**Great Lakes Maritime Research Institute**

*A University of Wisconsin - Superior and University of Minnesota Duluth Consortium*

# Structure of Bacterial Communities Associated with Accelerated Corrosive Loss of Port Transportation Infrastructure

***Final Report***

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## Executive Summary

Steel sheet piling material used for docks, bridges and bulkheads in the Duluth-Superior harbor has been reported to be corroding at an accelerated rate. Corroded areas on steel sheet pilings in this harbor have an orange rusty appearance characterized by blister-like, raised tubercles on the surface. These tubercles vary in diameter from a few millimeters to several centimeters and when removed, large and often deep pits are revealed. This pattern of corrosion is consistent with the appearance of microbiologically influenced corrosion (MIC). Using a community DNA fingerprinting method called T-RFLP, we demonstrated that bacterial communities on corroded steel sheet pilings in the most affected part of this harbor were different from bacterial communities on these structures at a less affected area just outside the harbor. *Siderooxidans lithoautotrophicus*, a microaerophilic chemotrophic bacterium that oxidizes $Fe^{2+}$ to $Fe^{3+}$, was repeated isolated from the corroding structures. Sequencing the 16S rDNA gene of bacterial clones indicated that the majority of bacteria on the surfaces of steel pilings at the corroded sites examined were from three bacterial phyla, the β- and α-Proteobacteria, and Cyanobacteria. This clonal analysis also indicated the presence of a bacterium most similar to an iron-reducing bacterium (*Rhodoferax ferrireducens*), which can grow well at the low temperatures (4°C) seasonally encountered in this harbor. Although we cannot provide conclusive evidence that these iron bacteria are the causative agents of the accelerated corrosion in this harbor, our preliminary results indicate that the corroding steel structures are covered by complex microbial biofilms that contain bacteria of the type responsible for corrosion of steel in other environments.

## Introduction

Steel sheet piling material used for docks, bridges and bulkheads in the Duluth-Superior harbor (DSH) has been reported to be corroding at an accelerated rate (Marsh et al. 2005). To date accelerated corrosion of this character and speed has not been observed by dive inspectors or reported by authorities at other ports and harbors in the Great Lakes. The increased rate of corrosion appears to have begun in the late 1970's in the DSH and will require expensive replacement if the cause and possible remedies cannot be identified. About 20 kilometers of steel sheet piling appear to be affected in the DSH, which may cost more than $100 million to replace (Marsh et al. 2005). The US Army Corps of Engineers, the Duluth Seaway Port Authority, and the Minnesota and Wisconsin Sea Grant Programs convened an expert panel in September 2004 to examine this corrosion issue (see Marsh et al. 2005). This panel made several recommendations including further analysis to check for microbiologically influenced corrosion (MIC) in the harbor.

The steel sheet piling reported to be corroding at an accelerated rate has an orange rusty appearance characterized by blister-like, raised tubercles on the surface. These tubercles vary in diameter from a few millimeters to several centimeters and when removed, large and often deep pits (6 to10 mm) are revealed in the sheet piling, which is sometimes perforated. This pattern of corrosion is consistent with the appearance of corrosion caused by iron-oxidizing bacteria (Hamilton 1985). However, microbiologically influenced corrosion is rarely caused by a single microbial group, but more often by consortia of microbes including sulfate-reducing and iron-oxidizing bacteria (Hamilton 1985, Rao et al. 2000, Starosvetsky et al. 2001). The corrosion in this harbor appears similar to accelerated low water corrosion (ALWC) reported during the past decade on marine steel pilings in the United Kingdom and Baltic Sea (Christie 2001, Graff and Seifert 2005), which is thought to be accelerated by the action of sulfate-reducing bacteria.

The Lake Superior corrosion problem is primarily seen in the lower part of the Duluth-Superior harbor (Marsh et al. 2005). Most of the corrosion is confined to the first 1.5 meters below the surface and decreases from 1.5 to 3 meters below the surface. Extensive zebra mussel colonization occurs on these pilings from about 3 meters to the bottom of the sheet pile where little or no corrosion is observed (Chad Scott, AMI Consulting Engineers, pers. comm.). Less corrosion has been found on steel sheet piling at the Oliver Bridge in the upper harbor near the mouth of the St. Louis River and on pilings exposed to Lake Superior water (Chad Scott, pers. comm.). Thus, there appears to be a dual gradient of corrosion from the upper harbor on one side and Lake Superior on the other side towards the middle of the DSH where the most extensive corrosion is seen. We examined microbial communities attached to sheet pilings at two corroded areas and one less affected site to determine if differences in microbial communities between these sites may indicate the participation of bacteria in this accelerated corrosion process.

The specific objectives of this project were to:

1. Sample microbial biofilm communities on steel pilings at two or more sites severely affected by corrosion in the Duluth-Superior harbor and at least one site where corrosion is minimal.
2. Extract DNA from these microbial biofilm communities

3. Compare the molecular diversity of bacterial biofilm communities on corroded and unaffected steel sheet pilings using a community DNA fingerprinting method (T-RFLP) to identify differences in biofilm communities at corroded and less affected sites.

## Methods

Samples were collected from the surfaces of steel pilings in the Duluth-Superior harbor in August and September 2006, and again in July and August 2007. Samples collected in 2007 are still being analyzed and information in this report is derived from the analysis of samples collected in 2006. Microbial community biofilm samples were collected from corroded structures (Fig. 1) by a commercial diver approximately 1 m below the waterline at Hallett Dock 5 (Aug. 1 and Sept. 14), Midwest Energy (Aug. 15), and the Duluth Entry (Sept. 15) during 2006 using a syringe brush sampler (Ksoll et al. 2007). Portions of these samples were examined using epifluorescence microscopy and DAPI staining (Porter and Feig, 1980), used to inoculate gradient tubes to isolate iron-oxidizing bacteria, and to extract DNA for bacterial community fingerprinting and cloning analyses.

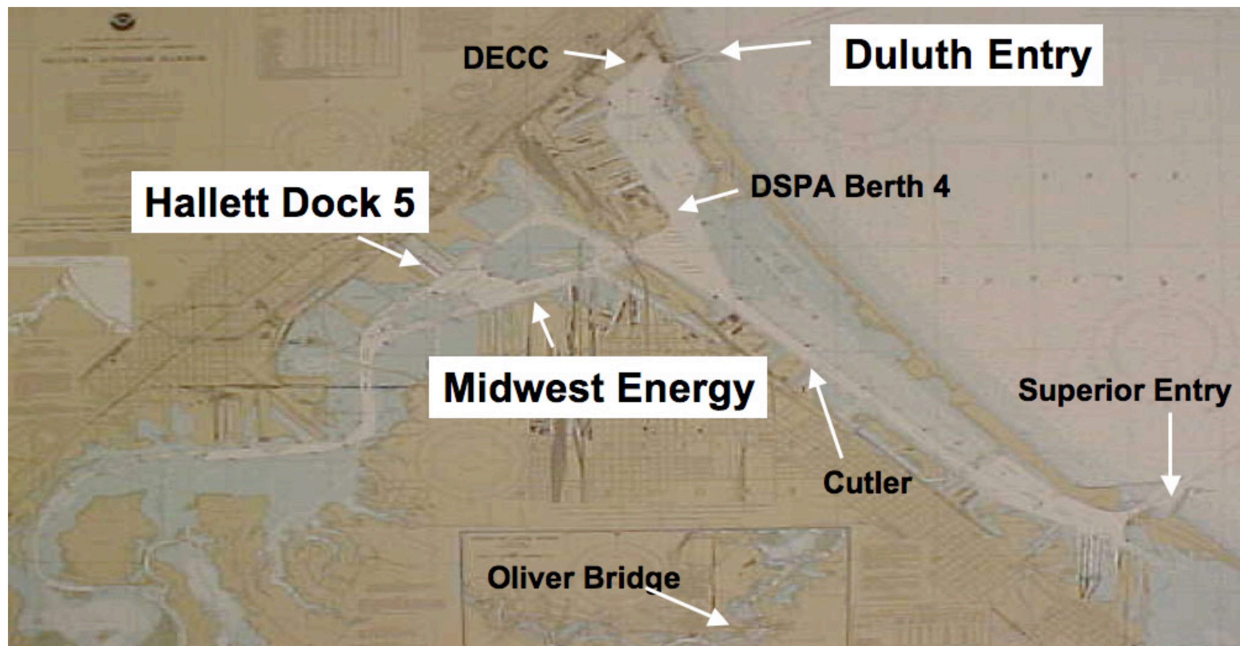


Fig. 1. Map of the Duluth-Superior harbor. Sites that were sampled during summer in 2006 and 2007 are shown in white boxes.

*Enrichment and isolation of iron-oxidizing bacteria –*
Iron-oxidizing bacteria were enriched and isolated using a gradient tube culturing technique (Emerson and Moyer 1997). Enrichments were carried out in opposing gradients of oxygen and $Fe^{2+}$ that were established in Hungate tubes. The tubes had a plug of either FeS or $FeCO_3$ overlaid with modified Wolfe's mineral medium (MWMM), a semisolid mineral salts bicarbonate-buffered medium and a headspace of air. The tubes were inoculated with 10 µl of suspension collected

with the syringe sampler by removing the top of a gradient tube, inserting a pipette tip containing the inoculum to the bottom agarose plug, and expelling the contents as the pipette tip was withdrawn from the gradient tube. After 14 to 21 days of growth, sterile pipette tips were used to remove samples from individual bands that had formed in the gradient tubes. These band samples were placed in different sterile microcentrifuge tubes. A portion of some samples was preserved in 2% formaldehyde and examined by epifluorescence microscopy after the cells had been stained with DAPI (Porter and Feig, 1980). DNA was extracted from another portion of each sample using a MoBio UltraClean Soil DNA Kit following the instructions of the manufacturer. The 16S rDNA gene was amplified using bacteria-specific PCR primers (27F, 1492R) using a BioRad DNA Engine thermal cycler (see conditions below). These 16S rDNA fragments were sequenced on an ABI DNA sequencer at the University of Minnesota BioMedical Genomics Center and the partial sequences compared to sequences of known bacteria in the GenBank public database using the BLASTn tool.

*Microbial community DNA extraction –*
Total DNA extracted directly from microbial biofilm samples was used for 16S rDNA-based T-RFLP analysis, and cloning and sequencing the 16S rRNA gene to identify bacterial members of these microbial communities. Total DNA was extracted using an UltraClean Soil DNA Kit (MoBio Laboratories, Inc.) following the instructions of the manufacturer. Nucleic acid concentrations and purity were determined spectrophotometrically by measuring absorbance with a Nanodrop spectrophotometer.

*T-RFLP community DNA fingerprinting –*
Terminal restriction fragment length polymorphism (T-RFLP) analysis, a community DNA fingerprinting technique, was used to distinguish bacterial communities collected from different sites based on the size of terminal fragments of 16S rRNA genes following restriction endonuclease digestion (Braker et al. 2001, Moeseneder et al. 1999). We used a PCR primer set (27F, 1492R) specific for the Bacteria domain (Lane 1991, Reysenbach et al. 1994) to directly amplify the 16S rDNA gene in DNA extracted from microbial biofilms on corroded surfaces. The forward primer was labeled on the 5' end with 6-carboxyfluorescein (6-FAM). All oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT). DNA samples were amplified using puReTaq Ready-To-Go PCR beads and 25 pmol of each primer with a BioRad DNA Engine thermal cycler. The reaction conditions were 35 cycles of: 94°C for 1.5 min, 60°C for 1.5 min, and 72 °C for 2 min. After 35 cycles, the samples were cooled to 4'C until further analysis. DNA from *E. coli* was used as a positive bacterial control. In addition to blanks (containing no DNA template), DNA from *Sulfolobus solfataricus* (ATCC 35091), an archaeal microorganism, was used as a negative control. PCR reaction mixtures containing bacterial rDNA products of the expected size (~ 1,500 base pairs, visualized on 1.5% agarose gels) were cleaned with a MoBio UltraClean PCR Clean-Up Kit. Cleaned PCR products (200 ng) were then digested at 37°C for at least 3 h with *Hae*III or *Rsa*I. The restriction fragments were precipitated, dried, and redissolved in 10 µl of sterile, nuclease free water. The sizes of the terminal restriction fragments in these digests were determined on an ABI DNA sequencer in the GeneScan mode at the University of Minnesota BioMedical Genomics Center. The pattern of terminal restriction fragments from each sample was imported into the BioNumerics statistical software package (Anonymous 2005). The molecular weights (in base pairs) of peaks in individual samples were normalized to internal molecular weight standards (ROX-1000 standard). Afterwards,

dendrograms comparing differences in the bacterial biofilm communities at the different sites in the harbor were constructed using Pearson correlations and the UPGMA clustering method. Similarities between different samples were calculated based on Pearson correlations of terminal restriction fragment patterns between 47 and 946 base pairs in size.

*Bacterial clones –*
A small bacterial clone library (fifty-seven partial 16S rDNA sequences) was created to identify the major bacterial groups associated with corroded steel piling materials in the Duluth-Superior harbor. Bacterial 16S rDNA was amplified in four microbial community samples from the Hallett Dock 5 site (collected on Aug 1 and Sept 14) and two samples from the Midwest Energy site (collected on Aug 15). We used the same bacterial PCR primers, materials, and PCR conditions as described above except the forward PCR primer was not labeled with 6-FAM. PCR reaction mixtures containing bacterial rDNA products of the expected size (~ 1,500 base pairs) were chosen for cloning using an Invitrogen TOPO-TA cloning kit (Keough et al. 2003, Jones et al. 2007). Twenty randomly selected transformant colonies appearing on selective media from each sample (except the Aug 1 sample from Hallett Dock 5) were isolated and screened by whole cell PCR. All colonies (n=110) were grown overnight in LB broth amended with 75 µg/ml ampicillin at room temperature. Cells from these cultures were frozen at -80ºC in glycerol stocks. Cloned 16S rDNA fragments from all the transformant cell lines were amplified using the primers M13F and M13R (included in the cloning kit), and then purified and concentrated using a MoBio UltraClean PCR Clean-Up Kit prior to sequencing. Inserts from clones were sequenced at the University of Minnesota BioMedical Genomics Center. These inserts were partially sequenced with the 27F primer to verify that the amplicons were 16S rDNA-like sequences (57 of 110 sequences). The partial 16S rDNA sequences of the clones were compared to 16S rDNA sequences in the public database GenBank using the BLASTn tool (www.ncbi.nlm.gov/BLAST/) to identify the closest bacterial relative.

## Results and Discussion

Scrapping samples from the corroded steel surface at Hallett Dock 5 demonstrated that complex microbial biofilms composed of filamentous algae, diatoms, and various bacteria cover the steel surfaces and corrosion tubercles (Fig. 2). Iron-oxidizing bacteria were cultivated from materials collected from corroding steel pilings at Hallett Dock 5 and Midwest Energy (Fig. 3A) but not the Duluth Entry site. 16S rDNA from some bacteria isolated from both sites was most similar (96%) to 16S rDNA from *Siderooxidans lithoautotrophicus*, a microaerophilic γ-proteobacterium that oxidizes $Fe^{2+}$ to $Fe^{3+}$ (Fig. 3B and C).

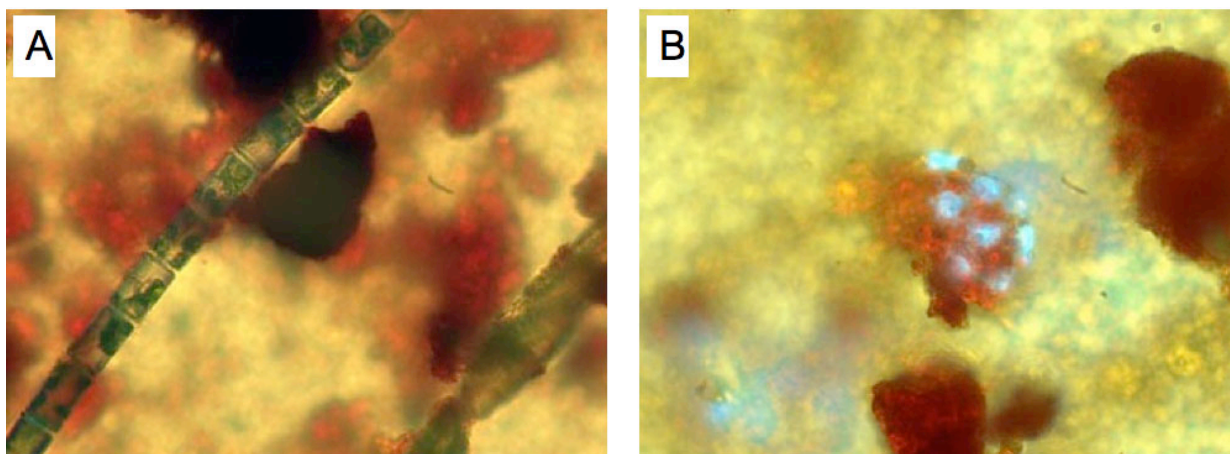


Fig. 2. Light photomicrographs of corrosion tubercle materials from the Duluth-Superior harbor. Note filamentous algae (A) and bacteria (blue color) on the surface of orange corrosion products (B). [photomicrographs by R. E. Hicks/UMD]

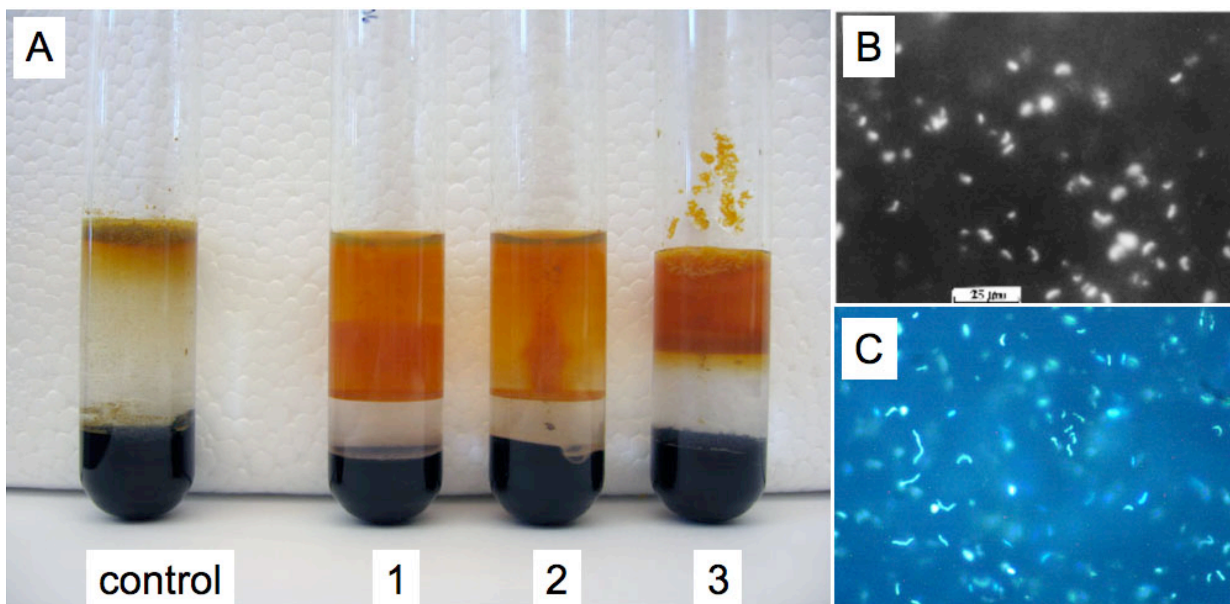


Fig. 3. Iron-oxidizing bacteria. (A) FeS gradient culture tubes inoculated with corrosion materials from Hallett Dock 5 collected on Sept. 14, 2006. (B) Photomicrograph of *Siderooxidans lithoautotrophicus* isolated by Emerson and Moyer (1997). (C) Photomicrograph of bacterial cells in the tube 2 band in panel A, whose 16S rDNA is 96% similar to *S. lithoautotrophicus*.

Community DNA fingerprints based on T-RFLP analysis indicated that bacterial communities on corroding steel pilings in the most affected part of the Duluth-Superior harbor were different from bacterial communities on steel sheet piling at the Duluth Entry (Fig. 4). At Hallett Dock 5, the composition of bacterial communities associated with corrosion tubercles was different than bacterial communities on adjacent areas of the steel sheet piling where tubercles were not present (Fig. 5).

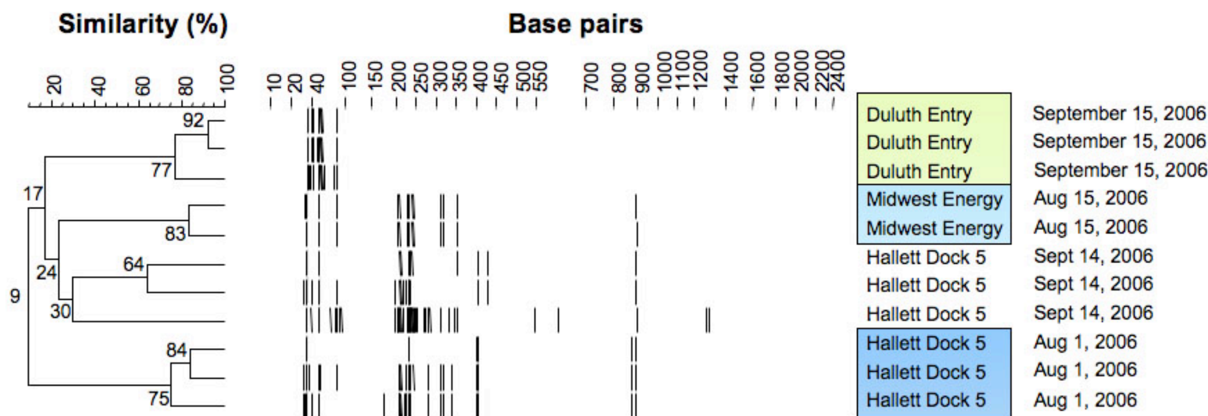


Figure 4. Similarity of bacterial biofilm communities at corroded sites (Hallett Dock 5, Midwest Energy) and a less affected area (Duluth Entry) in the Duluth Superior harbor during 2006. T-RFLP analysis of *Hae*III digests of bacterial 16S rDNA PCR products. Similarities are based on Pearson correlations of terminal restriction fragment patterns between 47 and 946 base pairs in size.

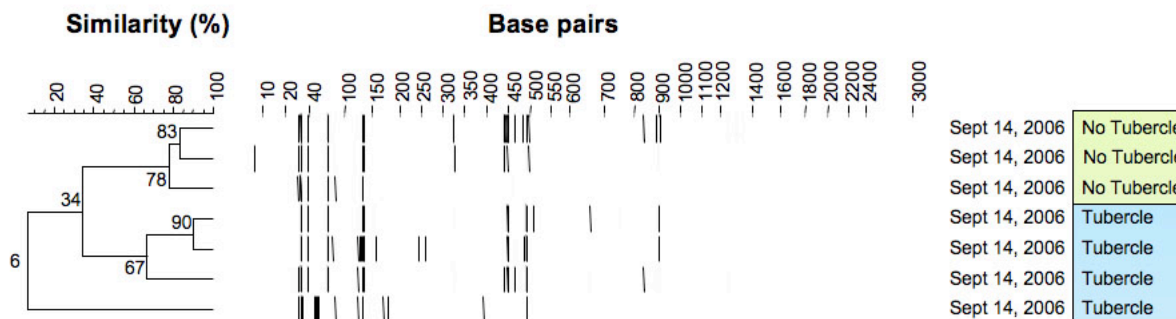


Fig. 5. Similarity of bacterial biofilm communities on corrosion tubercles and adjacent areas on steel sheet piling at Hallett Dock 5, September 14, 2006. T-RFLP analysis of *Rsa*I digests of bacterial 16S rDNA PCR products. Similarities are based on Pearson correlations of terminal restriction fragments patterns between 47 and 946 base pairs in size.

The bacterial clone library (57 clones) developed from 16S rDNA fragments amplified from Hallett Dock 5 and Midwest Energy community DNA samples demonstrated that most bacteria associated with the corroded steel surfaces were from three bacterial phyla, the β- and α-Proteobacteria, and Cyanobacteria (Fig. 6). One 16S rDNA clone from the β-Proteobacteria group was most similar to *Rhodoferax ferrireducens*. This iron-reducing bacterium grows well at low temperatures (~4°C) that are seasonally encountered in this harbor (Finneran et al. 2003).

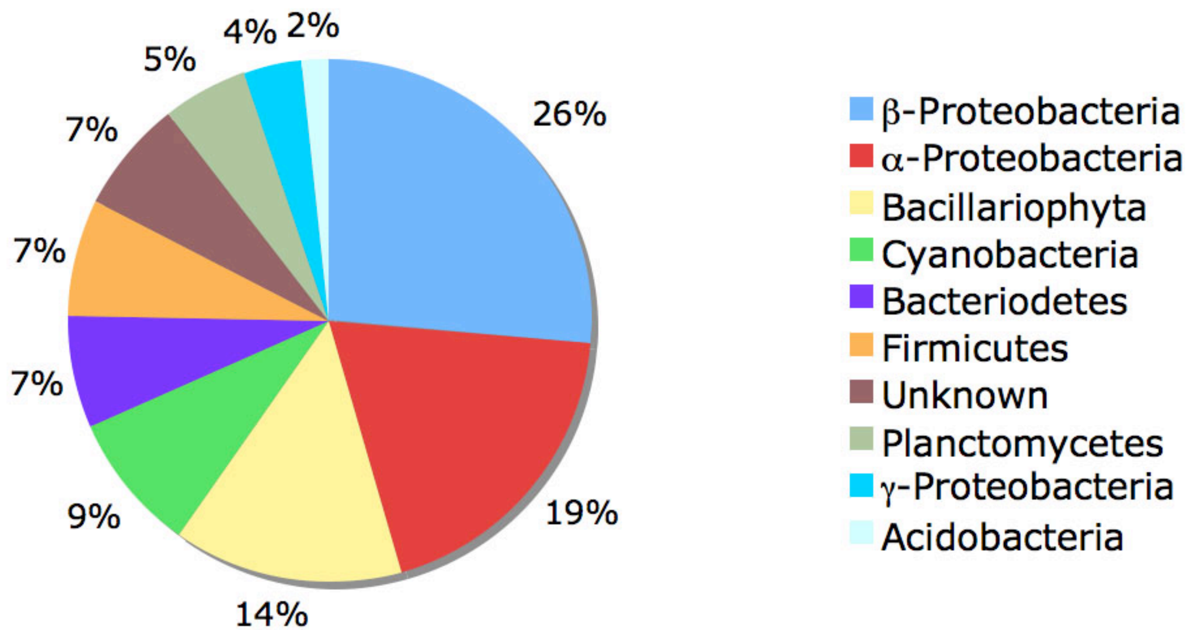


Fig. 6. Proportion of 16S rDNA clones from the Hallett Dock 5 (Aug 1, Sept 14) and Midwest Energy (Aug 15) in 2006 that are from different bacterial phyla (except the Bacillariophyta, which represent 16S rDNA clones from are eukaryotic diatom chloroplasts).

*Conclusions –*

- Corroded steel sheet piling surfaces and corrosion tubercles are covered by complex microbial biofilms.
- The diversity of bacterial biofilm communities is different on steel pilings at corroded and less affected sites.
- The composition of bacterial communities is different on corroded and uncorroded areas of steel sheet piling at Hallett Dock 5.
- The majority of bacteria on the surfaces of steel pilings at the corroded sites examined were from three bacterial phyla, the β- and α-Proteobacteria, and Cyanobacteria.
- Iron-oxidizing (*S. lithoautotrophicus*) and iron-reducing (*R. ferrireducens*) bacteria are present on corroding steel pilings in the Duluth-Superior harbor. Although we cannot provide conclusive evidence that these iron bacteria are causative agents of the accelerated corrosion of steel pilings in this harbor, our preliminary results indicate that the corroding steel structures are covered by complex microbial biofilms that contain bacteria of the type responsible for corrosion of steel in other environments.

## Potential Economic Impacts of the Research Results

Coating steel pilings is only a temporary solution and the best coatings may only prevent or reduce corrosion in this environment for 5 to 10 years (Chad Scott, AMI Consulting Engineers; pers. comm.). The upper portion of sheet pilings is frequently abraded by ice scouring during the winter months in this harbor. Corrosion similar to that seen on unprotected steel surfaces rapidly appears on coated sheet pilings after they are scratched or abraded. Thus, it is important to understand the mechanisms and agents responsible for this accelerated corrosion. Understanding the cause should be helpful in designing and testing mitigation practices, which could lead to improved control and remediation efforts.

Shipping through the Duluth-Superior harbor, the largest port by total cargo volume in the Laurentian Great Lakes, has a $200 million dollar annual impact on Minnesota's economy. Solving this accelerated corrosion issue should enhance the economic vitality of many companies whose 15 major cargo terminals ship ore, coal, and grain from this port. The results of this research should be valuable to the U.S. Army Corps of Engineers, the Duluth Seaway Port Authority, individual companies that have docks and slips in this harbor, and engineering and construction firms hired to repair or replace failing steel piling structures. A more thorough understanding of this accelerated corrosion process in Minnesota will be invaluable because preliminary inspections indicate that corrosion problems similar to the one observed in the Duluth-Superior harbor may be present in other ports on Lake Superior and possibly other Laurentian Great Lakes.

**Dissemination of Study Results**

1. Publications

   Hicks, R. E., J. M. Bergin, J. Ohaju, and B. Little. Association of *Siderooxidans lithoautotrophicus* with corroding steel pilings in a freshwater aquatic environment. Journal of Biofouling: in preparation.

2. Presentations

   Hicks, R. E., J. M. Bergin, J. Ohaju, and J. L. Kish. 2007. Microbial Aspects of Steel Sheet Piling Corrosion in the Duluth-Superior Harbor. Corrosion Issue Meeting-Army Corps of Engineers, Duluth, MN. May 22, 2007 (oral presentation)

   Hicks, R. E., J. M. Bergin, J. Ohaju, and J. Bostrom. 2007. Microbial Aspects of Steel Sheet Piling Corrosion in the Duluth-Superior Harbor. City of Duluth Harbor Technical Advisory Committee meeting, Duluth, MN. September 5, 2007 (oral presentation)

   Hicks, R. E., J. M. Bergin, J. Ohaju, and J. Bostrom. 2007. Microbial Aspects of Steel Sheet Piling Corrosion in the Duluth-Superior Harbor. GLMRI University Affiliates Meeting, Duluth, MN. September 28, 2007 (oral presentation)

   Hicks, R. E., J. M. Bergin, J. Ohaju, and J. L. Kish. 2007. Microbial Aspects of Sheet Steel Piling Corrosion in the Duluth-Superior Harbor. Making a Great Lake Superior conference, Duluth, MN. October 29-31, 2007 (poster presentation)

3. Other

   Bostrom, J. Microbial influenced corrosion of steel structures in a freshwater port (tentative title). M.S. Thesis, Integrated Biosciences Graduate Program, University of Minnesota Duluth (in progress)

## References


Anonymous. 2005. *BioNumerics Manual (v. 4.5)*. Applied Maths, Inc., Austin, TX. 323 p.

Braker, G., H. L. Ayala-Del-Rio, A. H. Devol, A. Fesefeldt, and J. M. Tiedje. 2001. Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nir*S) and 16S rRNA genes. Appl. Environ. Microbiol. 67:1893-1901.

Christie, J. B. 2001. *Concentration corrosion of berths and jetties, The threat to our maritime transportation infrastructure*. Aberdeen Harbour Board Report, Aberdeen, UK. 5 p.

Emerson, D., and C. Moyer. 1997. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Appl. Environ. Microbiol. 63:4784-4792.

Finneran, K. T., C. V. Johnsen, and D. R. Lovley. 2003. *Rhodoferax ferrireducens* sp. Nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). Int. J. Syst. Evol. Microbiol. 53:669-673.

Graff, M., and O. Seifert. 2005. *ALWC on a jetty: A case history from discovery to repair*. Second International Conference on Accelerated Low Water Corrosion, June 21-22, 2005, Liverpool, England. 5 p.

Hamilton, W. A. 1985. Sulfate-reducing bacteria and anaerobic corrosion. Ann. Rev. Microbiol. 39:195-217.

Jones, P. R., M. T. Cottrell, D. L. Kirchman, and S. C. Dexter. 2007. Bacterial community structure of biofilms on artificial surfaces in an estuary. Microb. Ecol. 53:153-162.

Keough, B., T. M. Schmidt, and R. E. Hicks. 2003. Archaeal nucleic acids in picoplankton from great lakes on three continents. Microb. Ecol. 46:238-248.

Ksoll, W. B, S. Ishii, M. J. Sadowsky, and R. E. Hicks. 2007. Presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior. Appl. Environ. Microbiol. 73:3771-3778.

Lane, D. J. 1991. 16S/23S rRNA sequencing. Pp. 115-175 in *Nucleic Acid Techniques in Bacterial Systematics*, E. Stackebrant and M. Goodfellow (eds.). John Wiley and Sons, Chichester. UK.

Marsh, C. P., J. Bushman, A. D. Beitelman, R. G. Buchheit, and B. J. Little. 2005. *Freshwater Corrosion in the Duluth-Superior Harbor - Summary of the Initial Workshop Findings*. Special publication ERDC/CERL SR-05-3, U.S. Army Corps of Engineers.

Moeseneder, M. M., J. M. Arrieta, G. Muyzer, C. Winter, and G. J. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65:4333-4339.

Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943-948.

Rao, T. S., T. N. Sairam, B. Viswanathan, and K. V. K. Nair. 2000. Carbon steel corrosion by iron-oxidizing and sulphate reducing bacteria in a freshwater cooling system. Corrosion Sci. 42:1354-1360.

Reysenbach, A. -L., G. S. Wickham, and N. R. Pace. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. 60:2113-2119.

Starosvetsky, D., R. Armon, J. Yahalom, and J. Starosvetsky. 2001. Pitting corrosion of carbon steel caused by iron bacteria. Int. Biodeterioration & Biodegradation 47:79-87.

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