



Title	Microbial community structures and in situ sulfate-reducing and sulfur-oxidizing activities in biofilms developed on mortar specimens in a corroded sewer system
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Citation	Water Research, 43(18), 4729-4739 <a href="https://doi.org/10.1016/j.watres.2009.07.035">https://doi.org/10.1016/j.watres.2009.07.035</a>
Issue Date	2009-10
Doc URL	<a href="http://hdl.handle.net/2115/45290">http://hdl.handle.net/2115/45290</a>
Type	article (author version)
File Information	satoh2009corrosion.pdf



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1  
2 **Microbial community structures and in situ sulfate-reducing and**  
3 **sulfur-oxidizing activities in biofilms developed on mortar specimens in a**  
4 **corroded sewer system**

5  
6 **Running title: Microbial communities and activities in sewer biofilms**

7  
8 By

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4

## 1 **ABSTRACT**

2

3       Microbially induced concrete corrosion (MICC) caused by sulfuric acid attack in  
4 sewer systems has been a serious problem for a long time. A better understanding of  
5 microbial community structures of sulfate-reducing bacteria (SRB) and sulfur-oxidizing  
6 bacteria (SOB) and their in situ activities is essential for the efficient control of MICC. In  
7 this study, the microbial community structures and the in situ hydrogen sulfide production  
8 and consumption rates within biofilms and corroded materials developed on mortar  
9 specimens placed in a corroded manhole was investigated by culture-independent 16S  
10 rRNA gene-based molecular techniques and microsensors for hydrogen sulfide, oxygen, pH  
11 and the oxidation-reduction potential. The dark-gray gel-like biofilm was developed in the  
12 bottom (from the bottom to 4 cm) and the middle (4 to 20 cm from the bottom of the  
13 manhole) parts of the mortar specimens. White filamentous biofilms covered the gel-like  
14 biofilm in the middle part. The mortar specimens placed in the upper part (30 cm above the  
15 bottom of the manhole) were corroded. The 16S rRNA gene-cloning analysis revealed that  
16 one clone retrieved from the bottom biofilm sample was related to an SRB, 12 clones and 6  
17 clones retrieved from the middle biofilm and the corroded material samples, respectively,  
18 were related to SOB. In situ hybridization results showed that the SRB were detected  
19 throughout the bottom biofilm and filamentous SOB cells were mainly detected in the upper  
20 oxic layer of the middle biofilm. Microsensor measurements demonstrated that hydrogen  
21 sulfide was produced in and diffused out of the bottom biofilms. In contrast, in the middle  
22 biofilm the hydrogen sulfide produced in the deeper parts of the biofilm was oxidized in the  
23 upper filamentous biofilm. pH was around 3 in the corroded materials developed in the

1 upper part of the mortar specimens. Therefore, it can be concluded that hydrogen sulfide  
2 provided from the bottom biofilms and the sludge settling tank was emitted to the sewer  
3 atmosphere, then oxidized to corrosive compounds in the upper and middle parts of the  
4 manhole, and only the upper part of the mortar specimens were corroded, because in the  
5 middle part of the manhole the generated corrosive compounds (e.g., sulfuric acid) was  
6 reduced in the deeper parts of the biofilm.

7

8 **Keywords:** Microbially induced concrete corrosion; sulfate-reducing bacteria;  
9 sulfur-oxidizing bacteria; in situ hydrogen sulfide production rates; in situ hydrogen sulfide  
10 consumption rates.

11

## 12 **1. Introduction**

13

14 Concrete corrosion is one of the most challenging problems faced by sewerage  
15 authorities, because it has an enormous economic impact for restoration of the damaged  
16 sewer systems (**Jensen et al., 2009; Vollertsen et al., 2008; Zhang et al., 2008**). In sewer  
17 systems the concrete corrosion is caused mainly by sulfuric acid generated by microbial  
18 sulfur oxidation because the atmospheric hydrogen sulfide ( $H_2S$ ) and oxygen ( $O_2$ )  
19 concentrations and moisture are relatively high. It is known as microbially induced concrete  
20 corrosion (MICC). Various microbial species and complex mechanisms are involved in the  
21 MICC. The general mechanism for the MICC in sewer systems has been described  
22 previously (**Jensen et al., 2009; Lahav et al., 2006; Vollertsen et al., 2008; Yamanaka et**  
23 **al., 2002; Zhang et al., 2008**). In the first step,  $H_2S$  is produced by sulfate-reducing bacteria

1 (SRB) present in waters and sediments under anaerobic conditions in the submerged part of  
2 sewer facilities. This H<sub>2</sub>S easily enters the sewer atmosphere by volatilization and dissolves  
3 in condensate on the surface of sewer facilities. Then, H<sub>2</sub>S is mainly chemically oxidized to  
4 thiosulfate (S<sub>2</sub>O<sub>3</sub><sup>-</sup>) and elemental sulfur (S<sup>0</sup>). Sulfur-oxidizing bacteria (SOB) present in the  
5 condensate on the concrete surface (e.g., the sewer crown) eventually oxidize the dissolved  
6 H<sub>2</sub>S and other sulfur compounds (e.g., S<sub>2</sub>O<sub>3</sub><sup>-</sup> and S<sup>0</sup>) to sulfuric acid, which corrodes the  
7 concrete.

8 Several measures to prevent H<sub>2</sub>S production have been developed (**Zhang et al.,**  
9 **2008**). SRB activity can be inhibited by pH elevation or inhibitors such as biocides, ozone,  
10 molybdate and formaldehyde (**Elovitz et al., 2000; Jayaraman et al., 1999; Nemati et al.,**  
11 **2001; Reinsel et al., 1996; Zhang et al., 2009**). Increasing ORP is achieved by addition of  
12 electron acceptor such as oxygen, nitrate or nitrite (**Bentzen et al., 1995; Hobson and**  
13 **Yang, 2000; Londry and Suflita, 1999; Ochi et al., 1998; Okabe et al., 2003a, b**). Other  
14 measures are concrete coatings (**Haile and Nakhla, 2009; Muynck et al., 2009**) and  
15 chemical and biological removal of H<sub>2</sub>S by addition of iron salts (**McComas et al., 2001**),  
16 H<sub>2</sub>O<sub>2</sub> (**Cadena and Peters, 1988**), chlorines, nitrate, or nitrate-reducing, sulfide-oxidizing  
17 bacteria (**De Gusseme et al., 2009**). To more efficiently control the MICC, it is necessary to  
18 understand microbial community structures, especially SRB and SOB species, in sewer  
19 systems.

20 The microbial community structures responsible for the MICC in sewer systems are  
21 very poorly understood because most of the previous studies were conducted with  
22 conventional culture-dependent techniques that could detect only a limited range of  
23 microorganisms in diversified microbial communities involved in the MICC (**Davis et al.,**

1 **1998; Harrison, 1984; Islander et al., 1991; Nielsen et al., 2008**). In contrast to these  
2 studies, **Vincke et al.** (2001) demonstrated by using conventional cultivation techniques  
3 and molecular tools that the density of acidophilic sulfur-oxidizing bacteria  
4 (*Acidithiobacillus thiooxidans*) was higher together with an increasing corrosion level.  
5 **Hernandez et al.** (2002) used fluorescent in situ hybridization (FISH) and successfully  
6 enumerated the number of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*  
7 in biofilms taken from the corroded crowns of sewer systems. Although molecular-based  
8 techniques have been useful for more accurately describing the microbial ecology of the  
9 microbial communities responsible for the MICC, these techniques do not necessarily  
10 provide us with information about microbial activities. This is because the majority of  
11 microorganisms have not yet been cultivated, and phylogeny and phenotype are not  
12 necessarily congruent with physiology.

13 Microbial activities responsible for the MICC should be analyzed in situ because  
14 sulfate reduction and sulfur oxidation occur in such a very thin layer as biofilm and  
15 condensate. A sulfur cycle in biofilms has been demonstrated by 16S rRNA gene-cloning  
16 analysis, FISH, microsensor measurements, and standard batch and reactor experiments (**Ito**  
17 **et al., 2002; Okabe et al., 1999b, 2002, 2003a, b, 2005; Santegoeds et al., 1998**).  
18 Especially, **Okabe et al.** (2007) investigated the succession of SOB in the bacterial  
19 community on corroding concrete in a sewer system in situ over 1 year with molecular  
20 techniques and microsensors. The results suggested that in the corroded materials with a  
21 thickness of about 10 mm, the production of sulfuric acid by SOB occurred mainly in the  
22 surface layer and the sulfuric acid produced penetrated through the corroded materials and  
23 then attacked the sound concrete below. However, in the previous studies the in situ

1 sulfate-reducing and sulfur-oxidizing activities within biofilms and corroded products on  
2 concrete surface in corroded sewer systems have not yet investigated.

3 The aim of this study was, therefore, to investigate the microbial community  
4 structures and the in situ H<sub>2</sub>S production and consumption rates within biofilms and  
5 corroded materials developed on mortar specimens installed in a corroded sewer system.  
6 Mortar specimens were placed in a severely corroded manhole of a real sewer system to  
7 investigate the MICC process under in situ conditions. The microbial community structures  
8 were analyzed by using 16S rRNA gene-cloning analysis and FISH, and the in situ H<sub>2</sub>S  
9 production and consumption rates were determined with microsensors. Moreover, the  
10 succession of the in situ H<sub>2</sub>S production and consumption rates in the biofilms was analyzed.  
11 In parallel, appearance of the mortar specimens was monitored and a corrosion rate of the  
12 mortar specimens was determined. The data sets resulting from these different approaches  
13 were integrated to elucidate the MICC mechanism in corroded sewer systems.

14

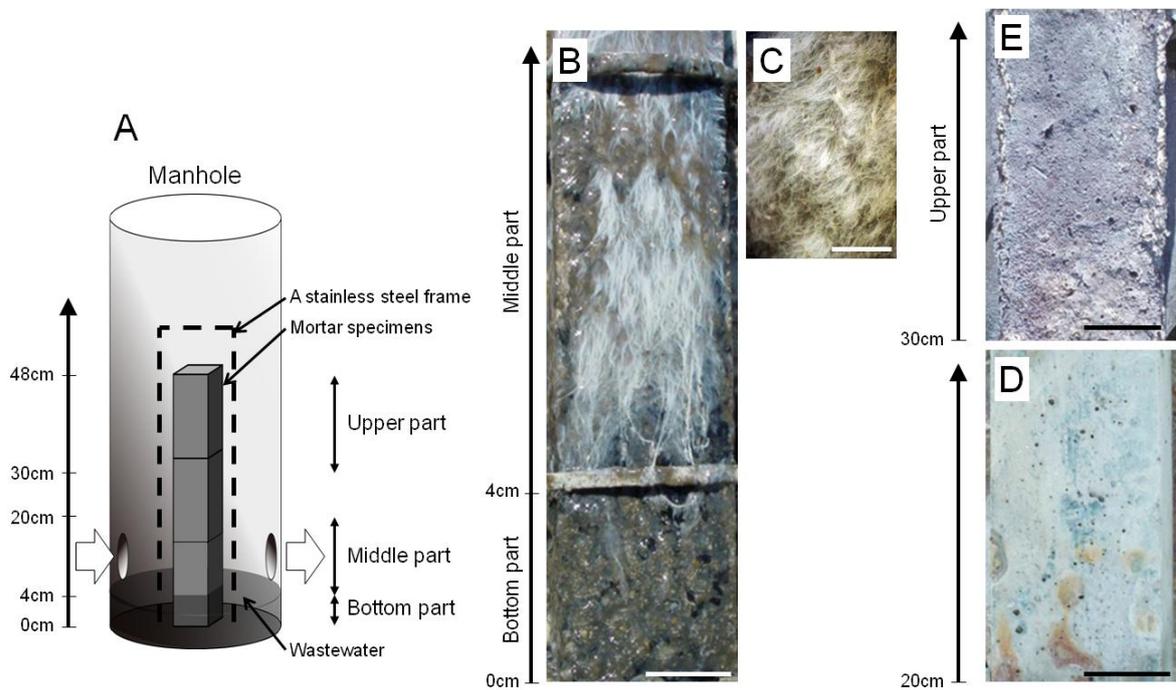
## 15 **2. Materials and methods**

16

### 17 **Mortar specimens.**

18 Mortar specimens were prepared by mixing cement, sand and water at a weight ratio  
19 of 2.5:5:1, according to the JIS (Japanese Industrial Standards) R 5201. The resulting paste  
20 was put into a frame (40 by 40 by 160 mm) and allowed to solidify. The specimens were  
21 cured in moist air for 24 h and then cured in water at 20°C for 14 days. The specimens were  
22 placed in a stainless steel frame and then installed in a manhole connecting to a sewer pipe  
23 from a sludge-thickening tank (Fig. 1) in Hachinohe, Japan, which has exhibited severe

1 corrosion of concrete sewer pipes. Returned water flowed into this manhole for 45 min of  
2 each hour. Three mortar specimens were piled vertically on the bottom of the manhole, so  
3 that the top of a set of specimens was 48 cm above the bottom of the manhole. Three sets of  
4 the piled mortar specimens were placed in the manhole.  
5



6  
7 **Fig. 1.** A schematic drawing (A) and photograph of the mortar specimens placed in a  
8 manhole which is severely corroded. (B) Biofilms developed in the bottom and the  
9 middle parts of the manhole. (C) A close-up view of white filamentous biofilms  
10 developed on the middle biofilm surface. (D) A sound mortar specimen 20 to 30 cm  
11 above the bottom of the manhole. (E) A corroded mortar specimen in the upper part of the  
12 manhole. White arrows in the panel A indicate flow direction of wastewater. Vertical  
13 axes indicate the distance from the bottom of the manhole. Scale bars indicate 20 mm (B,

1 D and E) and 2 mm (C).

2

### 3 **DNA extraction and PCR amplification.**

4 The mortar specimens were taken at day 105 after placing them in the manhole.  
5 Approximately 0.5 mL of the corroded materials and biofilm samples were collected from  
6 the mortar surface by scraping with a clean metal spatula. Each sample was immediately  
7 transferred to a separate, sterile 1.5 mL centrifuge tube containing sterilized and distilled  
8 water and then the tubes were shaken vigorously for 3 min for subsequent treatment (each of  
9 these samples is defined hereafter as a master sample). After large particles were allowed to  
10 settle for one minute, the master samples were directly used for 16S rRNA gene-cloning  
11 analysis. Total DNA was extracted directly from each master sample (an approximately 0.2  
12 mL subsample) with a FastDNA spin kit for soil (Bio 101; Qbiogene, Inc., Carlsbad, CA) as  
13 described in the manufacturer's instructions. 16S rRNA gene fragments were amplified  
14 from the extracted total DNA with *Taq* DNA polymerase (TaKaRa Bio, Inc., Ohtsu, Japan)  
15 by using bacterial primer sets 11F (**Weisburg et al., 1991**) and 1492R (**Weisburg et al.,**  
16 **1991**). The PCR products were electrophoresed on a 1% (wt/vol) agarose gel and purified  
17 with a WIZARD PCR Preps DNA purification system (Promega). To reduce the possible  
18 PCR bias, the 16S rRNA gene was amplified in duplicate tubes for each sample, and all four  
19 tubes were combined for the next cloning step.

20

### 21 **Cloning and sequencing of the 16S rRNA gene and phylogenetic analysis.**

22 The purified PCR products were ligated into a qCR-XL-TOPO vector with a TOPO  
23 XL PCR cloning kit (Invitrogen, Carlsbad, CA). The ligated products were transformed into

1 TOP10-competent *Escherichia coli* cells (Invitrogen). Plasmids were extracted from the  
2 cloned cells and purified with a Wizard Plus Minipreps DNA purification system (Promega).  
3 Nucleotide sequencing was performed with an automatic sequencer (3100 Avant genetic  
4 analyzer; Applied Biosystems). All sequences were checked for chimeric artifacts by using  
5 a CHECK\_CHIMERA program from the Ribosomal Database Project (**Maidak et al.,**  
6 **1997**). Partial sequences (approximately 600 bp) were compared with similar sequences of  
7 the reference organisms by a BLAST search (**Altschul et al., 1997**). Sequences with 97% or  
8 greater similarity were grouped into operational taxonomic units (OTUs) by a  
9 SIMILARITY\_MATRIX program from the Ribosomal Database Project (**Maidak et al.,**  
10 **1997**).

11

## 12 **Fluorescent in situ hybridization.**

13 For fluorescent in situ hybridization (FISH) analysis, subsamples of various volumes  
14 were taken from the master sample after large particles were allowed to settle for one minute  
15 and mixed with the equal amounts of fresh 8% paraformaldehyde solution (the final  
16 concentration was 4%). After fixation with a 4% paraformaldehyde solution for 4 h at 4°C,  
17 samples were washed three times with phosphate-buffered saline (10 mM sodium phosphate  
18 buffer, 130 mM sodium chloride [pH 7.2]) using sequential centrifugation (10 min at 10,000  
19 × g) and resuspension (**Peccia et al., 2000**). After the washing steps, samples were spotted  
20 onto a gelatin-coated glass slide with six glass surface windows separated by a hydrophobic  
21 coating (**Okabe et al., 1999a**). In situ hybridization was performed according to the  
22 procedure described by Amann (**Amann, 1995**) and Okabe et al. (**Okabe et al., 1999a**). The  
23 16S rRNA-targeted oligonucleotide probes used in this study were EUB338 (**Amann et al.,**

1 1990), EUB338II (Daims et al., 1999), and EUB338III (Daims et al., 1999) for mostly  
2 eubacteria, SRB385 (Amann et al., 1990) and SRB385Db (Rabus et al., 1996) for  
3 sulfate-reducing bacteria, G123T (Kanagawa et al., 2000) for *Thiothrix* spp., and SNA  
4 (Wagner et al., 1994) for *Sphaerotilus natans*. To detect all bacteria, probes EUB338,  
5 EUB338II, and EUB338III were used in an equimolar mixture (EUB338mix) (Daims et al.,  
6 1999). The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethyl  
7 rhodamine 5-isothiocyanate (TRITC). A model LSM510 confocal laser-scanning  
8 microscope (CLSM; Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (488  
9 nm) and a HeNe laser (543 nm) was used.

10

#### 11 **Microsensor measurement.**

12 For microsensor measurements, the mortar specimens were placed in the flow cell  
13 reactor (4.0 liter) that was filled with the synthetic medium. The synthetic medium consisted  
14 of MgCl<sub>2</sub> (450 μM), CaCl<sub>2</sub> (40 μM), NH<sub>4</sub>Cl (1,520 μM), MgSO<sub>4</sub> (1,000 μM), NaCl (1,000  
15 μM), K<sub>2</sub>HPO<sub>4</sub> (4,600 μM), KH<sub>2</sub>PO<sub>4</sub> (3,700 μM), NaHCO<sub>3</sub> (1,000 μM), glucose (500 μM),  
16 EDTA ([ethylenediaminetetraacetic acid] 270 μM), and Na<sub>2</sub>S•9H<sub>2</sub>O (various amounts). O<sub>2</sub>  
17 concentration in the medium was kept at a minimum for bottom biofilm measurements,  
18 approximately 50 μM for middle biofilm measurements, and approximately 150 μM for  
19 corroded material measurements by continuous bubbling with N<sub>2</sub> and air, which also  
20 resulted in sufficient mixing of the medium. The pH of the medium was adjusted to 7 to 8  
21 and the reactor was kept at room temperature (20 to 23°C) for all of the measurements. The  
22 biofilms were acclimated in the medium for at least 3 h before measurements to ensure that  
23 steady-state profiles were obtained. In contrast, the concentration profiles in the corroded

1 materials were measured within 1 h in order to avoid significant change in pH in the  
2 corroded materials due to diffusion out of H<sup>+</sup> from the corroded materials. The in situ  
3 steady-state concentration profiles of H<sub>2</sub>S, pH, ORP and O<sub>2</sub> in the biofilms and corroded  
4 materials were measured, using microsensors as described by **Okabe et al. (1999b)**. H<sub>2</sub>S  
5 (**Jeroschewski et al., 1996**), pH (**De Beer et al., 1997**), ORP (**Jang et al., 2005**) and O<sub>2</sub>  
6 (**Revsbech, 1989**) were prepared, calibrated, and used as described previously. At least  
7 three concentration profiles were measured for each chemical species.

8

### 9 **Chemical analyses.**

10 The COD concentrations were determined according to Standard Methods (**APHA,**  
11 **1995**). The SO<sub>4</sub><sup>2-</sup> concentrations were measured with an ion chromatograph (model DX-100  
12 with an AS4A column; Nippon DIONEX, Osaka, Japan) after filtration with  
13 0.2- $\mu$ m-pore-sized membrane filters (DISMIC-13CP; Advantec Co., Ltd.). The  
14 concentrations of T-H<sub>2</sub>S (total dissolved sulfide defined as the sum of H<sub>2</sub>S, HS<sup>-</sup> and S<sup>2-</sup>)  
15 were determined by the methylene blue method (**Cline, 1969**). For measurements of the  
16 gaseous H<sub>2</sub>S, gaseous samples were immediately transferred to 50 mL screw capped tubes  
17 containing 10 mL of a 10% w/v zinc acetate solution and the gaseous H<sub>2</sub>S was precipitated  
18 as ZnS. The ZnS concentrations were determined by the methylene blue method and then  
19 the gaseous H<sub>2</sub>S concentrations were calculated. The NH<sub>4</sub><sup>+</sup> concentrations were determined  
20 using ion chromatographs (DX-100, DIONEX, Sunnyvale, CA) equipped with an IonPac  
21 CS3 cation column. The ORP and pH were directly determined by using an ORP and a pH  
22 electrode, respectively.

23

### 1 3. Results

2

#### 3 **Chemical parameters of the returned water and appearance of mortar specimens.**

4

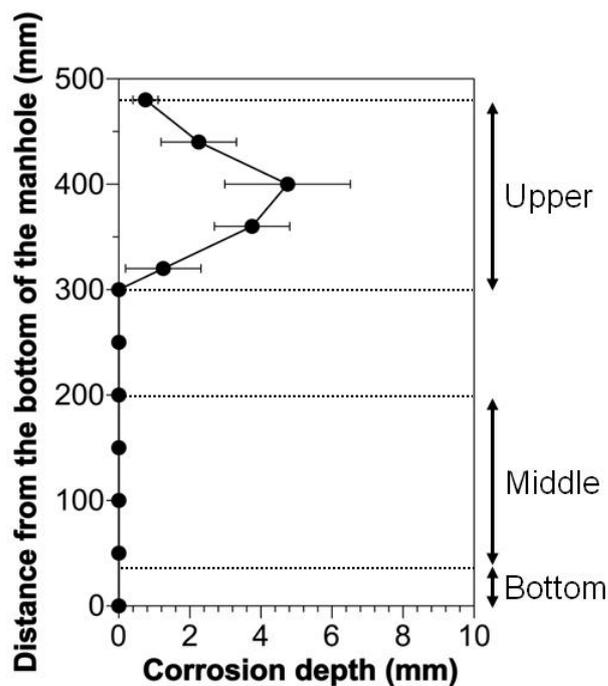
5 Average ( $\pm$  standard deviation) concentration of dissolved T-H<sub>2</sub>S in the returned water  
6 flowing through the manhole was  $236 \pm 174 \mu\text{M}$ . Dissolved oxygen of  $77 \pm 20 \mu\text{M}$  coexisted  
7 in the flowing water due to a high-turbulence flow. Consequently, ORP was relatively high  
8 ( $34 \pm 92 \text{ mV}$ ) although T-H<sub>2</sub>S was present. Because the water coming from a  
9 sludge-thickening tank was anaerobic, NH<sub>4</sub><sup>+</sup> ( $3900 \pm 1300 \mu\text{M}$ ) and chemical oxygen  
10 demand ( $7060 \pm 2375 \mu\text{M}$ ) concentrations were high. pH was  $6.9 \pm 0.2$ . The gaseous H<sub>2</sub>S  
11 concentration in the manhole atmosphere was  $30 \pm 20 \text{ ppmv}$ . Oxygen and carbon dioxide  
12 were at atmospheric concentrations.

13 Appearance of surface of the mortar specimens after 105 days of installation in the  
14 manhole was different from the bottom to the top of the manhole (Fig. 1). In the bottom part  
15 (from the bottom to 4 cm) the dark-gray gel-like biofilm was developed on the mortar  
16 specimens (Fig. 1B) where the mortar specimens were always submerged in the wastewater  
17 (see Materials and methods). The biofilm thickness was about 3 mm. In the middle part (4 to  
18 20 cm from the bottom of the manhole), where the mortar specimens were periodically  
19 submerged in the wastewater, the gel-like biofilm similar to that in the bottom part was  
20 developed (Fig. 1B). White filamentous biofilms covered the gel-like biofilm (Figs. 1B and  
21 1C). The mortar specimens placed 20 cm above the bottom of the manhole were always  
22 above the water line. The mortar specimens placed in the upper part (30 cm above the  
23 bottom of the manhole) were slightly corroded (Fig. 1E). Neither biofilm nor corroded

1 materials were found 20 to 30 cm above the bottom of the manhole (Fig. 1D). Although the  
2 reason for this has been unclear, one possible reason is lower abundance of SOB on the  
3 mortar surface just above the water level as reported by Vincke et al. (2001). Concrete  
4 surface of the manhole was severely corroded especially at a manhole opening and  
5 corrosion level decreased toward the bottom of the manhole, which agreed with the results  
6 for the mortar specimens.

7 After one year, the mortar specimens were washed using a brush to remove corroded  
8 materials, and corrosion depth (i.e., loss of the mortar specimens) were examined (Fig. 2).  
9 The thickness of the mortar specimen was 40 mm below 30 cm from the bottom of the  
10 manhole, indicating these parts were not corroded. In contrast, the upper parts of the  
11 specimen (i.e., 30 to 48 cm above the bottom of the manhole) were corroded. The part at 40  
12 cm from the bottom of the manhole was most severely corroded and the corrosion depth was  
13  $4.8 \pm 1.8$  mm. Thus, a corrosion rate was estimated to be  $4.8 \pm 1.8$  mm/year.

14



15

1 **Fig. 2** Corrosion depth of the mortar specimen after one year of installation in the manhole.

2

### 3 **Microbial community structures in biofilms and corroded materials.**

4 Microbial communities in the middle biofilms, bottom biofilms and the corroded  
5 materials developed on the mortar specimens were analyzed by 16S rRNA gene-cloning  
6 analysis. Three clone libraries (the bottom biofilm [68 clones], the middle biofilm [62  
7 clones], and the corroded materials [101 clones]) were constructed. The clones retrieved  
8 from the bottom biofilm samples were grouped into 36 OTUs and assigned to 7 phyla (Table  
9 1). The distribution of clones among phyla was as follows: *Betaproteobacteria*, 38%;  
10 *Actinobacteria*, 18%; *Alphaproteobacteria*, 15%; *Firmicutes*, 10%; *Gammaproteobacteria*,  
11 10%; *Bacteroidetes*, 7%; and *Deltaproteobacteria*, 1%. One clone was related to an  
12 anaerobic dehalogenating SRB, *Desulfomonile tiedjei*, with 95.0% similarity.

13 The clones retrieved from the middle biofilm samples comprised 4 phyla with 17  
14 OTUs (Table 2). In contrast to the bottom biofilm samples the phylum  
15 *Gammaproteobacteria* represented the most predominant phylum (55% of detection  
16 frequency). The second most abundant phylum was the *Betaproteobacteria* (24% of the total  
17 clones). The phylum *Alphaproteobacteria* represented 13% of the total clones, followed by  
18 the *Bacteroidetes* (8% of detection frequency). About 18% of clones (11/62) were related to  
19 *Thiothrix* sp. strain CT3 with 96.6% similarity and one clone was related to *Bosea*  
20 *thiooxidans* with 98.7% similarity.

21 The clones obtained from the corroded materials were grouped into 6 phyla that  
22 comprised 21 OTUs (Table 3). Similar to the middle biofilm sample, the phylum  
23 *Gammaproteobacteria* represented the most predominant phylum (57% of detection

1 frequency). Six clones were related to *Halothiobacillus neapolitanus*. The second most  
2 abundant phylum was the *Firmicutes* (17% of detection frequency), followed by the  
3 *Betaproteobacteria* (12% of detection frequency), the *Actinobacteria* (9% of detection  
4 frequency), the *Alphaproteobacteria* (4% of detection frequency), and the *Bacteroidetes*  
5 (1% of detection frequency).

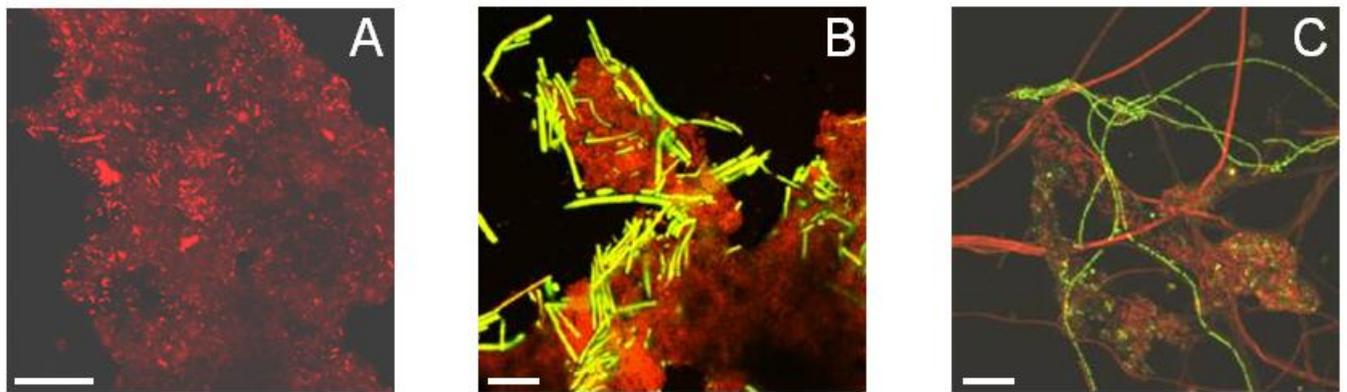
6

### 7 **Bacteria present in biofilms.**

8       According to 16S rRNA gene-cloning analysis, the SRB385 and SRB385Db probes  
9 specific for SRB (e.g., *Desulfovibrionales*, *Desulfobacterales* and *Desulfuromonales*) were  
10 used to investigate presence of SRB in the bottom biofilm. The bacteria hybridized with the  
11 SRB385 and SRB385Db probes were detected throughout the bottom biofilm (Fig. 3A).

12       Moreover, G123T and SNA probes were used to investigate presence of *Thiothrix* spp.  
13 and *Sphaerotilus natans* in the middle biofilms (Figs. 3B and 3C). The FISH results  
14 revealed that the G123T and SNA probes exclusively hybridized to filamentous cells, which  
15 appeared kinked or twisted. Very bright signals of the cells reflected high rRNA contents,  
16 indicating that they were active. The filamentous cells were mainly detected in the oxic  
17 biofilm surface, while they were hardly detected in the deeper parts of the middle biofilm.

18



1  
 2 **Fig. 3** Confocal laser scanning microscope images showing presence of sulfate-reducing  
 3 and sulfur-oxidizing bacteria in the bottom (panel A) and middle (panels B and C)  
 4 biofilms. (A) FISH with TRITC-labeled SRB385 and SRB385Db probes (red). (B) FISH  
 5 with FITC-labeled G123T probe (yellow) and TRITC-labeled EUB338mix probe (red).  
 6 (C) FISH with FITC-labeled SNA probe (green) and TRITC-labeled BET42a probe (red).  
 7 Scale bars indicate 20  $\mu\text{m}$ .

8

9 **Sulfide production and oxidation in biofilms and corroded materials.**

10 Steady-state concentration profiles of T-H<sub>2</sub>S, pH, O<sub>2</sub>, and ORP in the middle and  
 11 bottom biofilms and the corroded materials were measured with microsensors (Fig. 4). The  
 12 thickness of the bottom biofilm was about 1,000  $\mu\text{m}$  after 7 days of installation of the mortar  
 13 specimens in the manhole. A T-H<sub>2</sub>S concentration was 60  $\mu\text{M}$  at a depth of 700  $\mu\text{m}$  and  
 14 decreased toward the biofilm surface (Fig. 4A). T-H<sub>2</sub>S was still present (10  $\mu\text{M}$ ) at the  
 15 biofilm surface and diffused out of the bottom biofilm. The concentration gradient of T-H<sub>2</sub>S  
 16 was steeper in the upper parts of the biofilm, indicating high sulfate-reducing activity. ORP  
 17 significantly decreased in the upper parts of the biofilm and was unchanged in the deeper  
 18 parts, corresponding well with the concentration profile of T-H<sub>2</sub>S. pH was unchanged

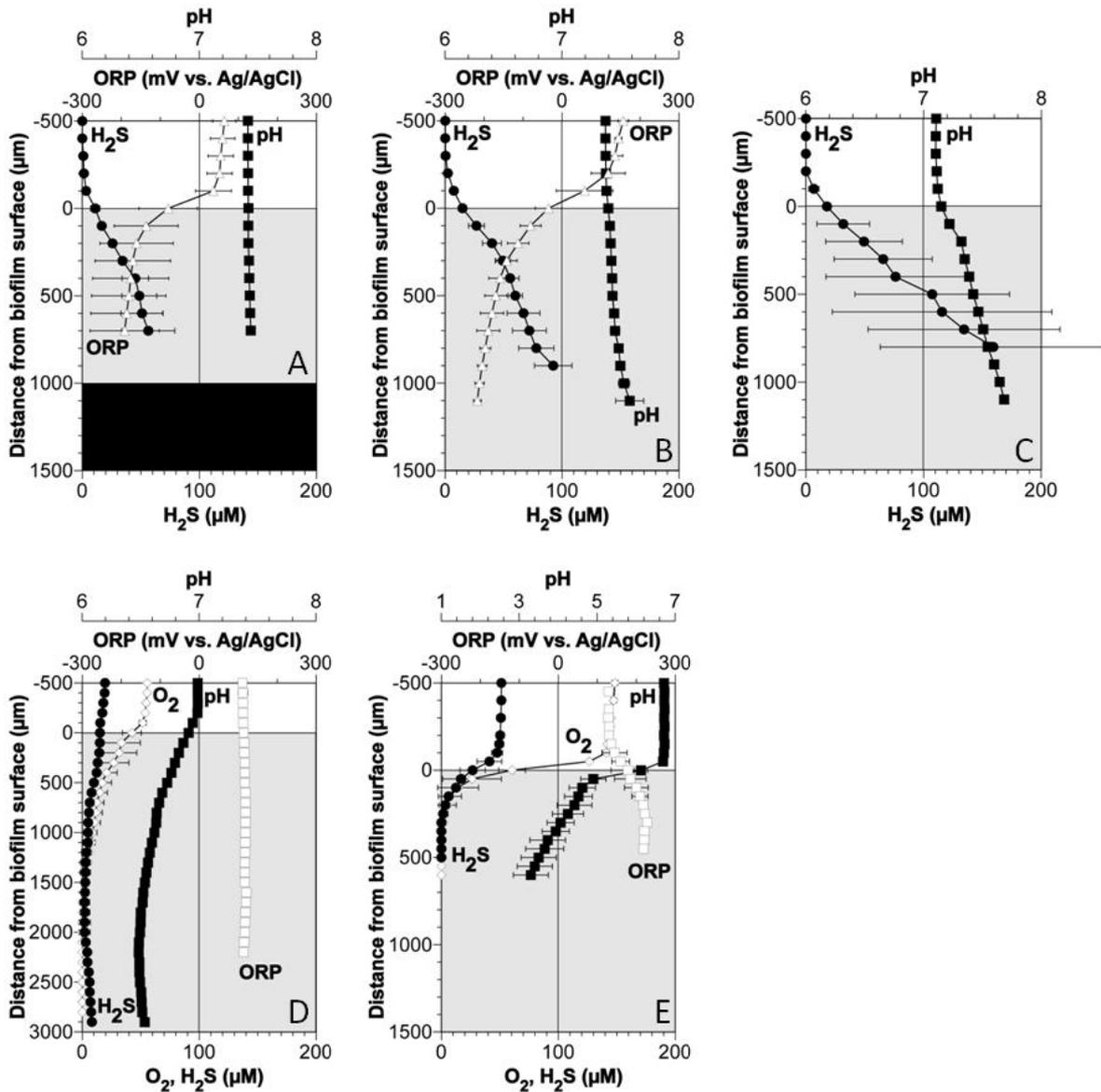
1 throughout the biofilm. The thickness of the bottom biofilm increased to ca. 1,500  $\mu\text{m}$  after  
2 28 days of installation of the mortar specimens (Fig. 4B). The maximal T-H<sub>2</sub>S concentration  
3 was higher (90  $\mu\text{M}$ ) than that in the 7-day-old biofilm, which demonstrated an increasing  
4 sulfate-reducing activity. A significant decrease in ORP reflected higher sulfate-reducing  
5 activity. After 105 days of installation of the mortar specimens, the bottom biofilm became  
6 thicker. The T-H<sub>2</sub>S concentration in the deeper parts of the biofilm increased up to 160  $\mu\text{M}$   
7 (Fig. 4C), indicating a further increase in sulfate-reducing activity. Relatively big standard  
8 deviations reflected heterogeneity of the biofilm structure.

9 Although the middle biofilm was very thin after 7 days of installation of the mortar  
10 specimens, the thickness of the middle biofilm was about 1,000  $\mu\text{m}$  after 28 days. A T-H<sub>2</sub>S  
11 concentration was less than 10  $\mu\text{M}$  at a depth of 700  $\mu\text{m}$  (data not shown), indicating that the  
12 sulfate-reducing activity was even lower than that in the bottom biofilm. After 105 days of  
13 installation of the mortar specimens, the middle biofilm grew thicker. However, A T-H<sub>2</sub>S  
14 concentration was very low and T-H<sub>2</sub>S production was restricted in the deeper 2000  $\mu\text{m}$  of  
15 the biofilm because of O<sub>2</sub> penetration into the upper parts of the biofilm (Fig. 4D). The  
16 T-H<sub>2</sub>S provided from the bulk solution as well as the deeper parts of the biofilm was  
17 oxidized in the upper oxic parts of the biofilm.

18 In the corroded materials the pH was around 3 in the deeper parts after 105 days of  
19 installation of the mortar specimens (Fig. 4E), indicating that the in situ pH level of the  
20 corroded materials was significant low as reported previously (**Okabe et al., 2007**).  
21 Increasing pH in the upper parts was attributed to diffusion out of H<sup>+</sup> from the corroded  
22 materials. The O<sub>2</sub> concentration drastically decreased within the uppermost 400  $\mu\text{m}$  by  
23 mainly a chemical O<sub>2</sub> consumption process. A T-H<sub>2</sub>S concentration drastically decreased

1 within the oxic parts due to chemical T-H<sub>2</sub>S oxidation. It was also likely that dissolved H<sub>2</sub>S  
 2 gas was emitted due to lower pH, because dissolved H<sub>2</sub>S rather than HS<sup>-</sup> became the  
 3 dominant species at lower pH taking into account the first dissociation constants for H<sub>2</sub>S  
 4 (pK<sub>a1</sub> = 7.04) (Zhang et al., 2008).

5



6

7 **Fig. 4.** Steady-state concentration profiles of T-H<sub>2</sub>S, pH, O<sub>2</sub>, and ORP in the bottom and  
 8 middle biofilms and the corroded materials. The bottom biofilms at day 7 (A), day 28 (B)  
 9 and day 105 (C), the middle biofilms at day 105 (D) and the corroded materials at day 105

1 (E). The profiles are average values ( $n = 3$ ) and error bars represent the standard  
2 deviations of triplicate measurements. Zero on the vertical axis corresponds to the surface  
3 of the granule.

#### 4 5 **4. Discussion**

##### 6 7 **Bottom biofilm.**

8 Microsensor measurements revealed that T-H<sub>2</sub>S was produced in and diffused out of  
9 the bottom biofilms (Figs. 4A, 4B and 4C). The net T-H<sub>2</sub>S flux ( $J(\text{H}_2\text{S})$ ) (i.e., a T-H<sub>2</sub>S  
10 emission rate) evaluated from the T-H<sub>2</sub>S concentration profiles was  $0.033 \pm 0.005$   
11  $\mu\text{mol}/\text{cm}^2/\text{h}$  at day 7 (Fig. 4A).  $J(\text{H}_2\text{S})$  increased to  $0.063 \pm 0.013 \mu\text{mol}/\text{cm}^2/\text{h}$  at day 28 (Fig.  
12 4B) and  $0.091 \pm 0.053 \mu\text{mol}/\text{cm}^2/\text{h}$  at day 105 (Fig. 4C). These rates were similar to those of  
13 microaerophilic wastewater biofilms (Ito et al., 2002; Okabe et al., 1999b), a trickling  
14 filter biofilm (Kühl and Jorgensen, 1992), biofilms grown in an activated sludge aeration  
15 basin (Santegoeds et al., 1998), and a *Beggiatoa* inhabited marine sediment (Preisler et al.,  
16 2007). This result indicates the successional development of SRB community in the bottom  
17 biofilm. One clone was affiliated with an anaerobic dehalogenating SRB, *Desulfomonile*  
18 *tiedjei*, with 95.0% similarity (Table 1). This bacterium was isolated from anaerobic sewage  
19 sludge (DeWeerd et al., 1990). Small number of clones related to SRB in the bottom  
20 biofilm clone library is probably due to low density of the SRB in the biofilm, inherent PCR  
21 biases, resulting from differences in the amplification efficiency of templates (Acinas et al.,  
22 2005) and in DNA extraction efficiency from environmental samples (Martin-Laurent et  
23 al., 2001), and the insufficient number of clones sequenced. However, we are convinced

1 that SRB present in the bottom biofilm produced T-H<sub>2</sub>S, because FISH results confirmed  
2 presence of SRB in the bottom biofilm (Fig. 3A).

3 The microbial community of the bottom biofilm, which consisted of 7 phylogenetic  
4 groups of clones at the phylum level, was most diversified among three samples (Table 1). It  
5 is probably explained by high organic compound and nutrient availability provided from  
6 wastewater. One clone was related to obligatory mixotrophic sulfur-oxidizing bacteria  
7 (SOB), *Thiothrix nivea*, with 94.4% similarity (Table 1). The versatile physiological ability  
8 of *T. nivea* to utilize reduced sulfur compounds and various organic compounds (acetate,  
9 malate, pyruvate, and oxaloacetate) (McGlannan and Makemson, 1990) might provide a  
10 competitive advantage in the bottom biofilm under microaerophilic conditions with high  
11 concentration of organic compounds (Okabe et al., 2007). *T. nivea* have been found in  
12 activated sludge treating paper and board mill wastes (Kim et al., 2002) and in  
13 H<sub>2</sub>S-containing flowing water (McGlannan and Makemson, 1990). However, the in situ  
14 T-H<sub>2</sub>S oxidation activities were not observed in the bottom biofilm (Figs. 4A, 4B and 4C),  
15 probably due to low abundance of SOB and scarcity of O<sub>2</sub>.

16

### 17 **Middle biofilm.**

18 Because the returned water was flowing into the manhole for 45 min of each hour, the  
19 middle biofilm was exposed periodically to the oxic sewer atmosphere and the returned  
20 water containing T-H<sub>2</sub>S. Periodic alternation resulted in significant growth of white  
21 filamentous bacteria on the surface of the middle biofilm (Figs. 1B and 1C). The 16S rRNA  
22 gene-cloning analysis revealed that the clones related to autotrophic SOB, *Thiothrix* sp.  
23 strain CT3, dominated the middle biofilm clone library at day 105 (Table 2). This bacterium

1 was isolated from an activated sludge treatment plant (Rossetti et al., 2003) and has been  
2 detected on mortar surface at the same sampling site (Okabe et al., 2007) and in wastewater  
3 biofilms growing under microaerophilic conditions (Okabe et al., 2005) where T-H<sub>2</sub>S was  
4 present. This strain has been reported as filamentous bacteria that grow on reduced sulfur  
5 compounds and accumulate sulfur granules internally (Rossetti et al., 2003). Hence, the  
6 biofilms colonized by *Thiothrix* sp. strain CT3 were characterized as white filamentous  
7 biofilms (Okabe et al., 2005). FISH analysis with the G123T probe confirmed that  
8 *Thiothrix*-like filamentous bacteria covered the biofilm surface (Fig. 3B). *Thiothrix* spp. can  
9 use nitrate as an electron acceptor (Nielsen et al., 2000). Although nitrate utilization in  
10 *Thiothrix* spp. was not investigated by a nitrate microsensor in this study, there was a  
11 possibility that they used nitrate generated in oxidation of ammonium in the oxic  
12 filamentous biofilms. In addition, one clone was related to chemolithoheterotrophic *Bosea*  
13 *thiooxidans* (Table 2), which is capable of oxidizing reduced inorganic sulfur compounds  
14 and grows on a wide range of organic substrates (Das et al., 1996). Thus, this strain might  
15 be also involved in T-H<sub>2</sub>S oxidation in the middle biofilm (Fig. 4D). The strain CT3 and *B.*  
16 *thiooxidans* can produce sulfate as the end product of oxidation of reduced sulfur  
17 compounds (Das et al., 1996; Rossetti et al., 2003).

18 microsensor measurements demonstrated that T-H<sub>2</sub>S was oxidized in the upper  
19 filamentous biofilm and subsequently pH decreased (Fig. 4D). Interestingly, T-H<sub>2</sub>S was  
20 regenerated in the deeper anaerobic parts of the biofilm and then the T-H<sub>2</sub>S generated was  
21 also completely oxidized in the upper filamentous biofilm (Fig. 4D). This result clearly  
22 indicated occurrence of simultaneous H<sub>2</sub>S production and consumption in the middle  
23 biofilm. Low sulfate-reducing activity in the deeper parts of the middle biofilm was

1 probably due to transport limitations of sulfate and organic compounds into the deeper layer  
2 of the biofilm. Based on these results, we concluded that the deeper gel-like layer of the  
3 middle biofilm around the water level played an important role to prevent deterioration of  
4 concrete caused by corrosive compounds (e.g., sulfuric acid) produced in the upper oxic  
5 layer of the middle biofilm. Hence, the mortar surface in the middle part of the manhole was  
6 preserved from corrosive attack, and concrete corrosion was restricted to the upper parts in  
7 the sewer system (e.g., the crown of sewer pipes). These results agreed with a previous  
8 report (Vincke et al., 2001). In addition, it is very likely that returned water diluted the  
9 corrosive compounds produced in the biofilm surface. However, the most severe damage of  
10 the real sewer pipes was found at the water level in the study site (data not shown). Similar  
11 findings have been reported in previous reports (Davis et al., 1998; Mori et al., 1992). It  
12 could be attributed with erosive impact by flow of wastewater and chemical reactions (e.g.,  
13 carbonation and chloride erosion).

14

#### 15 **Corroded materials.**

16 At day 105, mortar specimens placed 30 cm above the bottom of the manhole were  
17 slightly corroded (Fig. 1E). In the clone library of the corroded materials (Table 3), six  
18 clones were related to SOB, *Halothiobacillus neapolitanus*. Unlike *Thiothrix* spp. and *B.*  
19 *thiooxidans* that were detected in the middle biofilm, *H. neapolitanus* does not have an  
20 ability to grow mixotrophically but can grow in or adapt to a wider pH range (pH 4.5 to 8.5)  
21 (Kelly and Harrison, 1989). This is probably the reason why *H. neapolitanus* were  
22 detected in the corroded materials, where the concentration of organic carbon were  
23 relatively low and pH was around 3 (Fig. 4E). This result suggests that these SOB were

1 responsible for the production of corrosive compounds (e.g., sulfuric acid) on the mortar  
2 surface. **Okabe et al.** (2007) investigated the succession of SOB responsible for concrete  
3 corrosion at the same sampling site and demonstrated that the predominant SOB species  
4 shifted depending on the pH and trophic properties of each SOB. *H. neapolitanus* were  
5 detected in the slightly corroded concrete after 83 days, which agrees with our results. It  
6 indicates that the period we studied (i.e., 105 days) was not enough to reveal the succession  
7 of microbial community structures responsible for the MICC.

8       The 16S rRNA gene-cloning analysis revealed that the *Bacteria* other than SOB  
9 coexisted with SOB in the corroded materials (Table 3). Members of the phyla  
10 *Gammaproteobacteria*, *Firmicutes*, *Betaproteobacteria*, *Actinobacteria*, and  
11 *Alphaproteobacteria* dominated the clone library of the corroded materials. These phyla  
12 have been also found in corroded mortar samples at the same sampling site (**Okabe et al.**,  
13 **2007**) and corroded concrete walls of sewer pipes (**Vincke et al.**, **2001**). Most of these  
14 clones were related to heterotrophic bacteria (e.g., *Pseudoxanthomonas mexicana* (**Thierry**  
15 **et al.**, **2004**) and *Xanthomonas axonopodis* (**Malik et al.**, **2003**), acidophilic bacteria (e.g.,  
16 *Bacillus acidicola* (**Albert et al.**, **2005**), or halo-tolerant bacteria (e.g., *Halomonas*  
17 *gudaoense* (**Wang et al.**, **2007**)). Volatile organic compounds present in the sewer  
18 atmosphere might support the growth of these heterotrophic bacteria in the corroded  
19 materials.

20

## 21 **5. Conclusions**

22       The microbial community structures and the in situ H<sub>2</sub>S production and consumption  
23 rates in biofilms and corroded materials developed on mortar specimens in a corroded sewer

1 system was investigated by 16S rRNA gene-cloning analysis, FISH and microsensor  
2 measurements. The results in this study indicated that T-H<sub>2</sub>S was provided from the  
3 biofilms in the bottom part of the manhole. The T-H<sub>2</sub>S derived from the sludge settling tank  
4 was also the source of T-H<sub>2</sub>S. Then the dissolved H<sub>2</sub>S was emitted to the sewer atmosphere  
5 and oxidized to corrosive compounds on the mortar surface in the upper and middle parts of  
6 the manhole. However, only the upper part of the mortar specimens was corroded. In the  
7 biofilm developed around the air-water interface (i.e., the middle biofilm), the corrosive  
8 compounds generated in the upper filamentous biofilm were reduced in the deeper gel-like  
9 biofilm. This was the reason why the mortar surface in the middle part of the manhole was  
10 preserved from corrosive attack, and concrete corrosion was restricted to the upper parts in  
11 the sewer system (e.g., the crown of sewer pipes). In situ analyses of microbial community  
12 and their activities involved in the MICC process would provide us with valuable  
13 information to efficiently control, prevent, and/or predict MICC process in sewer systems.

14

## 15 **ACKNOWLEDGMENTS**

16 We thank the persons concerned with the wastewater treatment facility in Hachinohe,  
17 Japan, for allowing us to install the mortar specimens. This work was partially supported by  
18 a grant-in-aid (13650593) for developmental scientific research from the Ministry of  
19 Education, Science and Culture of Japan, and by funding from the Maeda Engineering  
20 Foundation.

21

## 22 **References**

23

- 1 Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., Polz, M.F., 2005. PCR-induced  
2 sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries  
3 constructed from the same sample. *Appl. Environ. Microbiol.* 71, 8966–8969.
- 4 Albert, R.A., Archambault, J., Rosselló-Mora, R., Tindall, B.J., Matheny, M., 2005.  
5 *Bacillus acidicola* sp. nov., a novel mesophilic, acidophilic species isolated from  
6 acidic *Sphagnum* peat bogs in Wisconsin. *Int. J. Syst. Evol. Microbiol.* 55(5),  
7 2125–2130.
- 8 Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J.,  
9 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search  
10 programs. *Nucleic Acids Res.* 25(17), 3389–3402.
- 11 Amann, R.I., 1995. In situ identification of microorganisms by whole cell hybridization  
12 with rRNA-targeted nucleic acid probes. In: Akkermans, A.D.L., van Elsas, J.D., de  
13 Bruijn, F.J., (Eds.), *Molecular Microbial Ecology Manual*. Kluwer Academic  
14 Publishers, Dordrecht, The Netherlands, pp. 1–15.
- 15 Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990.  
16 Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for  
17 analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56(6), 1919–1925.
- 18 APHA, 1995. *Standard Methods for the Examination of Water and Wastewater*, nineteenth  
19 ed. American Publishers Health Association, Washington DC, USA.
- 20 Bentzen, G., Smith, A.T., Bennet, D., Webster, N.J., Reinholt, F., Sletholt, E., Hobson, J.,  
21 1995. Controlled dosing of nitrate for prevention of H<sub>2</sub>S in a sewer network and the  
22 effects on the subsequent treatment process. *Water Sci. Technol.* 31(7), 293–302.
- 23 Cadena, F., Peters, R.W., 1988. Evaluation of chemical oxidizers for hydrogen-sulfide

- 1 control. J. Water Pollut. Control Fed. 60, 1259–1263.
- 2 Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters.  
3 Limnol. Oceanogr. 14(3), 454–458.
- 4 Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific  
5 probe EUB338 is insufficient for the detection of all bacteria: development and  
6 evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.
- 7 Das, S.K., Mishra, A.K., Tindall, B.J., Rainey, F.A., Stackebrandt, E., 1996. Oxidation of  
8 thiosulfate by a new bacterium, *Bosea thiooxidans* (strain BI-42) gen. nov., sp. nov.:  
9 analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing.  
10 Int. J. Syst. Bacteriol. 46(4), 981–987.
- 11 Davis, J.L., Nica, D., Shields, K., Roberts, D.J., 1998. Analysis of concrete from corroded  
12 sewer pipe. Int. Biodeter. Biodegr. 42(1), 75–84.
- 13 De Beer, D., Schramm, A., Santegoeds, C.M., Köhl, M., 1997. A nitrite microsensor for  
14 profiling environmental biofilms. Appl. Environ. Microbiol. 63, 973–977.
- 15 De Gusseme, B., De Schryver, P., De Cooman, M., Verbeken, K., Boeckx, P., Verstraete,  
16 W., Boon, N., 2009. Nitrate-reducing, sulfide-oxidizing bacteria as microbial oxidants  
17 for rapid biological sulfide removal. FEMS Microbiology Ecology 67, 151–161.
- 18 De Muynck, W., De Belie, N., Verstraete, W., 2009. Effectiveness of admixtures, surface  
19 treatments and antimicrobial compounds against biogenic sulfuric acid corrosion of  
20 concrete. Cement Concr. Compos. 31 163-170.
- 21 DeWeerd, K.A., Mandelco, L., Tanner, R.S., Woese, C.R., Suflita, J.M., 1990.  
22 *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating,  
23 sulfate-reducing bacterium. Arch. Microbiol. 154(1), 23–30.

- 1 Elovitz, M.S., von Gunten, U., Kaiser, H.P., 2000. Hydroxyl radical/ozone ratios during  
2 ozonation processes. II. The effect of temperature, pH, alkalinity and DOM properties.  
3 Ozone Sci. Eng. 22, 123–150.
- 4 Haile, T., Nakhla, G., 2009. Inhibition of microbial concrete corrosion by Acidithiobacillus  
5 thiooxidans with functionalised zeolite-A coating. Biofouling 25, 1–12.
- 6 Harrison, A.P., Jr., 1984. The acidophilic thiobacilli and other acidophilic bacteria that  
7 share their habitat. Annu. Rev. Microbiol. 38, 265–292.
- 8 Hernandez, M., Marchand, E.A., Roberts, D., Peccia, J., 2002. In situ assessment of active  
9 *Thiobacillus* species in corroding concrete sewers using fluorescent RNA probes. Int.  
10 Biodeterior. Biodegrad. 49, 271–276.
- 11 Hobson, J., Yang, G., 2000. The ability of selected chemicals for suppressing odour  
12 development in rinsing mains. Water Sci. Technol. 41(6), 165–173.
- 13 Islander, R.L., Devinsky, J.S., Mansfeld, F., Postyn, A., Shih, H., 1991. Microbial ecology  
14 of crown corrosion in sewers. J. Environ. Eng. 117, 751–770.
- 15 Ito, T., Okabe, S., Satoh, H., Watanabe, Y., 2002. Successional development of  
16 sulfate-reducing bacterial populations and their activities in a wastewater biofilm  
17 growing under microaerophilic conditions. Appl. Environ. Microbiol. 68(3),  
18 1392–1402.
- 19 Jang, A., Lee, J.-H., Bhadri, P.R., Kumar, S.A., Timmons, W., Beyette, F. R., Jr., Papautsky,  
20 I., Bishop, P.L., 2005. Miniaturized redox potential probe for in situ environmental  
21 monitoring. Environ. Sci. Technol. 39, 6191–6197.
- 22 Jayaraman, A., Mansfeld, F.B., Wood, T.K., 1999. Inhibiting sulfate-reducing bacteria in  
23 biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. J. Ind.

- 1 Microbiol. Biotechnol. 22, 167–175.
- 2 Jensen, H.S., Nielsen, A.H., Hvitved-Jacobsen, T., Vollertsen, J., 2009. Modeling of  
3 hydrogen sulfide oxidation in concrete corrosion products from sewer pipes. Water  
4 Environ. Res. 81, 365–373.
- 5 Jeroschewski, P., Steuckart, C., Köhl, M., 1996. An Amperometric microsensor for the  
6 determination of H<sub>2</sub>S in aquatic environments. Anal. Chem. 68, 4351–4357.
- 7 Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M., Wagner, M., 2000.  
8 Phylogenetic analysis of and oligonucleotide probe development for eikelboom type  
9 021N filamentous bacteria isolated from bulking activated sludge. Appl. Environ.  
10 Microbiol. 66(11), 5043–5052.
- 11 Kelly, D.P., Harrison, A.P., 1989. Genus *Thiobacillus*. In: Stanley, J.T., Bryant, M.P.,  
12 Pfennig, N., Holt, J., (Eds.), Bergey's manual of systematic bacteriology, vol. 3.  
13 Lippincott Williams & Wilkins, Baltimore, MD. pp. 1842–1858.
- 14 Kim, S.B., Goodfellow, M., Kelly, J., Saddler, G.S., Ward, A.C., 2002. Application of  
15 oligonucleotide probes for the detection of *Thiothrix* spp. in activated sludge plants  
16 treating paper and board mill wastes. Water Sci. Technol. 46(1–2), 559–564.
- 17 Köhl, M., Jørgensen, B.B., 1992. Microsensor measurements of sulfate reduction and  
18 sulfide oxidation in compact microbial communities of aerobic biofilms. Appl.  
19 Environ. Microbiol. 58, 1164–1174.
- 20 Lahav, O., Sagiv, A., Friedler, E., 2006 A different approach for predicting H<sub>2</sub>S<sub>(g)</sub> emission  
21 rates in gravity sewers. Water Res. 40, 259–266.
- 22 Londry, K.L., Suflita, J.M., 1999. Use of nitrate to control sulfide generation by  
23 sulfate-reducing bacteria associated with oil waste. J. Ind. Microbiol. Biotechnol. 22,

- 1 582–589.
- 2 Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., Woese, C.R., 1997.
- 3 The RDP (Ribosomal Database Project). *Nucleic Acids Res.* 25(1), 109–110.
- 4 Malik, A., Sakamoto, M., Hanazaki, S., Osawa, M., Suzuki, T., Tochigi, M., Kakii, K., 2003.
- 5 Coaggregation among nonflocculating bacteria isolated from activated sludge. *Appl.*
- 6 *Environ. Microbiol.* 69(10), 6056–6063.
- 7 Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G.,
- 8 Catroux, G., 2001. DNA extraction from soils: old bias for new microbial diversity
- 9 analysis methods. *Appl. Environ. Microbiol.* 67(5), 2354–2359.
- 10 McComas, C., Sublette, K.L., Jenneman, G., Bala, G., 2001. Characterization of a novel
- 11 biocatalyst for sulfide oxidation. *Biotechnol. Progr.* 17, 439–446.
- 12 McGlannan, M.F., Makemson, J.C., 1990.  $\text{HCO}_3^-$  fixation by naturally occurring tufts and
- 13 pure cultures of *Thiothrix nivea*. *Appl. Environ. Microbiol.* 56(3), 730–738.
- 14 Mori, T., Nonaka, T., Tazaki, K., Koga, M., Hikosaka, Y., Noda, S., 1992. Interactions of
- 15 nutrients, moisture and pH on microbial corrosion of concrete sewer pipes. *Water Res.*
- 16 26(1), 29–37.
- 17 Nemati, M., Mazutinec, T.J., Jenneman, G.E., Voordouw, G., 2001. Control of biogenic
- 18  $\text{H}_2\text{S}$  production with nitrate and molybdate. *J. Ind. Microbiol. Biotechnol.* 26,
- 19 350–355.
- 20 Nielsen, A.H., Vollertsen, J., Jensen, H.S., Wium-Andersen, T., Hvitved-Jacobsen, T., 2008.
- 21 Influence of pipe material and surfaces on sulfide related odor and corrosion in sewers.
- 22 *Water Res.* 42, 4206–4214.
- 23 Nielsen, P.H., de Muro, M.A., Nielsen, J.L., 2000 Studies on the in situ physiology of

- 1        *Thiothrix* spp. present in activated sludge. Environ. Microbiol. 2, 389-898.
- 2    Ochi, T., Kitagawa, M., Tanaka, S., 1998. Controlling sulfide generation in force mains by  
3        air injection. Water Sci. Technol. 37(1), 87–95.
- 4    Okabe, S., Satoh, H., Watanabe, Y., 1999a. In situ analysis of nitrifying biofilms as  
5        determined by in situ hybridization and the use of microelectrodes. Appl. Environ.  
6        Microbiol. 65, 3182–3191.
- 7    Okabe, S., Itoh, T., Satoh, H., Watanabe, Y., 1999b. Analyses of spatial distributions of  
8        sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Appl.  
9        Environ. Microbiol. 65(11), 5107–5116.
- 10   Okabe, S., Santegoeds, C.M., Watanabe, Y., De Beer, D., 2002. Successional development  
11        of sulfate-reducing bacterial populations and their activities in an activated sludge  
12        immobilized agar gel film. Biotechnol. Bioeng. 78(2), 119–130.
- 13   Okabe, S., Ito, T., Satoh, H., Watanabe, Y., 2003a. Effect of nitrite and nitrate on biogenic  
14        sulfide production in sewer biofilms determined by the use of microelectrodes. Water  
15        Sci. Technol. 47(11), 281–288.
- 16   Okabe, S., Santegoeds, C.M., De Beer, D., 2003b. Effect of nitrite and nitrate on in situ  
17        sulfide production in an activated sludge immobilized agar gel film as determined by  
18        use of microelectrodes. Biotechnol. Bioeng. 81(5), 570–577.
- 19   Okabe, S., Ito, T., Sugita, K., Satoh, H., 2005. Succession of internal sulfur cycles and  
20        sulfur-oxidizing bacterial communities in microaerophilic wastewater biofilms. Appl.  
21        Environ. Microbiol. 71(5), 2520–2529.
- 22   Okabe, S., Odagiri, M., Ito, T., Satoh, H., 2007. Succession of sulfur-oxidizing bacteria in  
23        the microbial community on corroding concrete in sewer systems. Appl. Environ.

1 Microbiol. 73(3), 971–980.

2 Peccia, J., Marchand, E.A., Silverstein, J., Hernandez, M., 2000. Development and  
3 application of small-subunit rRNA probes for assessment of selected *Thiobacillus*  
4 species and members of the genus *Acidiphilium*. Appl. Environ. Microbiol. 66,  
5 3065–3072.

6 Preisler, A., De Beer, D., Lichtschlag, A., Lavik, G., Boetius, A., Jørgensen, B.B., 2007  
7 Biological and chemical sulfide oxidation in a *Beggiatoa* inhabited marine sediment.  
8 ISME J. 1(4), 341–353.

9 Rabus, R., Fukui, M., Wilkes, H., Widdle, F., 1996. Degradative capacities and 16S  
10 rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic  
11 enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol.  
12 62(10), 3605–3613.

13 Reinsel, M.A., Sears, J.T., Stewart, P., 1996. Control of microbial souring by nitrate, nitrite  
14 or glutaraldehyde injection in a sandstone column. J. Microbiol. Biotechnol. 17,  
15 128–136.

16 Revsbech, N.P., 1989. An oxygen microelectrode with a guard cathode. Limnol. Oceanogr.  
17 34, 474–478.

18 Rossetti, S., Blackall, L.L., Levantesi, C., Uccelletti, D., Tandoi, V., 2003. Phylogenetic  
19 and physiological characterization of a heterotrophic, chemolithoautotrophic  
20 *Thiothrix* strain isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 53(5),  
21 1271–1276.

22 Santegoeds, C.M., Ferdelman, T.G., Muyzer, G., de Beer, D., 1998. Structural and  
23 functional dynamics of sulfate-reducing populations in bacterial biofilms. Appl.

1 Environ. Microbiol. 64(10), 3731–3739.

2 Thierry, S., Macarie, H., Iizuka, T., Geissdörfer, W., Assih, E.A., Spanevello, M., Verhe, F.,  
3 Thomas, P., Fudou, R., Monroy, O., Labat, M., Ouattara, A.S., 2004.  
4 *Pseudoxanthomonas mexicana* sp. nov. and *Pseudoxanthomonas japonensis* sp. nov.,  
5 isolated from diverse environments, and emended descriptions of the genus  
6 *Pseudoxanthomonas* Finkmann *et al.* 2000 and of its type species. Int. J. Syst. Evol.  
7 Microbiol. 54(6), 2245–2255.

8 Vincke, E., Boon, N., Verstraete, W., 2001. Analysis of the microbial communities on  
9 corroded concrete sewer pipes-a case study. Appl. Microbiol. Biotechnol. 57,  
10 776–785.

11 Vollertsen, J., Nielsen, A.H., Jensen, H.S., Wium-Andersen, T., Hvitved-Jacobsen, T., 2008.  
12 Corrosion of concrete sewers-the kinetics of hydrogen sulfide oxidation. Sci. Total  
13 Environ. 394, 162–170.

14 Wagner, M., Amann, R., Kampfer, P., Assmus, B., Hartmann, A., Hutzler, P., Springer, N.,  
15 Schliefer, K.-H., 1994. Identification and in situ detection of gram-negative  
16 filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17, 405–417.

17 Wang, Y.N., Cai, H., Yu, S.L., Wang, Z.Y., Liu, J., Wu, X.L., 2007 *Halomonas*  
18 *gudaonensis* sp. nov., isolated from a saline soil contaminated by crude oil. Int. J. Syst.  
19 Evol. Microbiol. 57(5), 911–915.

20 Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA  
21 amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

22 Yamanaka, T., Aso, I., Togashi, S., Tanigawa, M., Shoji, K., Watanabe, T., Watanabe, N.,  
23 Maki, K., Suzuki, H., 2002. Corrosion by bacteria of concrete in sewerage systems

1           and inhibitory effects of formates on their growth. *Water Res.* 36, 2636–2642.

2   Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., Verstraete, W.,  
3           2008. Chemical and biological technologies for hydrogen sulfide emission control in  
4           sewer systems: A review. *Water Res.* 42, 1–12.

5   Zhang, L., Mendoza, L., Marzorati, M., Verstraete, W., 2009. Inhibition of sulfide  
6           generation by dosing formaldehyde and its derivatives in sewage under anaerobic  
7           conditions. *Water Sci. Technol.* 59(6), 915-919.

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**TABLE 1.** Phylogenetic relatives and detection frequency of clones obtained from bottom biofilms developed on the concrete surface in the manhole

Closest relative (accession no. <sup>a</sup> )	No. of clones obtained
Total	68
<i>Alphaproteobacteria</i>	
<i>Rhodobacter blasticus</i> (DQ342322)	1
<i>Paracoccus aminovorans</i> (D32240)	1
Endosymbiont of <i>Acanthamoeba</i> sp. (EF140635)	4
<i>Paracoccus aminophilus</i> (D32239)	2
<i>Labrys methylaminiphilus</i> (DQ337554)	2
<i>Betaproteobacteria</i>	
<i>Aquaspirillum</i> sp. EMB325 (DQ372987)	3
<i>Acidovorax defluvii</i> (Y18616)	1
<i>Propionivibrio pelophilus</i> (AF016690)	1
<i>Comamonas denitrificans</i> (DQ836252)	1
<i>Rhodocyclus tenuis</i> (D16209)	1
<i>Acidovorax defluvii</i> (Y18616)	1
<i>Acidovorax temperans</i> (AF078766)	4
<i>Brachymonas denitrificans</i> (DQ836253)	3
<i>Hydrogenophaga defluvii</i> (AJ585993)	2
<i>Comamonas margaretae</i> (EF154518)	1
<i>Zoogloea ramigera</i> (X74913)	7
<i>Sterolibacterium denitrificans</i> (AJ306683)	1
<i>Gamma</i> proteobacteria	
<i>Xanthomonas axonopodis</i> (AB101447)	1
<i>Thiothrix nivea</i> (L40993)	1
<i>Pseudomonas geniculata</i> (EU239476)	1
<i>Legionella drancourtii</i> (X97366)	1
<i>Acinetobacter brisoui</i> (DQ832256)	2
<i>Acinetobacter johnsonii</i> (Z93440)	1
<i>Deltaproteobacteria</i>	
<i>Desulfomonile tiedjei</i> (AM086646)	1
<i>Actinobacteria</i>	
<i>Phycococcus</i> sp. WR48 (AB365792)	1
<i>Brooklawnia cerclae</i> (DQ196625)	1
<i>Tessaracoccus bendigoensis</i> (AF038504)	4
<i>Propionicicella superfundia</i> (DQ176646)	1
<i>Dermatophilus chelonae</i> (AJ243919)	2
<i>Phycosicoccus jejuensis</i> (DQ345443)	2
<i>Nocardioides aquiterrae</i> (AF529063)	1
<i>Bacteroidetes</i>	
<i>Paludibacter propionicigenes</i> (AB078842)	4
<i>Porphyromonadaceae bacterium</i> NML 060648 (EF184292)	1
<i>Firmicutes</i>	
<i>Megasphaera micronuciformis</i> (AF473833)	2
<i>Sporomusa silvacetica</i> (Y09976)	4
<i>Coprothermobacter proteolyticus</i> (X69335)	1

<sup>a</sup> Accession numbers are from the EMBL/GenBank/DDBJ databases.

**TABLE 2.** Phylogenetic relatives and detection frequency of clones obtained from middle biofilms developed on the concrete surface in the manhole

Closest relative (accession no. <sup>a</sup> )	No. of clones obtained
Total	62
<i>Alphaproteobacteria</i>	
<i>Afipia</i> genosp. 14 (U87785)	1
<i>Novosphingobium hassiacum</i> (AJ416411)	6
<i>Bosea thiooxidans</i> (AJ250796)	1
<i>Betaproteobacteria</i>	
<i>Comamonadaceae bacterium</i> (AJ505862)	10
<i>Acidovorax defluvii</i> (Y18616)	2
<i>Comamonas badia</i> (AB164432)	3
<i>Gammaproteobacteria</i>	
<i>Xanthomonas axonopodis</i> (AB101447)	14
<i>Xanthomonas axonopodis</i> (AB101447)	1
<i>Lysobacter brunescens</i> (AB161360)	1
<i>Acinetobacter johnsonii</i> (EF204266)	1
<i>Pseudoxanthomonas japonensis</i> (AB008507)	4
<i>Rhodanobacter ginsengisoli</i> (EF166075)	2
<i>Thiothrix</i> sp. strain CT3 (AF148516)	11
<i>Bacteroidetes</i>	
<i>Chryseobacterium daecheongense</i> (AJ457206)	2
<i>Flavobacterium saliperosum</i> (DQ021903)	1
<i>Flavobacterium columnare</i> (AJ491824)	1
<i>Flavobacteriaceae bacterium</i> YMS-2 (EF017801)	1

<sup>a</sup> Accession numbers are from the EMBL/GenBank/DDBJ databases.

**TABLE 3.** Phylogenetic relatives and detection frequency of clones obtained from corroded concrete developed on the concrete surface in the manhole

Closest relative (accession no. <sup>a</sup> )	No. of clones obtained
Total	101
<i>Alphaproteobacteria</i>	
<i>Novosphingobium hassiacum</i> (AJ416411)	2
<i>Rhodobacter blasticus</i> (DQ342322)	2
<i>Betaproteobacteria</i>	
<i>Ralstonia pickettii</i> (DQ908951)	2
<i>Comamonadaceae bacterium</i> (AJ505862)	6
Candidatus <i>Burkholderia vershuerenii</i> (AY277699)	4
<i>Gamma</i> proteobacteria	
<i>Halothiobacillus neapolitanus</i> (AF173169)	6
<i>Xanthomonas axonopodis</i> (AB101447)	17
<i>Pseudoxanthomonas mexicana</i> (AY124375)	26
<i>Halomonas desiderata</i> (X92417)	3
<i>Halomonas gudaoense</i> (DQ421808)	1
<i>Acinetobacter</i> sp. (Z93451)	3
<i>Thermomonas brevis</i> (AB355702)	2
<i>Actinobacteria</i>	
<i>Mycobacterium cookii</i> (AF480598)	1
<i>Beutenbergia cavernosa</i> (Y18378)	7
<i>Bifidobacterium pseudocatenulatum</i> (D86187)	1
<i>Firmicutes</i>	
<i>Bacillus acidicola</i> (AF547209)	6
<i>Salinibacillus</i> sp. BH128 (AY553085)	1
<i>Bacillus salarius</i> (AY667494)	1
<i>Bacillus</i> sp. BH164 (AY762977)	2
<i>Anaerospira hongkongensis</i> (AY372052)	7
<i>Bacteroidetes</i>	
Endosymbiont of <i>Acanthamoeba</i> sp. (AF215634)	1

<sup>a</sup> Accession numbers are from the EMBL/GenBank/DDBJ databases.