low nutrient medium and can exist as arthrospores for extended periods [54]. This allows them to survive on the surface of concrete for longer periods.

Our results showed the ability of *Streptomyces sp.* to reduce the pH to 3.4 (Figure 22). From IC we identified the mechanism through which *Streptomyces sp.* is producing acid. Initially, 4 mM thiosulfate was added to the growth medium, and complete oxidation of the thiosulfate produced approximately 8 mM sulfate (Figure 23). These results are consistent with oxidation of thiosulfate ($S_2O_3^{2-} + H_2O + 2O_2 \rightarrow 2H^+ + 2SO_4^{2-}$), which produces two moles of sulfate for every mole of thiosulfate that is oxidized.

To test whether the deterioration is unique to the *Streptomyces sp.* or is due to a combination of microbes, the effect of *Streptomyces sp.* on laboratory prepared concrete was studied. Since the high alkalinity of concrete can affect the growth of SOB [65, 73] and was shown to affected the pH of the mixed cultures (Figure 3), the concrete was

neutralized before inoculation by storing it in a CO_2 incubator for 3 months. Although the concrete was neutralized and did not significantly increase the pH of the culture, the alkalinity of the concrete still buffered the pH (Figure 24). A sudden drop in pH after first week was due to rapid conversion of thiosulfate to sulfate by *Streptomyces*. We expected that when there is a continuous supply of thiosulfate, the oxidation of thiosulfate to sulfate was maximized thereby maximizing the bio-deterioration of concrete. To test how the microbes respond to a new supply of thiosulfate, 1 g/L of thiosulfate was added to the *Streptomyces* culture at week 13. The addition of the thiosulfate dropped the pH to 4.3 over the course of the next two weeks. The pH then returned to near neutral as the acid presumably reacted with the concrete, which acted as a buffer to the solution (Figure 24). This results are in good agreement with literature results showing that thiosulfate should not be rate limiting in order to observe maximum production of sulfuric acid as well as maximum deterioration [65].

The IC results showed that in the *Streptomyces sp.* culture there was approximately 97 % oxidation of thiosulfate to sulfate within the first week of the addition of thiosulfate (Figure 25). When thiosulfate was again added to the culture at week 13 the concentration of thiosulfate gradually dropped from 4 mM to zero, and there was a corresponding increase in sulfate ion concentration to 8 mM (Figure 25).

The amount of calcium that leached from concrete was measured, and the calcium ion concentration increased initially and then stabilized, consistent with the pH measurements (Figure 24). When the pH dropped, the calcium ion concentration increased (Figure 24 and Figure 26). The leaching rate of calcium in *Streptomyces sp.* culture was up to 6-fold higher than that of the control (Figure 26). Consistent with the mixed culture studies, deep cracks were observed in the concrete cylinders exposed to *Streptomyces sp.* culture (Figure 27B).

1.5- CONCLUSIONS

The results of our study strongly support the initial idea that the deterioration observed at several Texas bridge sites was related to acid-producing microorganisms. Deterioration appears to be caused by a *Streptomyces* strain that is capable of lowering pH to approximately 3.5. IC on both the mixed and isolated cultures indicated that the microorganisms are reducing the pH by converting natural sulfur sources into sulfuric acid. Through ICP we also observed that the acid produced by the microbes attacked the concrete and solubilized calcium resulting in degradation. Physical degradation of concrete exposed to the microorganism was also observed through stereomicroscopy and SEM.

PART 2: MICROBIAL DIVERSITY AND COMMUNITY STRUCTURE OF CONCRETE SURFACES FROM BRIDGE COLUMNS

2.1- INTRODUCTION

Microbially induced concrete corrosion (MICC) is a significant problem causing the deterioration and premature failure of major infrastructures such as wastewater treatment facilities, hazardous waste storage facilities, sewer systems, leaking underground storage tanks, oilfield equipment's, bridge systems, etc. In the U.S., costs associated with maintaining an estimated 800,000 miles of wastewater collection infrastructure alone are approximately $4.5 billion per year [74]. Failure to adequately address the deteriorating infrastructure networks threatens our environment, public health, and safety.

Although the mechanism and extent of microbially-induced corrosion are still not fully understood, sulfate-reducing (SRB) and sulfide oxidizing (SOB) microbes have been implicated in corrosion processes [75]. The primary source of sulfur is sulfate (SO_4^{2-}), which can be reduced by SRB to hydrogen sulfide (H_2S) under anaerobic conditions. H_2S is transferred across the air-water interface to the aerobic concrete surfaces where chemoautotrophic bacteria, including SOB, convert the hydrogen sulfide and other sulfur compounds such as thiosulfate ($S_2O_3^{2-}$) and elemental sulfur (S^o) to biogenic sulfuric acid (H_2SO_4), which corroded the concrete.

Therefore the knowledge of microbial diversity of corroded concrete surfaces and their functional capacity is essential for effectively predicting, preventing and restoring corrosion process. Molecular tools developed in the last decades to overcome the limitations imposed by traditional cultivation techniques have revealed that the microbial diversity in natural environment is much diverse than has been previously reported. Earlier

studies using culture-dependent methods to characterize microorganisms on the concrete surfaces have implicated *Thiobacillus* sp and *Acidithiobacillus* sp as the key protagonists [27, 56, 76-78]. However, since most microorganisms are difficult to grow on artificial media, culture-based approaches often provide an incomplete microbiological description of environmental matrices [79].

Recently, studies have used clone libraries of the bacterial 16S rRNA gene to characterize the diversity and composition of microbial communities associated with MICC in sewer systems [50, 75, 78, 80]. These studies indicate that while *Acidithiobacillus* sp. can represent major components of some MICC communities, but they are not ubiquitously dominant. The finding that organisms other than *Acidithiobacillus* sp are key community members may also suggest that processes other than those currently known contribute to concrete corrosion.

Although while some of the microbial processes associated with MICC of sewer systems have been documented, only a few studies have examined microbial composition of deteriorated concrete from bridge columns. The primary goal of the present study was to study microbial diversity of corroded concrete surfaces from several bridge columns and compare. In the present study, concrete samples were collected from various bridge sites in Texas were visible deteriorated. Molecular microbial diversity analyses was accomplished by isolating community DNA and constructing 16S rRNA-gene clone libraries.

2.2- MATERIALS AND METHODS

2.2.1- Sampling

Concrete samples were taken from several bridges in Texas. Samples were collected from the concrete surface by scraping the surface with a clean metal chisel and

transferring it to separate sterilized containers and stored on ice. Samples were taken from surfaces that exhibited slight to more severe surface deterioration as well as from nearby clean surfaces at each site as control.

2.2.2- PCR Amplification of rDNA and cloning

Community DNA was isolated from microbes present in concrete surfaces at three sites including Madisonville, Tarkington, and State highway bridges using several commercially available extraction kits. The DNA extracts were pooled and amplified with the universal 16S rRNA primers 515F-1391R. PCR was carried out at 95 °C for 10 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for 45 s, and 72 °C for 2 min, followed by a 72 °C elongation step for 8 min. Triplicate PCR products were pooled before purification using ExoSAP-IT and cloning. Individual PCR-amplified rRNA genes were isolated by cloning with Topo-TA according to the manufacturer's instructions (Invitrogen, USA) and collected into libraries of 96 randomly chosen clones. Inserts were directly PCR amplified and Sanger sequenced using M13F and M13R primers.

2.2.3- 16S rRNA gene sequence analyses

Sequences were base called with phred score of 20, trimmed and checked for reasonable length. All analyses were performed using Mothur (v. 1.28.0) (Schloss et al. 2009). Sequences with ambiguous bases or homopolymers of more than 9 were removed. The screened sequences were then aligned against the greengenes database (gg_10_12). Aligned sequences were screened based on start and end position. Further stringent quality control was performed by removing sequences that did not have a minimum length as that of 90 % of the other sequences, as well as by filtering out the gaps in the alignment. Sequences were assigned to specific bacterial groups using the greengene's taxonomic database (gg_99) with 97 % sequence identity as the cut off point for each Operational

Taxonomic Unit (OTU) using the Bayesian classifier provided by Mothur (McDonald, et al. 2012; Schloss et al. 2009). Diversity calculations and rarefaction curves for each of the samples were also generated by Mothur. Species richness and diversity estimation was performed by calculating Chao1 and the Shannon index respectively at 97 % OTU.

2.3- RESULTS

2.3.1- Composition of microbial communities from concrete surfaces

The composition of microbial communities in corroded concrete samples collected at three different bridge sites in Texas was determined by analysis of rRNA gene clone libraries. Approximately 634, 429, 430 clones from Madisonville creek, Tarkington bayou, and State highway bridge columns, respectively were retrieved. Analysis revealed the presence diverse organisms belonging to both domain bacteria and archaea. Among bacteria, members of the phyla Actinobacteria (14-29 %), alpha-proteobacteria (13-21 %), cyanobacteria (9-21 %), chloroflexi (5-19 %) and Acidobacter (4-11 %) dominated the community at all sites. Among archaea, a large number of Crenarchaeota comprising 20% and 11 % of clones were retrieved from Madisonville and Tarkington samples, respectively (Table 4). Archaea were not detected in concrete from the State highway bridge column.

Phylum	Madisonville	Tarkington	State highway
Acidobactera	27	48	20
Actinobacteria	184	107	64
Alphaproteobacteria	131	62	56
Armatimonadetes	0	1	0
Bacteroidetes	12	10	2
Betaproteobacteria	8	6	9
Chloroflexi	31	29	83
Crenarchaeota	125	47	0
Cyanobacter	57	80	90
Deinococci	1	2	5
Deltaproteobacteria	6	15	47
Dictyoglomi	1	0	0
Euryarchaeota	9	0	0
Firmicutes	2	5	15
Gammaproteobacteria	8	0	5
Gemmatimonadetes	9	2	5
Lentisphaerae	1	0	0
Nitrospirae	12	2	7
Planctomycetes	8	13	9
TM7	0	0	1
unclassified	0	0	6
Verrucomicrobia	1	0	2
WPS-2	0	0	2
WS3	0	0	1
Total	**633**	**429**	**429**

Table 4: Relative abundance of microbial phyla in concrete samples collected at Madisonville, Tarkington, and State highway. Taxonomy assignments were done using Greengenes 16S rRNA database.

2.3.2- Rarefaction and similarity analyses

Rarefaction analysis allows for the comparison of observed levels of richness among samples [81]. To understand species richness, rarefaction analysis or accumulation plot was performed at each test sites by plotting the number of phylotypes (groups of clones with _97 % identity) observed against the number of clones sequenced (Figure 28). A flattening rarefaction curve suggests that a sufficient number of clones had been sequenced to represent the diversity of the sample. In our study, the rarefaction plots for all

samples did not reach a clear saturation or plateau indicating highly diverse microbial population existed in the corroded concrete and additional sampling maybe needed to reveal all the species present at the site.

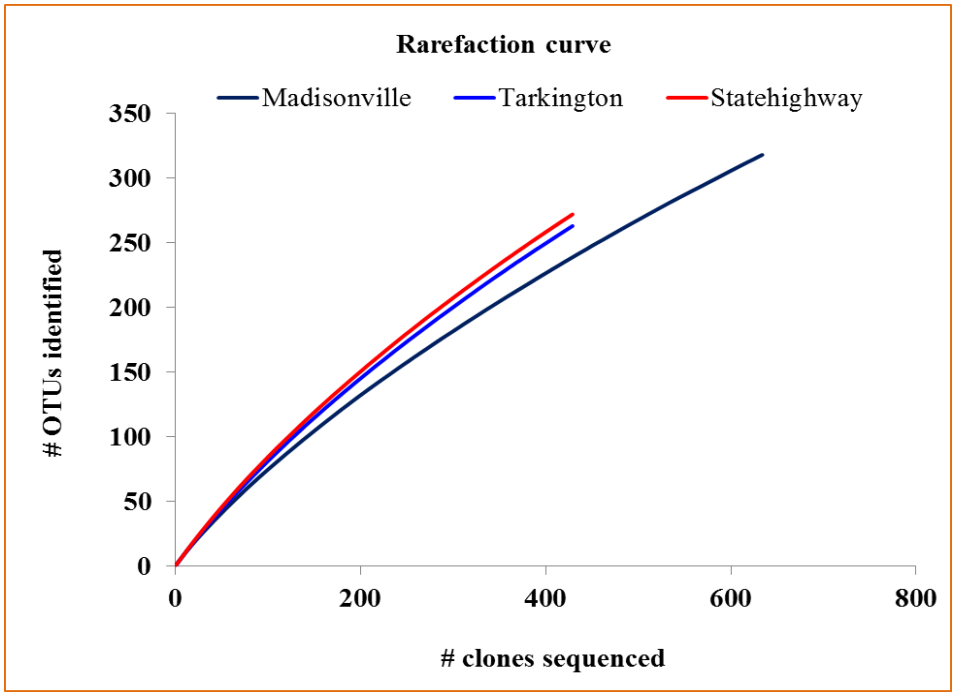
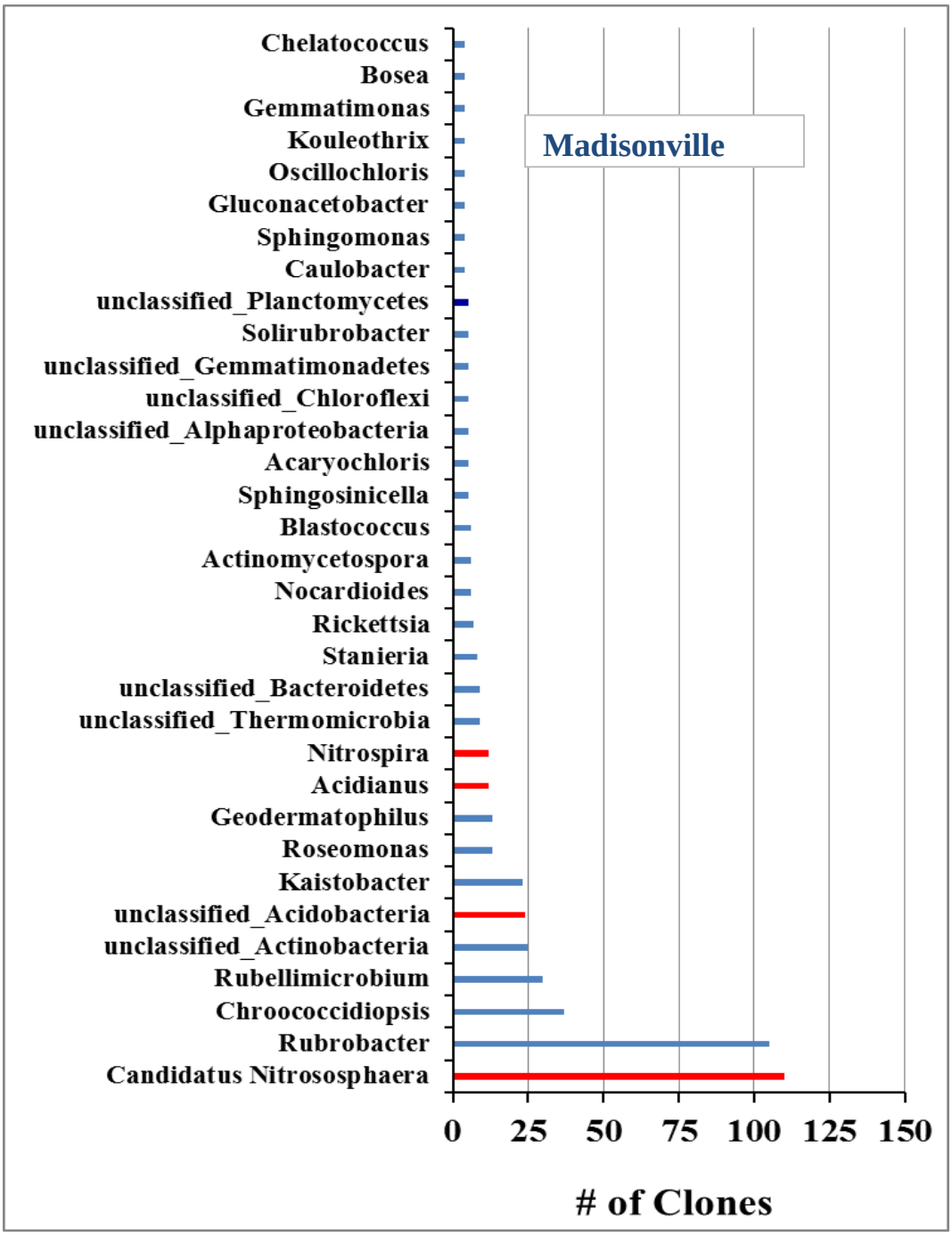
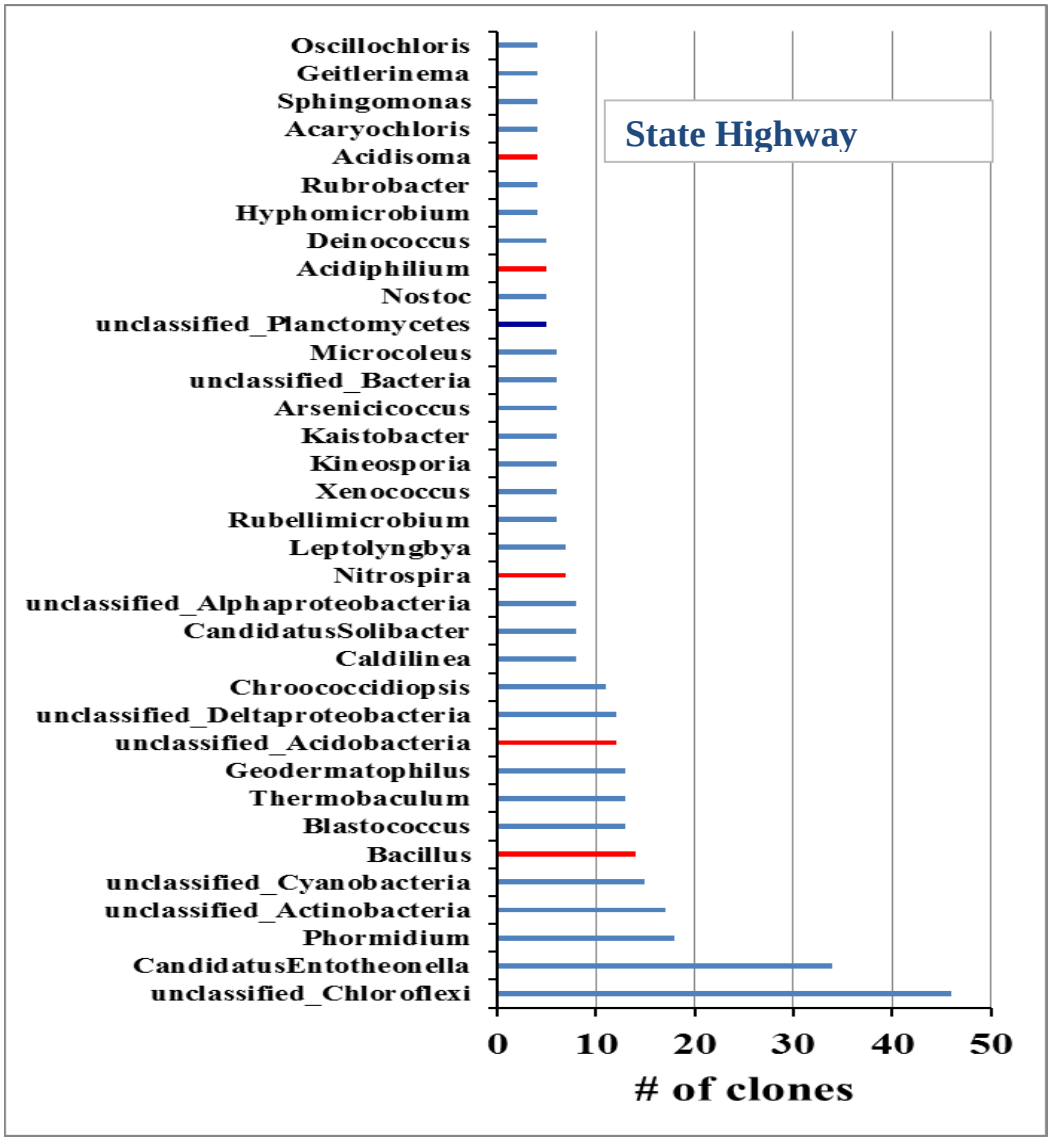


Figure 28: Rarefaction plots of microbial 16S rRNA gene sequences (observed at 97 % similarity) for each clone library.

Our clone library analysis at genus-level showed that hundreds of bacterial and archaeal species reside on surfaces and in cracks of concretes and many of these organisms have not previously been reported to be associated with concrete structures (Figure 29, 30, 31). Among the clones, only few putative acid producing organisms such as *Acidobacter* sp, *Acidisoma* sp, *Acidiphilium* sp and Nitrospira group were found to present at the three bridge sites at the time of analysis. This could be due to many factors including (i) low numbers of acid-producing organisms, and (ii) lack of nutrients and electron donors to support acid producing chemolithotrophs. The concrete corrosion is a highly dynamic process involving the interaction a variety of phototrophic, heterotrophic and chemolithotrophic organisms that undergo temporal succession driven by environmental conditions [50, 75, 77]. We believe the availability of reduced sulfur compounds such as

elemental sulfur, hydrogen sulfide and thiosulfate in the environment can play important role in the growth and acid production by sulfur oxidizing microorganisms. In order to test this hypothesis, we have setup laboratory-scale microcosms with concrete collected at Madisonville creek, Tarkington bayou, and State highway bridge sites. Microcosms were supplemented with thiosulfate as the electron donor (energy source) and no carbon source was provided in order to promote the growth of sulfur oxidizing chemolithotrophs. Microcosms were incubated at 30^{o}C in the dark and samples were withdrawn on weekly basis and monitored for pH, thiosulfate, and sulfate. Also, microbial diversity analysis was performed at the conclusion of the experiments to test if a shift in microbial community composition has occurred.


Madisonville
Chelatococcus
Bosea
Gemmatimonas
Kouleothrix
Oscillochloris
Gluconacetobacter
Sphingomonas
Caulobacter
unclassified_Planctomycetes
Solirubrobacter
unclassified_Gemmatimonadetes
unclassified_Chloroflexi
unclassified_Alphaproteobacteria
Acaryochloris
Sphingosinicella
Blastococcus
Actinomycetospora
Nocardioides
Rickettsia
Stanieria
unclassified_Bacteroidetes
unclassified_Thermomicrobia
Nitrospira
Acidianus
Geodermatophilus
Roseomonas
Kaistobacter
unclassified_Acidobacteria
unclassified_Actinobacteria
Rubellimicrobium
Chroococcidiopsis
Rubrobacter
Candidatus Nitrososphaera
0
25
50
75
100
125
150
# of Clones


State Highway
Oscillochloris
Geitlerinema
Sphingomonas
Acaryochloris
Acidisoma
Rubrobacter
Hyphomicrobium
Deinococcus
Acidiphilium
Nostoc
unclassified_Planctomycetes
Microcoleus
unclassified_Bacteria
Arsenicicoccus
Kaistobacter
Kineosporia
Xenococcus
Rubellimicrobium
Leptolyngbya
Nitrospira
unclassified_Alphaproteobacteria
CandidatusSolibacter
Caldilinea
Chroococcidiopsis
unclassified_Deltaproteobacteria
unclassified_Acidobacteria
Geodermatophilus
Thermobaculum
Blastococcus
Bacillus
unclassified_Cyanobacteria
unclassified_Actinobacteria
Phormidium
CandidatusEntotheonella
unclassified_Chloroflexi
0
10
20
30
40
50
# of clones

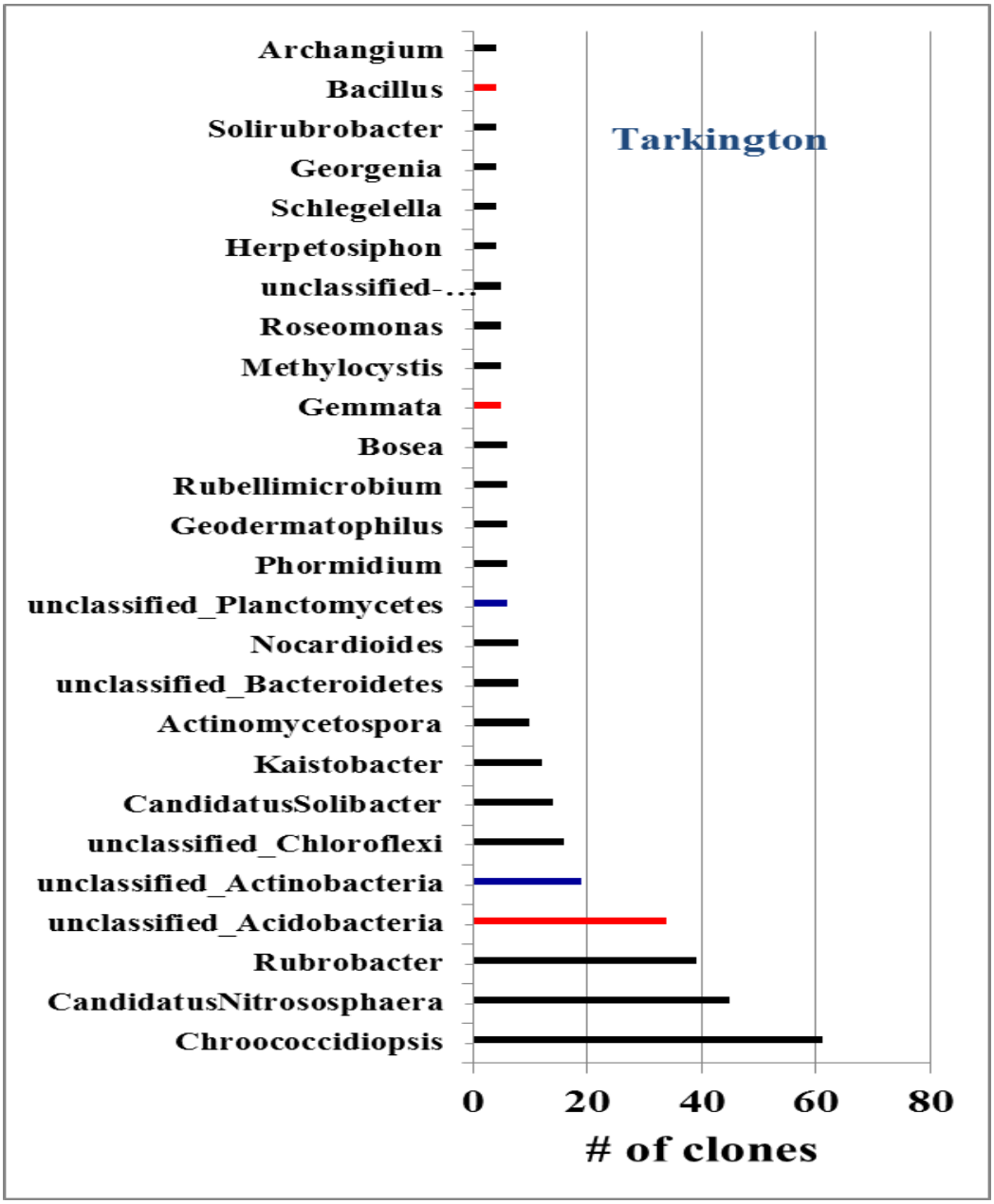


Figure 29: Abundance of microbial genera (97 % sequence similarity cutoff) determined by 16S rRNA-gene sequencing of clones generated from DNA isolated from concrete samples collected at the three sites. The organisms shown in red are putative acid producers.

Results (Figure 30) show a marked decrease in pH from 6.7 to 2.8 within 4 weeks and this decrease was coupled to the oxidation of thiosulfate to sulfate (sulfuric acid at low pH). These results indicate the presence of sulfur-oxidizing microorganisms in concrete samples taken from different bridges and are responsible for acid production. Microorganisms were able to oxidized 40 mM thiosulfate to roughly 75 mM sulfate within 5 weeks suggesting almost stoichiometric conversion (94 %) of the added energy source.

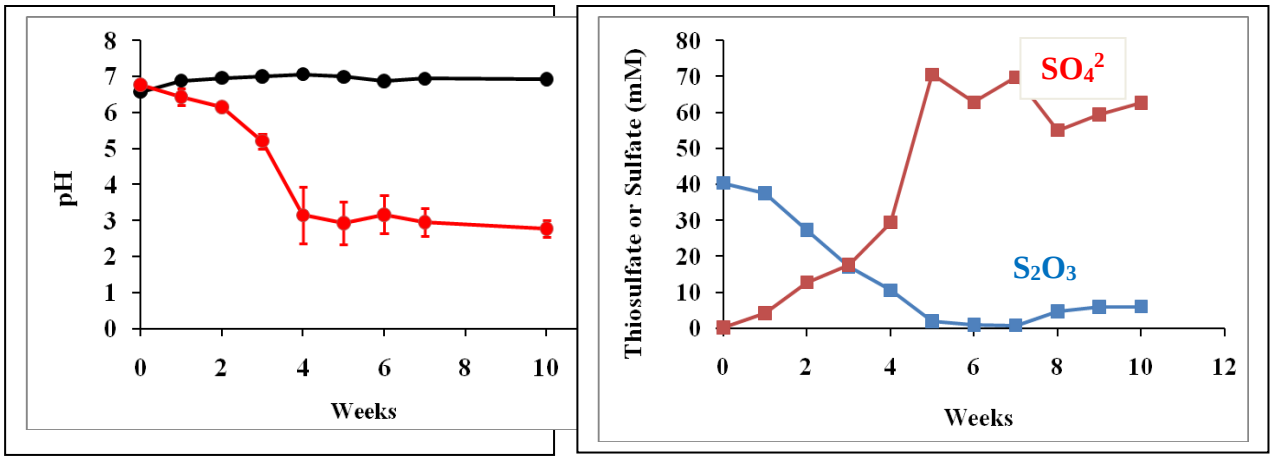


Figure 30: (A) Time dependent pH change in culture bottles containing concrete and thiosulfate. (B) Oxidation of thiosulfate to sulfate [$S_2O_3^{2-} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8e^- + 10H^+$] by the microbes present in the concrete.

2.3.3- Microbial community shift at low pH

Community DNA was isolated from cultures that had produced acidic pH due to the oxidation of thiosulfate (Figure 30). The 16S rRNA genes from the microbial community were amplified by PCR and clone libraries were developed as described before. Approximately 178, 161, 110 clones from Madisonville creek, Tarkington bayou, and State highway bridge columns, respectively were retrieved and diversity was analyzed at the genus level. Figure 31 shows microbial community structure in State highway microcosms at low pH. Analysis clearly indicates a shift in microbial community structure had occurred due to the oxidation of thiosulfate to sulfate (or sulfuric acid at low culture pH). Several acid producing bacteria including *Thiobacillus thioparus*, *Alicyclobacillus ferrooxydans*, *Alicyclobacillus pomorum*, *Alicyclobacillus acidocaldarius*, *Alicyclobacillus* sp. and *Bacillus* sp., formed the dominant members of the community at low pH (Figure 31) compared to microbial communities in the same concrete in the absence of the energy source, thiosulfate (Figure 29). At low pH roughly 70 % of the OTUs were comprised of acid producers. Similar observations were made with Madisonville and Tarkington microcosms (data not shown). These results clearly indicate complex microbial interactions and succession due to the availability of reduced sulfur compounds during concrete corrosion.

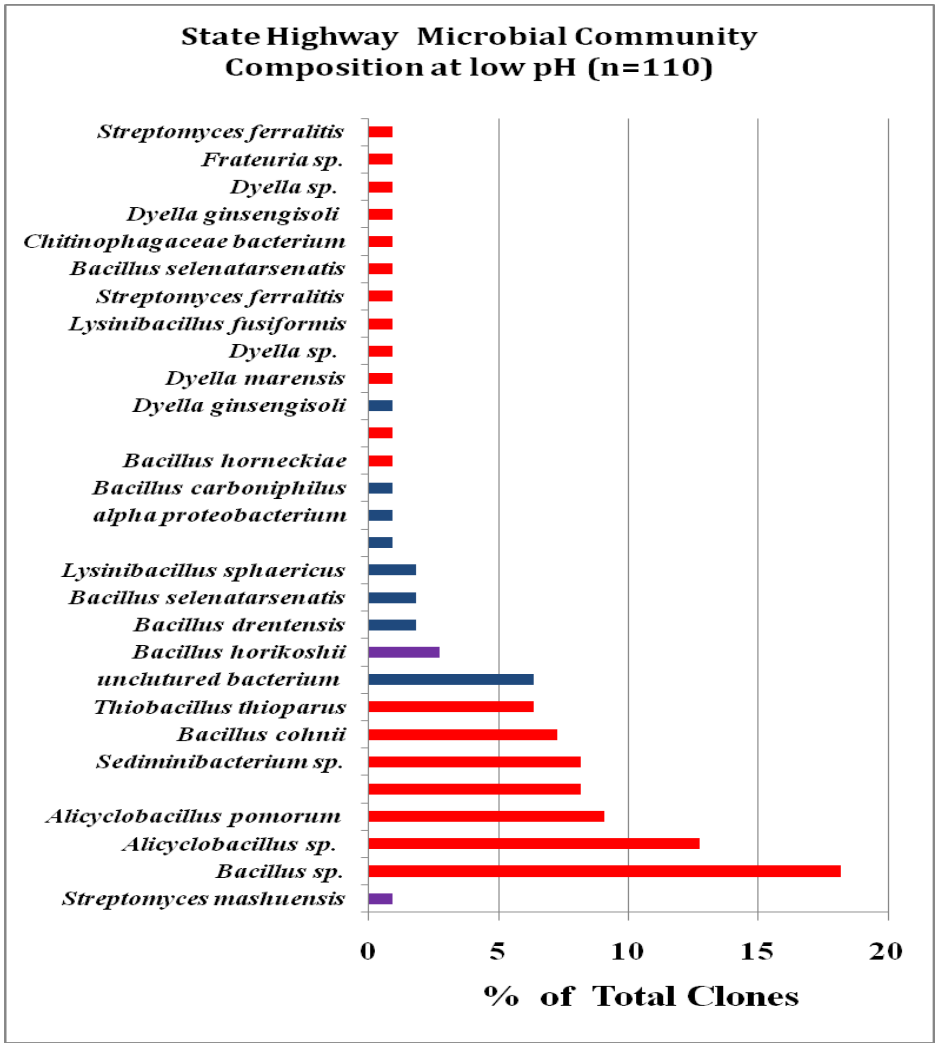


Figure 31: Abundance of OTUs (genus-level taxonomic classification) during active corrosion process caused by the oxidation of thiosulfate to sulfuric acid in microcosms containing concrete from State highway site. OTU classification was performed at ≥97.

2.4- DISCUSSION

Microbial induced corrosion of concrete is a significant global problem incurring losses in the order of billions of dollars per year. The microbial communities responsible for the deterioration of concrete structures are poorly understood because most of the previous studies were conducted with conventional culture-dependent techniques that could detect only a limited range of microorganisms. A better understanding of the microbial diversity and community structure of corroded concrete is needed to develop new approaches to mitigate microbial corrosion. Recent advances in molecular-based

approaches were shown to be very useful in detecting the presence of microbes that can't be cultivated using standard laboratory techniques. The objective of this study was to apply recent molecular tools to better characterize the microbial population associated with concrete and their dynamics during corrosion process.

Approximately, 400 to 600 16S rRNA-gene sequences representing microbial communities at three different bridge columns were analyzed. Highly diverse bacterial and archael community existed at all three sites. Among bacteria, actinobacteria, alpha-proteobacteria, cyanobacteria, chloroflexi and acidobacter dominated the community. Among archaea, a large number of crenarchaeota were present in concrete from Madsonville, Tarkington sites, and no archaea were detected at the Statehighway site. Despite the presence of highly diverse communities at all three sites, only a handful of organisms were known acid producers. These results suggest that a broad spectrum of microbial taxa exist on corroded concrete and many of these have not been previously reported to be associated with corrosion process. These observations are consistent with other studies performed using concrete from sewer pipes and wasterwater systems (Cayford, 2012; Li et al 2012; Santo Domingo, 2011).

In this study we also investigated the mechanism of concrete corrosion and succession of microbial community during active corrosion process. Microcosms prepared with concrete and thiosulfate showed the oxidation of thiosulfate to sulfuric acid and significant decrease in pH. Analysis of microbial diversity before and after the oxidation of thiosulfate showed that the entire microbial community structure shifted from a highly diverse to a highly rich community dominated by acid producers. These results clearly suggest that the corrosion is a highly dynamic and temporal process. Therefore, a comprehensive understanding of the microbial diversity and dynamics is important for prediction and prevention of microbial concrete corrosion on bridge columns and other infrastructures.

2.5- CONCLUSIONS

The present molecular microbial diversity study reveals that a variety of chemoautotrophic and heterotrophic microbial species resided on surfaces and in cracks of concretes. However, the microbial community structure is highly dependent on the available growth conditions. For example, the availability of sulfur compounds such as H_2S, $S_2O_3^{2-}$, and other reduced sulfur compounds leads to the increased growth of sulfur oxidizing acid producers forming the dominant population of the community. In our study, we found that during active corrosion process (at low pH) more than 60 % of the population was comprised of known sulfur oxidizing bacteria such as *Thiobacillus thioparas*, *Alicyclobacillus sp*. , *Alicyclobacillus acidocaldarius*, *Alicyclobacillus pomorum*, and *Bacillus* sp. These results are consistent with other studies dealing with corrosion of sewer pipes and wastewater treatment facilities suggesting that sulfur oxidizers are mainly responsible for concrete corrosion. This information is important for developing mitigation strategies.

REFERENCES

1. Nielsen, A.H., T. Hvitved-Jacobsen, and J. Vollertsen, *Kinetics and stoichiometry of sulfide oxidation by sewer biofilms.* Water Research, 2005. **39**(17): p. 4119-4125.

2. Parker, C.D., *Mechanisms of corrosion of concrete sewers by hydrogen sulfide.* Sewage and Industrial Wastes, 1951. **23**(12): p. 1477-1485.

3. Sand, W. and E. Bock, *Concrete corrosion in the Hamburg Sewer system.* Environmental Technology Letters, 1984. **5**(12): p. 517-528.

4. Satoshi Okabe, M. Odagiri, Tsukasa Ito, and H. Satoh, *Succession of Sulfur-Oxidizing Bacteria in the Microbial Community on Corroding Concrete in Sewer Systems.* Applied Environmental Microbiology, 2007 **73**(3): p. 971–980.

5. Bielefeldt, A., M. Gutierrez-Padilla, S. Ovtchinnikov, J. Silverstein, and M. Hernandez, *Bacterial Kinetics of Sulfur Oxidizing Bacteria and Their Biodeterioration Rates of Concrete Sewer Pipe Samples.* Journal of Environmental Engineering, 2009. **136**(7): p. 731-738.

6. Bock, E. and W. Sand, *The microbiology of masonry biodeterioration.* Journal of Applied Bacteriology 1993. **74**: p. 503-514.

7. Ciferri, O., *Microbial Degradation of Paintings.* Applied and Environmental Microbiology, 1999. **65**(3): p. 879-885.

8. Suihko, M., H. Alakomi, A. Gorbushina, I. Fortune, J. Marquardt, and M. Saarela, *Characterization of Aerobic Bacterial and Fungal Microbiota on Surfaces of Historic Scottish Monuments.* Systematic and Applied Microbiology, 2007. **30**: p. 494-508.

9. Warscheid, T. and J. Bramms, *Biodeterioration of Stone: a Review*. International Biodeterioration and Biodegradation, 2000. **46**: p. 343-368.

10. Parker, C.D., *The Corrosion of Concrete .2. The Function of Thiobacillus-Concretivorus (Nov-Spec) in the Corrosion of Concrete Exposed to Atmospheres Containing Hydrogen Sulphide.* Australian Journal of Experimental Biology and Medical Science, 1945. **23**(2): p. 91-98.

11. Sand, W., E. Bock, and D.C. White, *Biotest System for Rapid Evaluation of Concrete Resistance to Sulfur-Oxidizing Bacteria.* Materials Performance, 1987. **26**(3): p. 14-17.

12. Giannantonio, D.G., J.C. Kurth, K.E. Kurtis, and P.A. Sobecky, *Effects of concrete properties and nutrients on fungal colonization and fouling.* International Biodeterioration and Biodegradation, 2008. **30**: p. 1-8.

13. Trejo, D., P.d. Figueiredo, M. Sanchez, C. Gonzalez, S. Wei, and L. Li, *Analysis and Assessment Of Microbial Biofilm-Mediated Concrete Deterioration,* in *Texas Tansportation System, The Texas A and M University System*2008, Texas Tansportation System, The Texas A and M University System

14. Crispim, C.A. and C.C. Gaylarde, *Cyanobacteria and Biodeterioration of Cultural Heritage: A Review.* Microbial Ecology, 2005. **49**(1): p. 1-9.

15. Crispim, C.A., P.M. Gaylarde, and C.C. Gaylarde, *Algal and Cyanobacterial Biofilms on Calcareous Historic Buildings.* Current Microbiology, 2003. **46**(2): p. 0079-0082.

16. Gaylarde, C., M. Ribas Silva, and T. Warscheid, *Microbial impact on building materials: an overview.* Materials and Structures, 2003. **36**(5): p. 342-352.

17. Saiz-Jiminez, X.A. C., and J. Ortega-calvo, *Mechanisms of stone deterioration by photosynthesis-based epilithic biofilms.* European Commission Environment, 1995: p. 25-62.

18. Olmstead, W.M. and H. Hamlin, *Converting Portions of the Los Angles Outfall Sewer into Septic Tank.* Engineering News, 1900. **44**: p. 317.

19. Parker, C.D., *The Corrosion of Concrete 1. The Isolation of a Species of Bacterium Associated with the Corrosion of Concrete Exposed to Atmospheres Containing Hydrogen sulfide.* Australian Journal of Experimental Biology and Medical Sciences, 1945. **23**: p. 81-90.

20. Parker, C.D., *Mechanics of Corrosion of Concrete Sewers by Hydrogen Sulfide.* Sewage and Industrial Wastes, 1951. **23**(12): p. 1477-1485.

21. Parker, C.D. and J. Prisk, *The Oxidation of Inorganic Compounds of Sulphur by Various Sulphur Bacteria.* Journal of General Microbiology, 1953. **8**(3): p. 344-364.

22. Milde, K., W. Sand, W. Wolff, and E. Bock, *Thiobacilli of the Corroded Concrete Walls of the Hamburg Sewer System.* Journal of General Microbiology, 1983. **129**(May): p. 1327-1333.

23. Sand, W., *Microbial mechanisms of deterioration of inorganic substrates-A general mechanistic overview.* International Biodeterioration and Biodegradation 1997. **40**: p. 183-190.

24. Bock, E. and W. Sand, *The Microbiology of Masonry Biodeterioration.* Journal of Applied Bacteriology, 1993. **74**: p. 503-514.

25. Bastidas-Arteaga, E., M. Sánchez-Silva, A. Chateauneuf, and M.R. Silva, *Coupled reliability model of biodeterioration, chloride ingress and cracking for reinforced concrete structures.* Structural Safety, 2008. **30**(2): p. 110-129.

26. Diercks, M., W. Sand, and E. Bock, *Microbial corrosion of concrete.* Cellular and Molecular Life sciences, 1991. **47**: p. 514-516.

27. Parker, C.D., *Species of Sulphur Bacteria Associated with the Corrosion of Concrete.* Nature, 1947. **159**(4039): p. 439-440.

28. Sand, W. and E. Bock, *Biodeterioration of Mineral Materials by Microorganisms -*

Biogenic Sulfuric and Nitric-Acid Corrosion of Concrete and Natural Stone. Geomicrobiology Journal, 1991. **9**(2-3): p. 129-138.

29. Saiz-Jiminez, C., X. Arino, and J. Ortega-calvo, *Michanisms of Stone Deterioration by Photosynthesis-based Epithilic Biofilms.* European Commission Environment, 1995: p. 25-62.

30. Pinheiro, S.M.M. and S.R. M, *Alteration of the Concrete Microstructure Promoted by Biodeterioration Mechanisms.* Paper presented at the second international RILEM workshop on microbial impact on building materials, 2003.

31. Pinheiro, S.M.M. and M. Ribas Silva, *Alteration of the Concrete Microstructure Promoted by Biodeterioration Mechanisms.* . Paper presented at the second international RILEM workshop on microbial impact on building materials., 2003.

32. Videla, H.A., P.S. Guiament, and S. Gomez de Saravia, *Assessment of Microbiological and Atmospheric Effects on Rock Decay.* Corrosion 2003, 2003. **03175**: p. 1-13.

33. Pinheiro, S.M.M. and R.M. Silva, *Microorganisms and Aesthetic Biodeterioration of Concrete and Mortar.* Paper presented at he third international RILEM workshop on microbial impact on building materials, 2004.

34. de la Torre, M.A. and G.Gomez-Alarcon, *Manganese and iron oxidation by fungi isolated from building stone.* Microbial Ecology, 1994. **27**: p. 177-188.

35. Wakefeild, R.D. and M.S. Jones, *An introduction to Stone Colonizing Microorganisms and Biodeterioration of Building Stone.* Quarterly Journal of Engineering Geology, 1998. **31**: p. 301-313.

36. Bock, E. and W. Sand, *The Microbiology of Masonry Biodeterioration.* Journal of Applied Bacteriology, 1993. **74**(5): p. 503-514.

37. Gaylarde, C., R.M. Silva, and T. Warscheid, *Microbial Impact on Building Materials: an Overview.* Materials and Structures, 2003. **36**: p. 342-352.

38. Diercks, M., W. Sand, and E. Bock, *Microbial Corrosion of Concrete.* Cellular and Molecular Life Sciences, 1991. **47**: p. 514-516.

39. Jensen HS, Nielsen AH, Hvitved-Jacobsen T, and V. J., *Modeling of hydrogen sulfide oxidation in concrete corrosion products from sewer pipes.* Water Environmental Research, 2009. **81**(4): p. 365-373.

40. Berndt, M.L., *Evaluation of coatings, mortars and mix design for protection of concrete against sulphur oxidising bacteria.* Construction and Building Materials, 2011. **25**(10): p. 3893-3902.

41. De Muynck, W., N. De Belie, and W. Verstraete, *Effectiveness of admixtures, surface treatments and antimicrobial compounds against biogenic sulfuric acid corrosion of concrete.* Cement and Concrete Composites, 2009. **31**(3): p. 163-170.

42. Alum, A., A. Rashid, B. Mobasher, and M. Abbaszadegan, *Cement-based biocide*

coatings for controlling algal growth in water distribution canals. Cement and Concrete Composites, 2008. **30**(9): p. 839-847.

43. Giannantonio, D.J., J.C. Kurth, K.E. Kurtis, and P.A. Sobecky, *Effects of concrete properties and nutrients on fungal colonization and fouling.* International Biodeterioration & Biodegradation, 2009. **63**(3): p. 252-259.

44. Park, S.-K., J.-H.J. Kim, J.-W. Nam, H.D. Phan, and J.-K. Kim, *Development of anti-fungal mortar and concrete using Zeolite and Zeocarbon microcapsules.* Cement and Concrete Composites, 2009. **31**(7): p. 447-453.

45. Valentini, F., A. Diamanti, and G. Palleschi, *New bio-cleaning strategies on porous building materials affected by biodeterioration event.* Applied Surface Science, 2010. **256**(22): p. 6550-6563.

46. De Muynck, W., A.M. Ramirez, N. De Belie, and W. Verstraete, *Evaluation of strategies to prevent algal fouling on white architectural and cellular concrete.* International Biodeterioration & Biodegradation, 2009. **63**(6): p. 679-689.

47. Jensen, H.S., A.H. Nielsen, Hvitved-Jacobsen T, and J. Vollertsen, *Modeling of hydrogen sulfide oxidation in concrete corrosion products from sewer pipes.* Water Environ Research, 2009 **81**(4): p. 365-73.

48. Vollertsen, J., A.H. Nielsen, H.S. Jensen, T. Wium-Andersen, and T. Hvitved-Jacobsen, *Corrosion of concrete sewers—The kinetics of hydrogen sulfide oxidation.* Science of The Total Environment, 2008. **394**(1): p. 162-170.

49. Bielefeldt, A.R., M.G.D. Gutierrez-Padilla, S. Ovtchinnikov, J. Silverstein, and M. Hernandez, *Bacterial kinetics of sulfur oxidizing bacteria and their biodeterioration rates of concrete sewer pipe samples* Journal of Environmental Engineering, 2010. **136**(7): p. 731-738.

50. Okabe, S., O. Mitsunori, I. Tsukasa, and S. Hisashi, *Succession of sulfur-oxidizing bacteria in the microbial community on corroding concrete in sewer systems.* Applied Environmental Microbiology., 2007 **73**(3): p. 971–980.

51. Parker, C.D., *Mechanics of corrosion of concrete sewers by hydrogen sulfide.* Sewage and Industrial Wastes, 1951. **23**(12): p. 1477-1485

52. Wei, S., M. Sanchez, D. Trejo, and C. Gillis, *Microbial mediated deterioration of reinforced concrete structures.* International Biodeterioration and Biodegradation, 2010. **64**(8): p. 748-754.

53. Pepe, O., L. Sannino, S. Palomba, M. Anastasio, G. Blaiotta, F. Villani, and G. Moschetti, *Heterotrophic microorganisms in deteriorated medieval wall paintings in southern Italian churches.* Microbiological Research, 2010. **165**(1): p. 21-32.

54. Suihko, M.-L., H.-L. Alakomi, A. Gorbushina, I. Fortune, J. Marquardt, and M. Saarela, *Characterization of aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments.* Systematic and Applied Microbiology, 2007. **30**(6): p. 494-508.

55. Abdel-Kareem, O., *Fungal deterioration of historical textiles and approaches for their control in Egypt.* E-Preservations Science, 2010. **7**: p. 40-47.

56. Nica, D., J.L. Davis, L. Kirby, G. Zuo, and D.J. Roberts, *Isolation and characterization of microorganisms involved in the biodeterioration of concrete in sewers.* International Biodeterioration & Biodegradation, 2000. **46**(1): p. 61-68.

57. Lahav, O., A. Sagiv, and E. Friedler, *A different approach for predicting H2S(g) emission rates in gravity sewers.* Water Research, 2006. **40**(2): p. 259-266.

58. Robert L. Islander, A.M. Joseph S. Devinny, ASCE2, , Florian Mansfeld, Adam Postyn, and H. Shih, *Microbial ecology of crown corrosion in sewers* Journal of Environmental Engineering, 1991. **117**(6): p. 751-770.

59. Mori, T., T. Nonaka, K. Tazaki, M. Koga, Y. Hikosaka, and S. Noda, *Interactions of nutrients, moisture and pH on microbial corrosion of concrete sewer pipes.* Water Research, 1992. **26**(1): p. 29-37.

60. Tian, B. and M.D. Cohen, *Does gypsum formation during sulfate attack on concrete lead to expansion?* Cement and Concrete Research, 2000. **30**(1): p. 117-123.

61. Davis, J.L., D. Nica, K. Shields, and D.J. Roberts, *Analysis of concrete from corroded sewer pipe.* International Biodeterioration & Biodegradation, 1998. **42**(1): p. 75-84.

62. Parker, C.D. and J.Prisk, *The oxidation of inorganic compounds of sulphur by various sulphur bacteria.* Journal of General Microbiology, 1953. **8**(3): p. 344-364.

63. Sand, W. and E. Bock, *Biodeterioration of mineral materials by microorganisms—biogenic sulfuric and nitric acid corrosion of concrete and natural stone.* Geomicrobiology Journal, 1991. **9**(2-3): p. 129-138.

64. Sand, W., E.Bock, and D.C.White, *Biotest system for rapid evaluation of concrete resistance to sulfur oxidizing bacteria.* . Materials Performance 1987. **26**(3): p. 14-17.

65. Orli, A., Gabi Bar-Nes, Z. Yehuda, and S. Alex, *Accelerated biodegradation of cement by sulfur-oxidizing bacteria as a bioassay for evaluating immobilization of low-level radioactive waste.* Applied Environmental Microbiology., 2004 **70**(10): p. 6031-6036.

66. Steinhauer, E.S., C.R. Omelon, and P.C. Bennett, *Limestone corrosion by neutrophilic sulfur-oxidizing bacteria: A coupled microbe-mineral system.* Geomicrobiology Journal, 2010. **27**(8): p. 723-738.

67. Silva, R.M. and R.M. Pinho, *Applicationof Mineralogical Calculation to The Study of Concrete Deterioration by X-ray Diffraction and Scanning Electron Microscopy.* Proceedings of The Twentieth International Conference on Cement microscopy, 1998(175-185).

68. Hughes, T.L., C.M. Methven, T.G.J. Jones, S.E. Pelham, P. Fletcher, and C. Hall, *Determining cement composition by Fourier transform infrared spectroscopy.* Advanced Cement Based Materials, 1995. **2**(3): p. 91-104.

69. Parker, C.D., *The corrosion of concrete.1. The isolation of a species of bacterium associated with the corrosion of concrete exposed to atmospheres containing hydrogen sulfide.* Australian journal of Experimental Biology and Medical Science, 1945. **23**(2): p. 81-90.

70. Parker, C.D., *Species of sulphur bacteria associated with the corrosion of concrete.* Nature, 1947. **159**(4039): p. 439-440.

71. Sand, W. and E. Bock, *Biodeterioration of ceramic materials by biogenic acids.* International Biodeterioration & Biodegradation, 1991. **27**(2): p. 175-183.

72. Satoh, H., M. Odagiri, T. Ito, and S. Okabe, *Microbial community structures and in situ sulfate-reducing and sulfur-oxidizing activities in biofilms developed on mortar specimens in a corroded sewer system.* Water Research, 2009. **43**: p. 4729-4739.

73. Cwalina, B., *Biodeterioration of concrete.* Architecture Civil Engineering Environment, 2008. **4**: p. 133-140.

74. *USEPA (United States Environmental Protection Agency): State of Technology Review Report on Rehabilitation of Wastewater Collection and Water Distribution Systems. EPA/600/R-09/048. Cincinnati, OH:*
. Office of Research and Development, 2009.

75. Satoh H, Odagiri M, Ito T, and O. S., *Microbial community structures and in situ sulfate-reducing and sulfur-oxidizing activities in biofilms developed on mortar specimens in a corroded sewer system.* Water Res., 2009 **43**(18): p. 4729-4739.

76. Islander, R., J. Devinny, F. Mansfeld, A. Postyn, and H. Shih, *Microbial ecology of crown corrosion in sewers.* Journal of Environmental Engineering, 1991. **117**(6): p. 751-770.

77. Roberts, D.J., D. Nica, G. Zuo, and J.L. Davis, *Quantifying microbially induced deterioration of concrete: initial studies.* International Biodeterioration & Biodegradation, 2002. **49**(4): p. 227-234.

78. Vincke E, Boon N, and V. W., *Analysis of the microbial communities on corroded concrete sewer pipes—a case study.* Appl. Microbiol. Biotechnol. , 2001. **57**: p. 776 – 785.

79. Amann RI, Ludwig W, and S. K-H, *Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation.* Microbiol Rev 1995. **59**: p. 143–169.

80. Santo Domingo JW, Revetta RP, Iker B, Gomez-Alvarez V, Garcia J, Sullivan J, and W. J., *Molecular survey of concrete sewer biofilm microbial communities.* Biofouling. , 2011. **27**(9): p. 993-1001.

81. Hughes, J.B. and J.J. Hellmann, *The Application of Rarefaction Techniques to Molecular Inventories of Microbial Diversity*, in *Methods in Enzymology*, R.L. Jared, Editor. 2005, Academic Press. p. 292-308.