Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ugmb20

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Published online: 05 Mar 2012.

To cite this article: Hui Li , Dianfeng Liu , Bin Lian , Yuan Sheng & Hailiang Dong (2012) Microbial Diversity and Community Structure on Corroding Concretes, Geomicrobiology Journal, 29:5, 450-458, DOI: 10.1080/01490451.2011.581328

To link to this article: http://dx.doi.org/10.1080/01490451.2011.581328

*Geomicrobiology Journal*, 29:450–458, 2012

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451.2011.581328

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# Microbial Diversity and Community Structure on Corroding Concretes

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**The objective of this study was to analyze bacterial diversity in two different concrete samples to understand the dominant types of bacteria that may contribute to concrete corrosion. Two concrete samples, HN-1 from the sunny side and HN-2 from dark and damp side, were collected from Zijin Mountain in Nanjing and genomic DNA was extracted. The partial bacterial 16S rRNA gene fragment was PCR amplified and two clone libraries were constructed. Amplified ribosomal DNA restriction analysis (ARDRA) was performed by digestion of the 16S rRNA gene and each unique restriction fragment polymorphism pattern was designated as an operational taxonomic unit (OTU). Phylogenetic trees of bacterial 16S rDNA nucleotide sequences were constructed. Sample HN-1 and HN-2 contained 21 OTUs and 26 OTUs, respectively. Proteobacteria and Planctomycetes were the predominant bacteria in both samples, and they are distributed among *Herbaspirillum, Archangium*, Phyllobacteriaceae and Planctomycetaceae. Cyanobacteria and *Rubrobacter* sp. are dominant in HN-1; while Acidobacteriaceae, *Adhaeribacter* sp. and *Nitrospira* sp. are predominant in HN-2. This distribution pattern was consistent with local environmental conditions of these two samples. The inferred physiological characteristics of these bacteria, based on relatedness of the DNA clone sequences to cultivated species, revealed different mechanisms of concrete corrosion depending on the local environmental conditions.**

**Keywords** 16S rRNA gene, amplified ribosomal DNA restriction analysis (ARDRA), concrete, diversity of bacterial communities, phylogenetic analysis

## INTRODUCTION

Concrete is one of the most widely used building materials in construction industry due to its many advantages such as strength, long life cycle, fire resistance, low price and low maintenance cost, and endurance to weathering. However, the concrete structures are subject to corrosion damage because of microbial weathering, resulting in huge economic loss (Zhang and Zhang 2006). For example, 10–20% construction materials are damaged in Germany due to microbial corrosion (Thaer 2001).

In Los Angeles, USA, 208 km out of 1900 km concrete sewers suffered from microbial corrosion, and the cost of replacement or restoration would be up to 400 million U.S. dollars (Thaer 2001). In China, a survey of domestic sewage treatment facilities revealed that a large number of sewage treatment facilities suffer severe damages due to microbial corrosion, and they could not achieve their designed life expectancy. Significant corrosion has also been observed in some newly operated sewage treatment projects (Han et al. 2000).

To understand the mechanism of microbial weathering and to look for a fundamental solution for the corrosive damage of concrete structures, many microbial studies have been conducted (De Graef et al. 2005; Gaylarde et al. 2003; Le et al. 2006; Ma et al. 2006; Sand 2001; Zhang and Zhang 2006; Zhu 2010). Microbial corrosion may be caused by the following agents: inorganic acid (Diercks et al. 1991; Sand 2001), organic acid (Cho and Mori 1995; Ma et al. 2006) or complex compounds (Chen 1996); salt stress (Chen 1996); microbial metabolites such as $H_2S$, NO and $NO_2$ (Hamilton 1985); the formation of microbial biofilms (Laiz et al. 2009); and the action of extracellular

Received 10 December 2010; accepted 4 April 2011.

This research was jointly supported by National Natural Science Foundation of China (Grant No. 40773069), National Science Fund for Creative Research Groups (Grant No. 41021062) and the Priority Academic Program Development of Jiangsu Higher Education Institutions. We thank two anonymous reviewers for their insightful comments and suggestions.

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enzymes and emulsifiers (Chen 1996). Ma et al. (2006) reported that microorganisms (such as iron reducing and oxidizing bacteria, sulfate-reducing bacteria) can corrode concrete through their growth and production of organic acids.

Chemoautotrophic bacteria can use inorganic compounds to produce inorganic acids to corrode concrete. For instance, sulfur-oxidizing bacteria can produce sulfuric acid from oxidization of hydrogen sulfide, and ammonia-oxidizing bacteria can oxidize ammonium into nitrite and nitrate. Chemoheterotrophic bacteria, such as denitrifying bacteria, sulfate-reducing bacteria, ammonification bacteria, however, can degrade organic materials in concrete to carbon dioxide, nitrate, sulfate, and phosphate under anaerobic conditions; to methane, carbon dioxide, ammonia, hydrogen sulfide under aerobic conditions (Zhang and Zhang 2006), leading to corrosion by weak acids. In addition, small algae (cyanobacteria, green algae) usually grow on concrete surface, and their acidic metabolites can also cause corrosion of concrete (Le et al. 2006). Based on the preceding observations and findings, the dominant mechanism of microbial corrosion of concrete appears to be via production of inorganic and organic acids.

To develop strategies to prevent and control microbial corrosion of concrete, the diversity of microbial community and the mechanism of corrosion should be understood. Because the current knowledge of microbial composition is inadequate, the control measures, such as concrete modification, surface coating, and biological protection methods (Wang and Yan 2009), need to be modified or amended. For example, the selection of concrete coating materials and their effects on other properties of concrete, the type and content of fungicides used as a functional component of concrete, and their stability, all depend on a full understanding of microbial composition on concrete (Zhang and Zhang 2006).

Traditional method for analysis of microbial diversity is based on cultivation of microorganisms in pure culture, which is laborious, time consuming, and costly. Microbial community in concrete is not only very diverse, but also largely non-cultivable (Øvreås and Torsvik 1998). Therefore, the traditional cultivation method can only reveal a fraction of microbial species causing corrosive damage of concrete. Molecular biology methods, however, can avoid the limitations of the traditional culture methods, and reveal the true microbial diversity. Among the molecular methods, the 16S rRNA gene sequence analysis has been widely used in the literature. As microbial 16S rRNA gene sequences accumulate in public database such as GenBank, this technology has become a powerful tool in bacterial identification, classification, and community diversity analysis for environmental samples (Liu et al. 2008).

In the present study, two concrete samples were collected from artificial barriers of two different micro-environments of Zijin Mountain in Nanjing City, China. Two bacterial 16S rRNA gene clone libraries were constructed. The bacterial community composition and the diversity were analyzed by Amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequence analysis in order to determine the role of various species of bacteria causing microbial corrosion of concrete.

## MATERIALS AND METHODS

### Sampling

Concrete samples were collected from Nanjing Zijin Mountain, Jiangsu Province, China, in June, 2009. The area has a subtropical humid climate, with an average temperature of 15.3°C and annual precipitation of 1106.5 mm. The time between mid June and early July is a rainy season. Two representative concrete samples were sampled. HN-1 was collected from an artificial barrier with adequate sunshine; while HN-2 was taken from an artificial barrier on the shady side of the mountain, relatively dark and damp. The concrete samples were placed in sterile bags, sealed, and stored in a 4°C refrigerator for 4 hours before analysis.

## METHODS

### Extraction of Genomic DNA

Total genomic DNA was extracted from the concrete samples using OMEGA's soil DNA extraction kit (Tingbest Biological Technology Co., Ltd. Nanjing, China) in accordance with the instructions of the kit.

### PCR Amplification and Gel Purification of Bacterial 16S rDNA Fragment

Bacterial universal PCR primers used for amplification were 16S-fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′, and 16S-rD1: 5′-ACGGTTACCTTGTTACGACTT-3′ (Weisburg et al. 1991). PCR were carried out in 50 $\mu$L of reactions containing 5 $\mu$L of 10 $\times$ buffer, 4 $\mu$L of $Mg^{2+}$ (25 mmol/L), 4 $\mu$L of dNTPs (2.5 mmol/L), 2 $\mu$L of upstream and downstream primers (10 pmol/L), 1 $\mu$L of template DNA (total DNA), 2 $\mu$L of TaqDNA polymerase (5U/$\mu$L), and 30 $\mu$L of sterile $ddH_2O$ with the following conditions: first denaturation at 94°C for 5 min, and then 10 cycles at 94°C for 1 min, 65°C down to 55°C, each cycle decreased 1°C for 1 min, 72°C for 2 min; another 20 cycles at 94°C for 1 min; 55°C for 1 min, 72°C for 2 min; 72°C for another 10 min. PCR products were detected by electrophoresis in 1.0% agarose gel.

### Ligation, Transformation, and Colony PCR Amplification

Purified PCR products were ligated into pGEM-Teasy vector (TaKaRa Inc.). The ratio of the concentration of target DNA and pGEM-Teasy vector was 2:1. The ligation reactions (10 $\mu$L) containing 5 $\mu$L of 2 $\times$ rapid ligation buffer, 2 $\mu$L of the purified PCR product, 1 $\mu$L of pGEM-Teasy vector, 1 $\mu$L of T4DNA ligase, and 1$\mu$L of double-distilled water, were incubated at 16°C overnight. The plasmid carrying the target 16S rDNA fragment was cloned into *Escherichia coli* DH5$\alpha$ competent cells.

After cloning, the recombinant transformants were screened using blue-white screening LB medium containing X-Gal, IPTG, and 0.8 mL/L ampicillin, cultured at 37°C for 12–16 hrs. White colonies were picked and grown in LB liquid medium containing 0.8 mL/L ampicillin, at 37°C and 220 rpm for overnight. Colony PCR was conducted using the cultured *E. coli* cells as template with universal primer T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ACGATTTAGGTGACACTATAG-3′) (Liu et al. 2008), under the same conditions as described here.

### *Hinf* I Digestion and ARDRA Analysis

The colony PCR products were digested by *Hin*f I restriction endonuclease (TaKaRa Company). *Hin*f I enzyme digestion system containing 1$\mu$L of 10 × T buffer, 5 $\mu$L of plasmid DNA, 0.5 $\mu$L of Hinf I enzyme, 3.5 $\mu$L of double-distilled water, was incubated at 37°C for 3–4 hrs. The digested products were separated by electrophoresis in a 2% agarose (Biowest, Spain) gel, and visualized at a gel imaging system (LELDOC-II, UVP Inc.) to identify the various restriction patterns. The ARDRA patterns of all positive clones were analyzed and classified into a number of operational taxonomic units (OTUs). The number of OTUs for each concrete sample and the number of positive clones belonging to each OTU type were calculated, and one representative clone for every OTU type was selected for nucleotide sequencing (Shanghai Bio Engineering Co., Ltd.).

### DNA Nucleotide Sequence Analysis

Nucleotide sequences of 16S rRNA gene clones were compared with those in NCBI GenBank database by the BLASTn searching tool. One to three gene sequences with the highest sequence similarity to the query sequence were retrieved from GenBank. The target 16S rRNA gene sequence and the retrieved sequences from the data base were compared by ClustalX program (Larkin et al. 2007). The sequences were identified with Chimera_Check (Cole et al. 2005) and removed if discovered.

Some un-related sequences were discarded based on the preliminary phylogenetic analysis results. Finally, a streamlined group of sequences were again aligned by ClustalX algorism according to the default settings. Sequences were edited and the fuzzy sites were removed or corrected by reference to the secondary structure of 16S rRNA gene. Multiple sequence alignment was carried out and a neighbor joining (N-J) phylogenetic tree with 1000 bootstrap was then constructed using the Tamura-Nei model by Mega 4.0 (Tamura et al. 2007) package.

The C + G contents of our sequences were slightly higher than A + T contents, therefore, both nucleotide conversion/tranversion and variation were taken into account when the neighbor-joining phylogenetic trees for two concrete samples were constructed (Figure 2 and Figure 3). The nucleotide sequences have been submitted to GenBank (HM565019-HM565065).

### Statistical Analysis

Coverage (C) of the constructed clone libraries was calculated as follows: C = 1-(*n1/N*), where *n1* is the number of phylotypes that occurred only once in the clone library and *N* is the total number of clones analyzed (Jiang et al. 2009). Shannon index (H), Simpson diversity index (D) and evenness (E) of sample HN-1 and HN-2 were calculated using the Bio-Dap software (Thomas and Clay 2000). Shannon index and Simpson index reflects the microbial species richness and the degree of dominance (Luo et al. 2009), respectively.

## RESULTS

### 16S rRNA Gene Clone Libraries

Two hundred clones were randomly selected from each 16S rRNA gene clone library for sequencing. One hundred and forty-five clones from HN-1 were identified containing the target inserts. ARDRA analysis revealed a total of 21 OTUs, among which 5 OTUs were dominant types (including more than 10 clones). The largest OTU contained 25 clones, while 7 OTUs had only a single clone. The coverage (Good 1953) of the HN-1 clone library was 95.2%. One hundred and fifty-three clones from the HN-2 clone library carried the right inserts, and ARDRA analysis revealed 26 OTUs, of which 7 OTUs were dominant types. The largest OTU was composed of 17 clones, whereas 6 OTUs contained only one clone. The coverage of the HN-2 clone library was 96.1%. These results suggested that both clone libraries had higher degree of clone coverage. In addition, the rarefaction curves for the 16S rRNA gene sequences from the two clone libraries were saturated, indicating that the number of clones analyzed was sufficient to reflect the diversity and to determine bacterial community structure (Figure 1).

### Bacterial Population Structure

Sample HN-2 not only contained more bacterial species, but also those species were distributed more uniformly than those in sample HN-1 (Table 1). The difference in Simpson index value suggests that the two samples contained different predominant bacteria, which is consistent with the results of bacterial community structure analysis described here.

TABLE 1
The diversity of bacterial populations in sample HN-1and HN-2

| | HN-1 | HN-2 |
|---|---|---|
| H | 2.93 | 3.07 |
| E | 0.84 | 0.86 |
| D | 0.93 | 0.95 |

H stands for Shannon index, E represents Evenness, and D represents Simpson index.

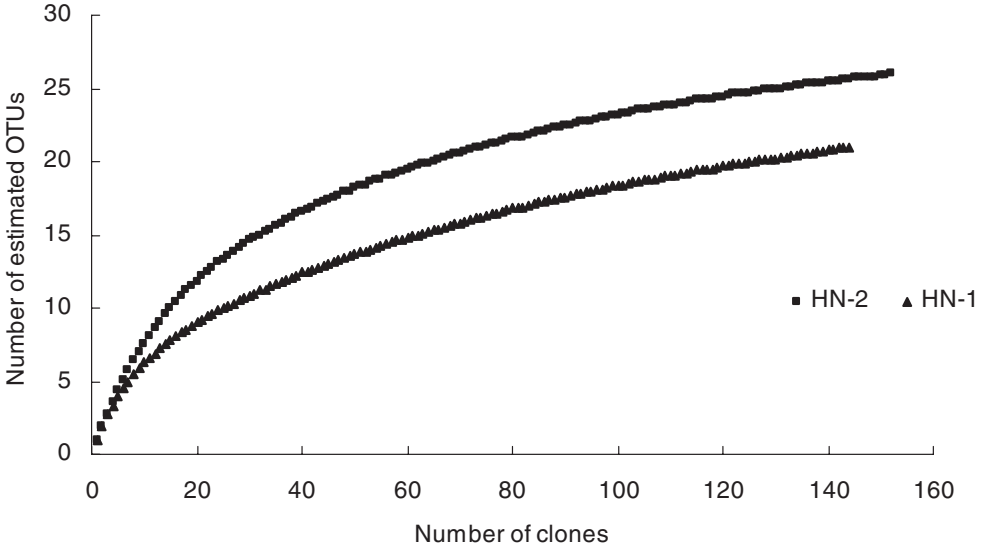


FIG. 1. Rarefaction curves for bacterial 16S rRNA gene clone sequences for sample HN-1 and HN-2 showing that these curves are nearly saturated.



## Bacterial Community Structure

The BLASTn results showed that the difference between the majority of our clone sequences and the related sequences currently in the NCBI database is less than 5%. The bacterial clones for HN-1 were classified into eight large clusters: *Proteobacteria* (41.4%), *Planctomycetes* (20%), *Cyanobacteria* (17.2%), *Actinobacteria* (3.4%), *Bacteroidetes* (4.8%), *Acidobacteria* (9%), *Firmicutes* (2.8%), and *Nitrospirae (1.4%)* (Figures 2 and 4).

Five OTUs, namely H-39, H-101, H-197, H-83, H-99 from the HN-1 clone library, contained a large proportion of 16S rRNA gene sequences, accounting for 19.3%, 17.2%, 16.6%, 14.5% and 6.9% of the total clones, respectively. Based on the position in the phylogenetic tree and the sequence similarity, H-39 belongs to the phylum *Planctomycetes*; H-101 is closely related to the phylum Cyanobacteria; H-197 can be assigned to the order *Burkholdales*, the class *betaroteobacteria*, and the phylum Pro*teobacteria*; H-83 is similar to *Archangium gephyra* of the class *deltaproteobacteria*, the phylum Proteobacteria; H-99 belongs to the *Rubrobacter* sp. of the class *Actinomyces*, the phylum *Actinobacteria*.

Nine major clusters were identified in the phylogenetic tree of the HN-2 clone library, which are Acidobacteria (27.5%), Bacteroidetes (20.3%), Planctomycetes (11.8%), Proteobacteria (11.8%), Nitrospirae (1.1%), Bacillariophyta (7.8%), Actinobacteria (6.5%), Chloroflexi (2.6%), and Gemmatimonadetes (0.6%) (Figures 3 and 5).

RADRA analysis revealed 8 OTUs in the HN-2 clone library, namely N-181, N-191, N-89, N-44, N-111, N-54, N-78, N-9, accounting for 11.1%, 11.1%, 10.5%, 8.5%, 7.8%, 7.8%, 7.2%, and 7.2%, respectively. Based on the position in the phylogenetic tree and the sequence similarity, N-181 and N-44 are related to the phylum Acidobacteria. N-9 is close to the genus *Acidobacterium*. N-191 belongs to the genus *Adhaeribacter* sp. of the class Sphingobacteriium, the phylum *Bacteroidetes*. N-89 belongs to the genus *Nitrospira* of the Family Nitrospirae, the order Nitrospirae, the class Nitrospirae, and the Phylum Nitrospirae. N-111 is related to bacteria in the genus *Planctomyces* of the Family Planctomycetaceae, the order Planctomycetales, the class Planctomycetia and the phylum Planctomycetesm. N-54 is close to bacteria in the genus *Fragilaria* of the family Fragilariaceae, the order Fragilariales, the class Fragilariophyceae and the phylum Bacillariphyta. N-78 belongs to *Aliihoeflea aestuarii* of the Family Phyllobacteriaceae, the order Rhizobiales, the class $\alpha$-Proteobacteria, and the phylum Proteobacteria.

## DISCUSSION

Analysis of bacterial diversity in sample HN-1 and HN-2 revealed that bacteria of the phylum Proteobacteria were abundant in both samples, i.e., in the genus *Herbaspirillum* of *Betaproteobacteria*; the species *Archangium gephyra* of *Deltaproteobacteria* and the species *Aliihoeflea aestuarii* of *Alphaproteobacteria.* These results suggest that Proteobacteria were abundant in concretes regardless of their exposure to the sun and wet/dry condition. The corrosive damage of concrete may be related to the nitrogen cycle (such as nitric acid production), as some of these identified species play important roles in nitrogen cycling (Ma et al. 2006; Zakria et al. 2007).

Another important group of bacteria present in both samples belong to the genus *Gemmata* of the family Planctomycetaceae and it accounted for 20% and 11.8% in HN-1 and HN-2, respectively. The phylum Planctomycetes is abundant in soil and water (Buckley et al. 2006); however, the exact type and quantity of this pylum in soil are affected by soil pH and nitrate content (Buckley et al. 2006). This phylum contains anammox bacteria; obligately anaerobic, autotrophic bacteria that are capable of

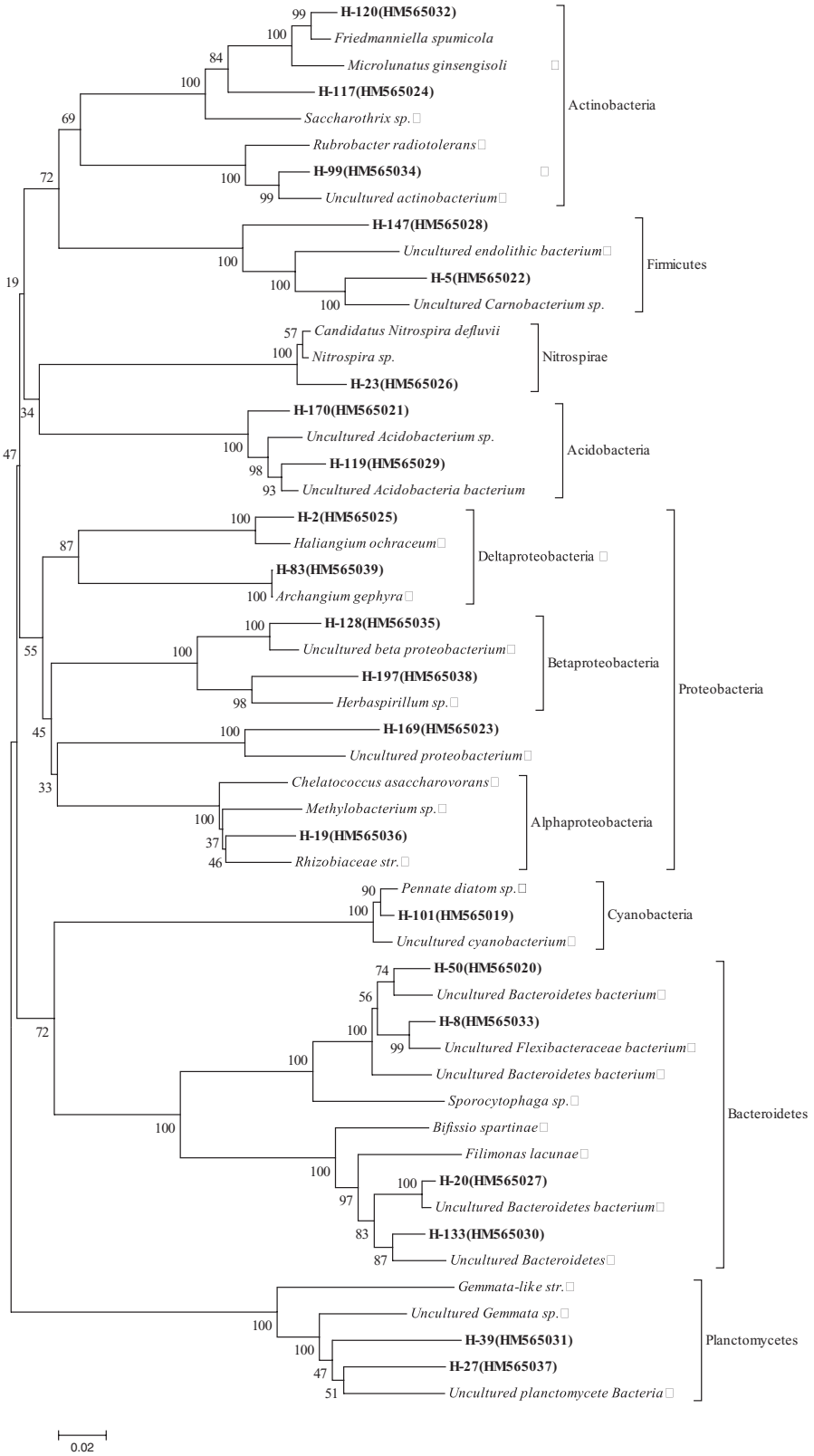


FIG. 2. A neighbor joining phylogenetic tree showing the phylogenetic relationships of the bacterial 16S rRNA gene sequences cloned from sample HN-1 to those related sequences from the GenBank database. The sequences obtained in this study were indicated in bold; H-120, 117 . . . 27 represent different clones of sample HN-1. The reference sequences were from GenBank database. Scale 0.02 represents the distance of evolution. Bootstrap replications of 1,000 were used.

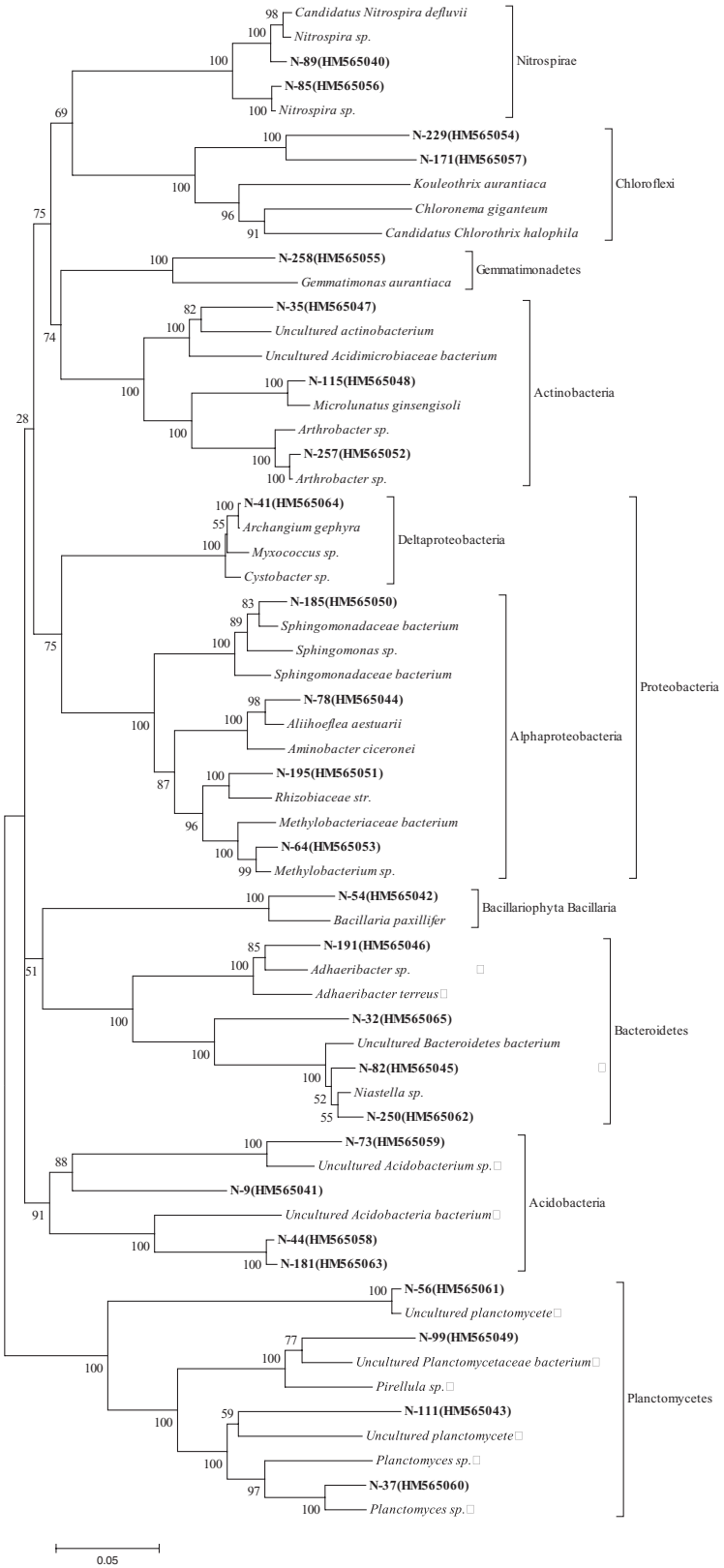


FIG. 3. A neighbor joining phylogenetic tree showing the phylogenetic relationships of the bacterial 16S rRNA gene sequences from sample HN-2 to those related sequences from the GenBank database. The sequences obtained in this study were indicated in bold; N-89, 85 . . . 37 represent different clones of sample HN-2. The reference sequences were from GenBank database. Scale 0.05 represents the distance of evolution. Bootstrap replications of 1000 were used.

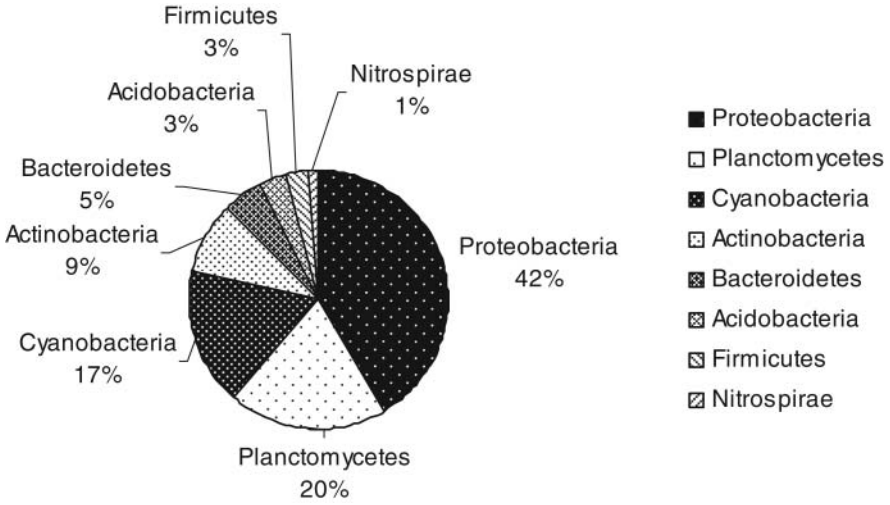


FIG. 4. The relative proportions of various bacterial groups in sample HN-1 based on occurrence of clone sequences within that group.



oxidizing ammonia with nitrite as electron acceptor. These bacteria may have impacted concrete corrosion through the formation of biofilms and production of organic compounds/acids. Similar to Proteobacteria, Planctomycetes can grow and reproduce in the concrete regardless of the amount of sunlight or humidity.

Cyanobacteria were present in sample HN-1 but absent in sample HN-2 (Figure 5). This difference may be caused by different micro-environments between the two samples. Cyanobacteria and green algae are phototrophic, and thrive under conditions with plenty of sunlight and water (Le et al. 2006). Sample HN-1 was collected from a concrete exposed to sufficient sunlight, a necessary condition for growth. However, both the surface and the deep section of HN-1 would have contained low nutrient levels, which would have forced cyanobacteria to break down the concrete to extract nutrition and form biofilms, consequently eroding the concrete.

Because HN-2 was collected from a concrete on the dark side of a mountain road thus, the lack of adequate light accounted for the absence of cyanobateria in this sample. These data suggest that cyanobacteria are likely an important component of bacterial population on the sunny side and would corrode concrete. In a dark and damp environment, cyanobacteria may not play a major role in concrete corrosion.

Actinobacteria were present as one of the important groups of bacteria, accounting for 9% and 6.5% in HN-1 and HN-2, respectively. Most actinobacteria have substrate and air mycelia which would help to penetrate into fractures in concrete. This

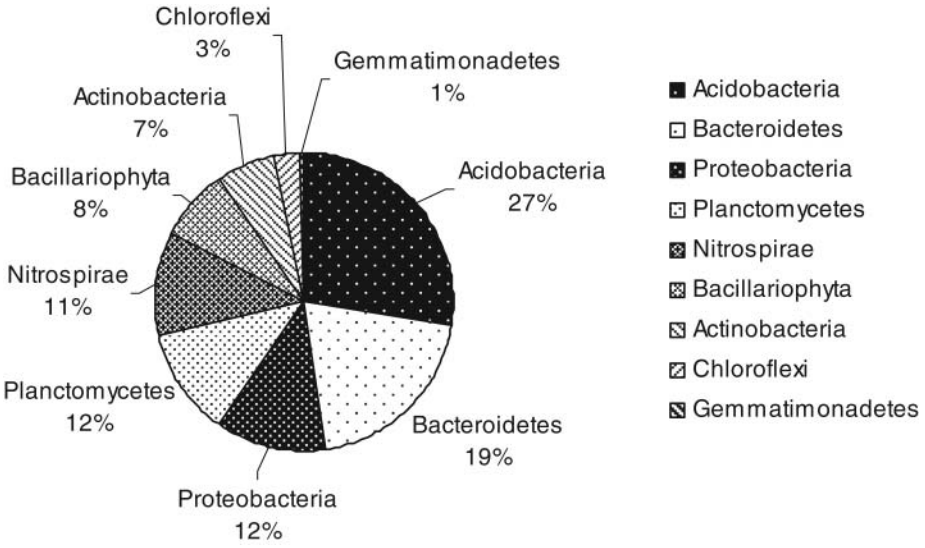


FIG. 5. The relative proportions of various bacterial groups in sample HN-2 based on occurrence of clone sequences within that group.

type of mechanical action would result in weathering of concrete (Jongmans et al. 1997; Lian et al. 2008). Furthermore, some species of Actinobacteria, such as *Rubrobacter* sp., was found to be related to the color fading of the lime wall paintings (Schabereiter-Gurtner et al. 2001). *Rubrobacter* sp. has been found to cause erosion of rocks via three mechanisms: formation of biofilms on rock surface, penetration of bacterial cells into rock cracks, and production of the crystals (struvite, $NH_4Mg(H_2O)_6[PO_4]$) on biofilms (Laiz et al. 2009). The continuity of the biofilm would be interrupted by emerging crystals in a process similar to the formation of efflorescences, and when dried, the biofilm would remove mineral grains, producing a mechanical deterioration (Laiz et al. 2009).

Biological corrosion of the rock paintings by Actinobacteria may be related to light, humidity and other environmental conditions (Nugari et al. 2009). Whereas photosynthetic bacteria grow densely in the damp places, heterotrophic Actinobacteria are typically found in the lower humidity areas (Nugari et al. 2009). The distribution of *Rubrobacter* sp. in the two studied samples is consistent with this physiological characteristic of Actinobacteria.

Acidobacteria were predominant in sample HN-2 and accounted for 27.5% of the clones, while they only accounted for 3.4% of the clones in sample HN-1. This relative abundance difference between the two samples suggests that Acidobacteria may be one of the main groups of bacteria impacting concrete corrosion in the shady and damp environment, but not in the sunny environment.

Nitrospirae bacteria accounted for 11.1% and 1.4% of the clones in sample HN-2 and HN-1, respectively. This group of bacteria belongs to the genus *Nitrospira*. According to Diercks et al. (1991), the bacteria in this genus can oxidize ammonia to nitrate through nitrification, which can cause severe acid corrosion of concrete. Some nitrifying *Nitrospira* sp. cannot survive without water, and ultraviolet radiation has a lethal effect. In the dark, damp micro-environment, *Nitrospirae* sp. can grow and reproduce more easily than under sunny and dry conditions. Therefore, similar to Acidobacteria, Nitrospirae may also be a major group that can cause the corrosion of concrete in the shady and wet environment.

In summary, the present study showed that plenty of bacterial species resided on surfaces and in cracks of concretes. The Phyla Proteobacteria and Planctomycetes accounted for a large proportion of the clones in both sample HN-1 and HN-2, and thus were expected to play an important role in the corrosion of concrete. Because the two concrete samples were under different micro-environment conditions, their bacterial population structure, especially the predominant bacteria groups were different. The shady and damp environment favored the growth of some Acidobacteria, Nitrospirae, and Bacteroides. Acidobacteria and Nitrospirae in this environment could produce acids to corrode concrete. However, we acknowledge that positive confirmation of bacterial role in corrode corrosion requires isolation of these bacteria and direct functional testing against corrosion.

## CONCLUSIONS

Concrete samples harbored diverse bacterial communities. The predominant bacterial groups are: Proteobacteria, Planctomycetes, Cyanobacteria, Actinobacteria, Acidobacteria, Bacteroidetes and Nitrospirae. Proteobacteria and Planctomycetes are predominant groups and may be important in corroding concrete by producing inorganic and organic acids.

Appropriate sunlight is conducive to the growth and reproduction of Cyanobacteria and Actinobacteria, and corrosion of concrete by these groups could have been via mechanical damage and biochemical erosion through the formation of the biofilm and the production of acidic metabolites. The shady and moist environment with less light favors the growth of Acidobacteria, Bacteroidetes and Nitrospirae, and the mechanism of concrete corrosion by these bacteria are possibly by the production of inorganic and organic acids.

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