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

Running title: Microbial communities and activities in sewer biofilms

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ABSTRACT

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

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

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

1. Introduction

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

(SRB) present in waters and sediments under anaerobic conditions in the submerged part of sewer facilities. This H_2S easily enters the sewer atmosphere by volatilization and dissolves in condensate on the surface of sewer facilities. Then, H_2S is mainly chemically oxidized to thiosulfate ($S_2O_3^-$) and elemental sulfur (S^0). Sulfur-oxidizing bacteria (SOB) present in the condensate on the concrete surface (e.g., the sewer crown) eventually oxidize the dissolved H_2S and other sulfur compounds (e.g., $S_2O_3^-$ and S^0) to sulfuric acid, which corrodes the concrete.

Several measures to prevent H_2S production have been developed (**Zhang et al., 2008**). SRB activity can be inhibited by pH elevation or inhibitors such as biocides, ozone, molybdate and formaldehyde (**Elovitz et al., 2000; Jayaraman et al., 1999; Nemati et al., 2001; Reinsel et al., 1996; Zhang et al., 2009**). Increasing ORP is achieved by addition of electron acceptor such as oxygen, nitrate or nitrite (**Bentzen et al., 1995; Hobson and Yang, 2000; Londry and Suflita, 1999; Ochi et al., 1998; Okabe et al., 2003a, b**). Other measures are concrete coatings (**Haile and Nakhla, 2009; Muynck et al., 2009**) and chemical and biological removal of H_2S by addition of iron salts (**McComas et al., 2001**), H_2O_2 (**Cadena and Peters, 1988**), chlorines, nitrate, or nitrate-reducing, sulfide-oxidizing bacteria (**De Gusseme et al., 2009**). To more efficiently control the MICC, ii is necessary to understand microbial community structures, especially SRB and SOB species, in sewer systems.

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

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

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

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

The aim of this study was, therefore, to investigate the microbial community structures and the in situ H_2S production and consumption rates within biofilms and corroded materials developed on mortar specimens installed in a corroded sewer system. Mortar specimens were placed in a severely corroded manhole of a real sewer system to investigate the MICC process under in situ conditions. The microbial community structures were analyzed by using 16S rRNA gene-cloning analysis and FISH, and the in situ H_2S production and consumption rates were determined with microsensors. Moreover, the succession of the in situ H_2S production and consumption rates in the biofilms was analyzed. In parallel, appearance of the mortar specimens was monitored and a corrosion rate of the mortar specimens was determined. The data sets resulting from these different approaches were integrated to elucidate the MICC mechanism in corroded sewer systems.

2. Materials and methods

Mortar specimens.

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

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

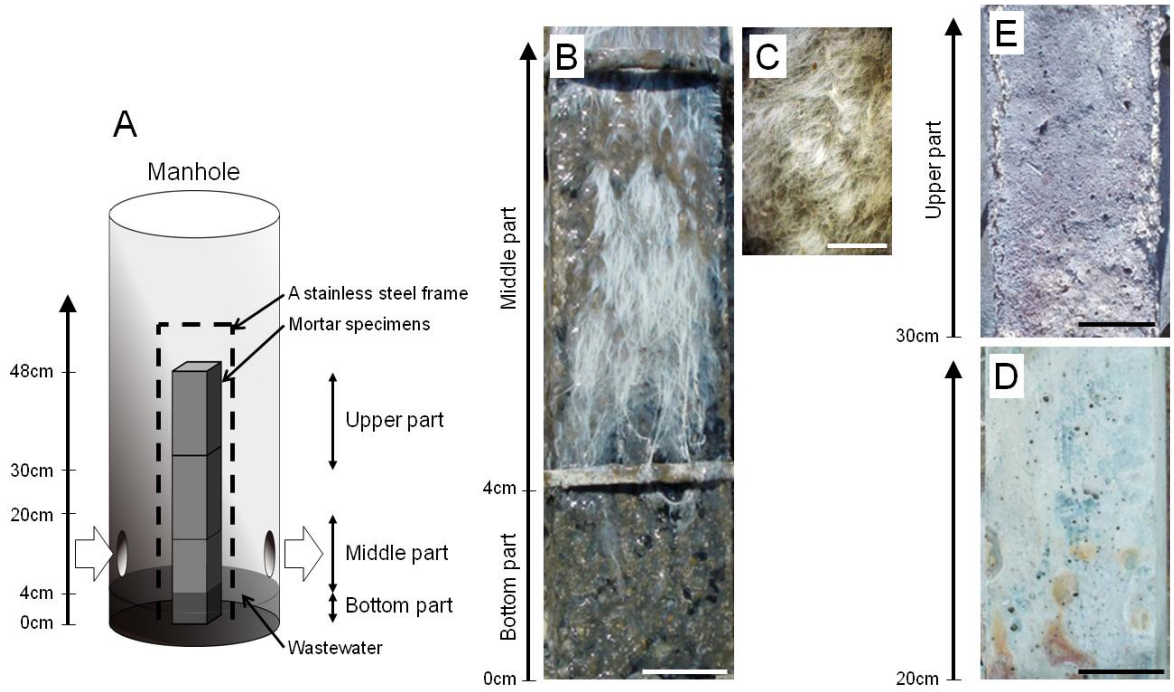

Fig. 1. A schematic drawing (A) and photograph of the mortar specimens placed in a manhole which is severely corroded. (B) Biofilms developed in the bottom and the middle parts of the manhole. (C) A close-up view of white filamentous biofilms developed on the middle biofilm surface. (D) A sound mortar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

D and E) and 2 mm (C).

DNA extraction and PCR amplification.

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

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

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

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

Fluorescent in situ hybridization.

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

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

Microsensor measurement.

For microsensor measurements, the mortar specimens were placed in the flow cell reactor (4.0 liter) that was filled with the synthetic medium. The synthetic medium consisted of $MgCl_2$ (450 µM), $CaCl_2$ (40 µM), NH_4Cl (1,520 µM), $MgSO_4$ (1,000 µM), NaCl (1,000 µM), K_2HPO_4 (4,600 µM), KH_2PO_4 (3,700 µM), $NaHCO_3$ (1,000 µM), glucose (500 µM), EDTA ([ethylenediaminetetraacetic acid] 270 µM), and $Na_2S•9H_2O$ (various amounts). O_2 concentration in the medium was kept at a minimum for bottom biofilm measurements, approximately 50 µM for middle biofilm measurements, and approximately 150 µM for corroded material measurements by continuous bubbling with N_2 and air, which also resulted in sufficient mixing of the medium. The pH of the medium was adjusted to 7 to 8 and the reactor was kept at room temperature (20 to 23°C) for all of the measurements. The biofilms were acclimated in the medium for at least 3 h before measurements to ensure that steady-state profiles were obtained. In contrast, the concentration profiles in the corroded

materials were measured within 1 h in order to avoid significant change in pH in the corroded materials due to diffusion out of H^+ from the corroded materials. The in situ steady-state concentration profiles of H_2S, pH, ORP and O_2 in the biofilms and corroded materials were measured, using microsensors as described by **Okabe et al.** (**1999b**). H_2S (**Jeroschewski et al., 1996**), pH (**De Beer et al., 1997**), ORP (**Jang et al., 2005**) and O_2 (**Revsbech, 1989**) were prepared, calibrated, and used as described previously. At least three concentration profiles were measured for each chemical species.

Chemical analyses.

The COD concentrations were determined according to Standard Methods (**APHA, 1995**). The SO_4^{2-} concentrations were measured with an ion chromatograph (model DX-100 with an AS4A column; Nippon DIONEX, Osaka, Japan) after filtration with 0.2-μm-pore-sized membrane filters (DISMIC-13CP; Advantec Co., Ltd.). The concentrations of T-H_2S (total dissolved sulfide defined as the sum of H_2S, HS^- and S^{2-}) were determined by the methylene blue method (**Cline, 1969**). For measurements of the gaseous H_2S, gaseous samples were immediately transferred to 50 mL screw capped tubes containing 10 mL of a 10% w/v zinc acetate solution and the gaseous H_2S was precipitated as ZnS. The ZnS concentrations were determined by the methylene blue method and then the gaseous H_2S concentrations were calculated. The NH_4^+ concentrations were determined using ion chromatographs (DX-100, DIONEX, Sunnyvale, CA) equipped with an IonPac CS3 cation column. The ORP and pH were directly determined by using an ORP and a pH electrode, respectively.

3. Results

Chemical parameters of the returned water and appearance of mortar specimens.

Average (± standard deviation) concentration of dissolved T-H_2S in the returned water flowing through the manhole was 236 ± 174 µM. Dissolved oxygen of 77 ± 20 µM coexisted in the flowing water due to a high-turbulence flow. Consequently, ORP was relatively high (34 ± 92 mV) although T-H_2S was present. Because the water coming from a sludge-thickening tank was anaerobic, NH_4^+ (3900 ± 1300 µM) and chemical oxygen demand (7060 ± 2375 µM) concentrations were high. pH was 6.9 ± 0.2. The gaseous H_2S concentration in the manhole atmosphere was 30 ± 20 ppmv. Oxygen and carbon dioxide were at atmospheric concentrations.

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

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

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

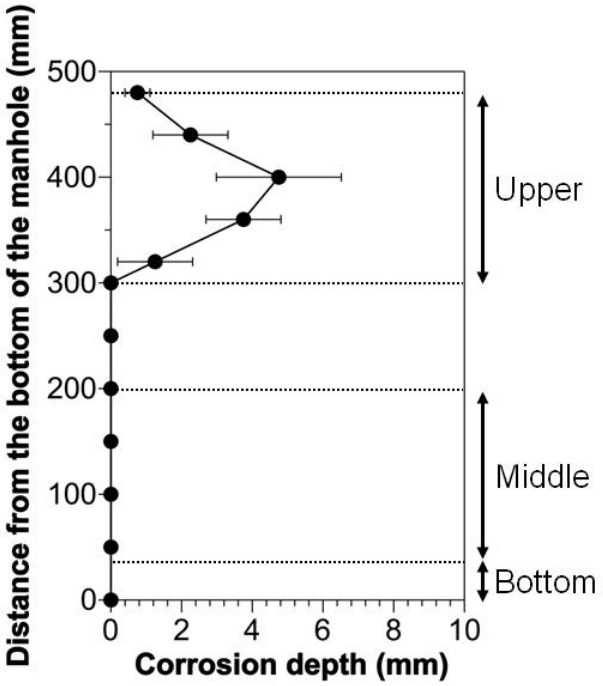

Fig. 2 Corrosion depth of the mortar specimen after one year of installation in the manhole.

Microbial community structures in biofilms and corroded materials.

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

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

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

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

Bacteria present in biofilms.

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

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

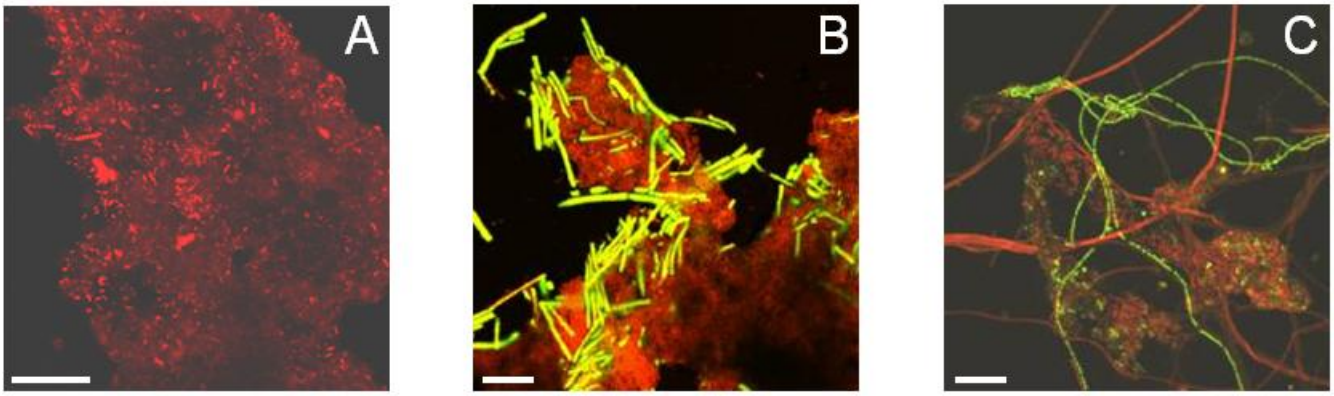

Fig. 3 Confocal laser scanning microscope images showing presence of sulfate-reducing and sulfur-oxidizing bacteria in the bottom (panel A) and middle (panels B and C) biofilms. (A) FISH with TRITC-labeled SRB385 and SRB385Db probes (red). (B) FISH with FITC-labeled G123T probe (yellow) and TRITC-labeled EUB338mix probe (red). (C) FISH with FITC-labeled SNA probe (green) and TRITC-labeled BET42a probe (red). Scale bars indicate 20 µm.

Sulfide production and oxidation in biofilms and corroded materials.

Steady-state concentration profiles of T-H_2S, pH, O_2, and ORP in the middle and bottom biofilms and the corroded materials were measured with microsensors (Fig. 4). The thickness of the bottom biofilm was about 1,000 µm after 7 days of installation of the mortar specimens in the manhole. A T-H_2S concentration was 60 µM at a depth of 700 µm and decreased toward the biofilm surface (Fig. 4A). T-H_2S was still present (10 µM) at the biofilm surface and diffused out of the bottom biofilm. The concentration gradient of T-H_2S was steeper in the upper parts of the biofilm, indicating high sulfate-reducing activity. ORP significantly decreased in the upper parts of the biofilm and was unchanged in the deeper parts, corresponding well with the concentration profile of T-H_2S. pH was unchanged

throughout the biofilm. The thickness of the bottom biofilm increased to ca. 1,500 μm after 28 days of installation of the mortar specimens (Fig. 4B). The maximal T-H_2S concentration was higher (90 μM) than that in the 7-day-old biofilm, which demonstrated an increasing sulfate-reducing activity. A significant decrease in ORP reflected higher sulfate-reducing activity. After 105 days of installation of the mortar specimens, the bottom biofilm became thicker. The T-H_2S concentration in the deeper parts of the biofilm increased up to 160 μM (Fig. 4C), indicating a further increase in sulfate-reducing activity. Relatively big standard deviations reflected heterogeneity of the biofilm structure.

Although the middle biofilm was very thin after 7 days of installation of the mortar specimens, the thickness of the middle biofilm was about 1,000 μm after 28 days. A T-H_2S concentration was less than 10 μM at a depth of 700 μm (data not shown), indicating that the sulfate-reducing activity was even lower than that in the bottom biofilm. After 105 days of installation of the mortar specimens, the middle biofilm grew thicker. However, A T-H_2S concentration was very low and T-H_2S production was restricted in the deeper 2000 μm of the biofilm because of O_2 penetration into the upper parts of the biofilm (Fig. 4D). The T-H_2S provided from the bulk solution as well as the deeper parts of the biofilm was oxidized in the upper oxic parts of the biofilm.

In the corroded materials the pH was around 3 in the deeper parts after 105 days of installation of the mortar specimens (Fig. 4E), indicating that the in situ pH level of the corroded materials was significant low as reported previously (**Okabe et al., 2007**). Increasing pH in the upper parts was attributed to diffusion out of H^+ from the corroded materials. The O_2 concentration drastically decreased within the uppermost 400 μm by mainly a chemical O_2 consumption process. A T-H_2S concentration drastically decreased

within the oxic parts due to chemical T-H_2S oxidation. It was also likely that dissolved H_2S gas was emitted due to lower pH, because dissolved H_2S rather than HS^- became the dominant species at lower pH taking into account the first dissociation constants for H_2S (pK_{a1} = 7.04) (**Zhang et al., 2008**).

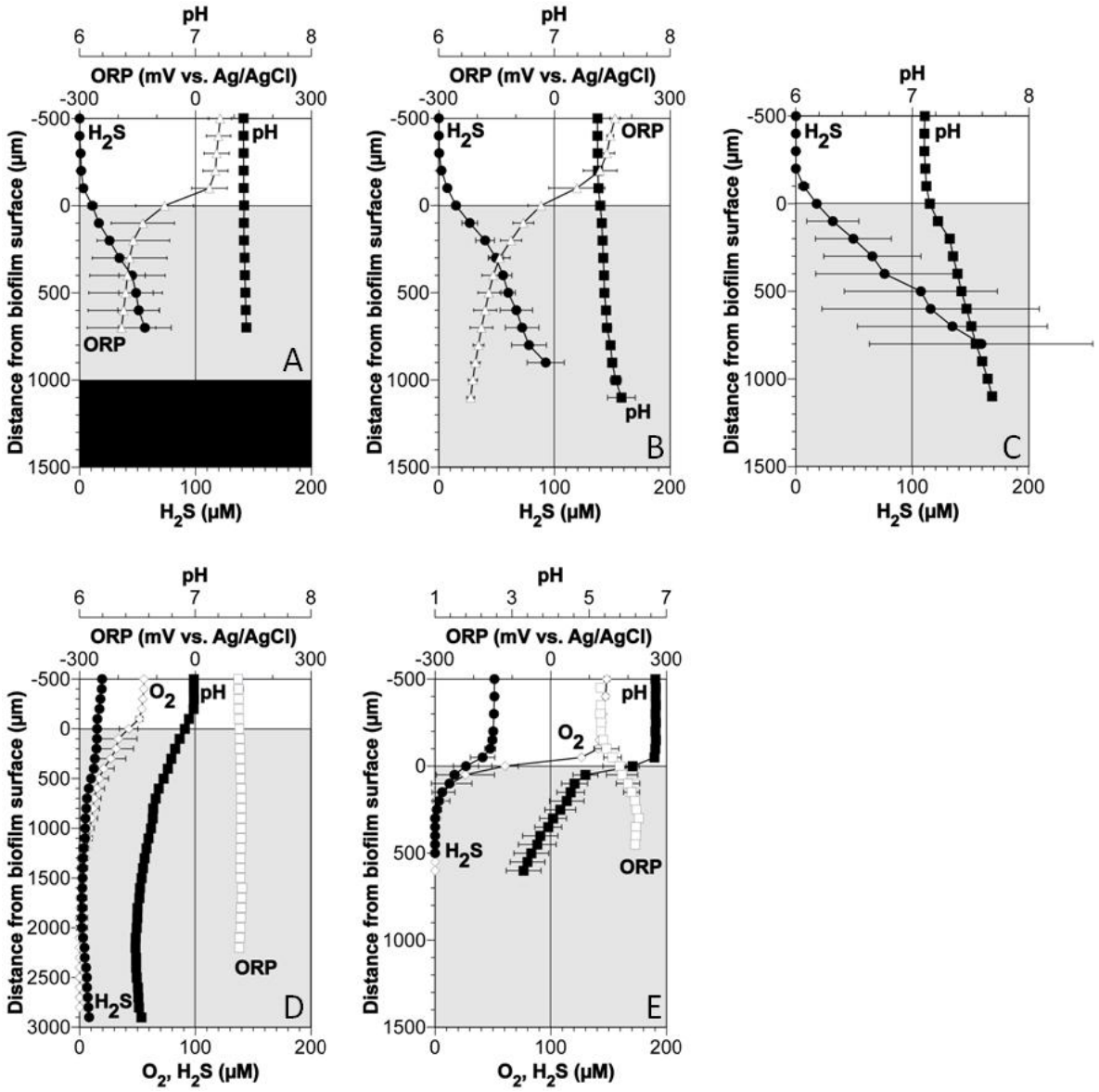

Fig. 4. Steady-state concentration profiles of T-H_2S, pH, O_2, and ORP in the bottom and middle biofilms and the corroded materials. The bottom biofilms at day 7 (A), day 28 (B) and day 105 (C), the middle biofilms at day 105 (D) and the corroded materials at day 105

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

4. Discussion

Bottom biofilm.

Microsensor measurements revealed that T-H_2S was produced in and diffused out of the bottom biofilms (Figs. 4A, 4B and 4C). The net T-H_2S flux (J(H_2S) (i.e., a T-H_2S emission rate) evaluated from the T-H_2S concentration profiles was 0.033 ± 0.005 μmol/cm^2/h at day 7 (Fig. 4A). J(H_2S) increased to 0.063 ± 0.013 μmol/cm^2/h at day 28 (Fig. 4B) and 0.091 ± 0.053 μmol/cm^2/h at day 105 (Fig. 4C). These rates were similar to those of microaerophilic wastewater biofilms (**Ito et al., 2002; Okabe et al., 1999b)**, a trickling filter biofilm (**Kühl and Jorgensen, 1992**), biofilms grown in an activated sludge aeration basin (**Santegoeds et al., 1998**), and a *Beggiatoa* inhabited marine sediment (**Preisler et al., 2007**). This result indicates the successional development of SRB community in the bottom biofilm. One clone was affiliated with an anaerobic dehalogenating SRB, *Desulfomonile tiedjei*, with 95.0% similarity (Table 1). This bacterium was isolated from anaerobic sewage sludge (**DeWeerd et al., 1990**). Small number of clones related to SRB in the bottom biofilm clone library is probably due to low density of the SRB in the biofilm, inherent PCR biases, resulting from differences in the amplification efficiency of templates (**Acinas et al., 2005**) and in DNA extraction efficiency from environmental samples (**Martin-Laurent et al., 2001**), and the insufficient number of clones sequenced. However, we are convinced

that SRB present in the bottom biofilm produced T-H_2S, because FISH results confirmed presence of SRB in the bottom biofilm (Fig. 3A).

The microbial community of the bottom biofilm, which consisted of 7 phylogenetic groups of clones at the phylum level, was most diversified among three samples (Table 1). It is probably explained by high organic compound and nutrient availability provided from wastewater. One clone was related to obligatory mixotrophic sulfur-oxidizing bacteria (SOB), *Thiothrix nivea*, with 94.4% similarity (Table 1). The versatile physiological ability of *T. nivea* to utilize reduced sulfur compounds and various organic compounds (acetate, malate, pyruvate, and oxaloacetate) (**McGlannan and Makemson, 1990**) might provide a competitive advantage in the bottom biofilm under microaerophilic conditions with high concentration of organic compounds (**Okabe et al., 2007**). *T. nivea* have been found in activated sludge treating paper and board mill wastes (**Kim et al., 2002**) and in H_2S-containing flowing water (**McGlannan and Makemson, 1990**). However, the in situ T-H_2S oxidation activities were not observed in the bottom biofilm (Figs. 4A, 4B and 4C), probably due to low abundance of SOB and scarcity of O_2.

Middle biofilm.

Because the returned water was flowing into the manhole for 45 min of each hour, the middle biofilm was exposed periodically to the oxic sewer atmosphere and the returned water containing T-H_2S. Periodic alternation resulted in significant growth of white filamentous bacteria on the surface of the middle biofilm (Figs. 1B and 1C). The 16S rRNA gene-cloning analysis revealed that the clones related to autotrophic SOB, *Thiothrix* sp. strain CT3, dominated the middle biofilm clone library at day 105 (Table 2). This bacterium

was isolated from an activated sludge treatment plant (**Rossetti et al., 2003**) and has been detected on mortar surface at the same sampling site (**Okabe et al., 2007**) and in wastewater biofilms growing under microaerophilic conditions (**Okabe et al., 2005**) where T-H_2S was present. This strain has been reported as filamentous bacteria that grow on reduced sulfur compounds and accumulate sulfur granules internally (**Rossetti et al., 2003**). Hence, the biofilms colonized by *Thiothrix* sp. strain CT3 were characterized as white filamentous biofilms (**Okabe et al., 2005**). FISH analysis with the G123T probe confirmed that *Thiothrix*-like filamentous bacteria covered the biofilm surface (Fig. 3B). *Thiothrix* spp. can use nitrate as an electron acceptor (**Nielsen et al., 2000**). Although nitrate utilization in *Thiothrix* spp. was not investigated by a nitrate microsensor in this study, there was a possibility that they used nitrate generated in oxidation of ammonium in the oxic filamentous biofilms. In addition, one clone was related to chemolithoheterotrophic *Bosea thiooxidans* (Table 2), which is capable of oxidizing reduced inorganic sulfur compounds and grows on a wide range of organic substrates (**Das et al., 1996**). Thus, this strain might be also involved in T-H_2S oxidation in the middle biofilm (Fig. 4D). The strain CT3 and *B. thiooxidans* can produce sulfate as the end product of oxidation of reduced sulfur compounds (**Das et al., 1996; Rossetti et al., 2003**).

microsensor measurements demonstrated that T-H_2S was oxidized in the upper filamentous biofilm and subsequently pH decreased (Fig. 4D). Interestingly, T-H_2S was regenerated in the deeper anaerobic parts of the biofilm and then the T-H_2S generated was also completely oxidized in the upper filamentous biofilm (Fig. 4D). This result clearly indicated occurrence of simultaneous H_2S production and consumption in the middle biofilm. Low sulfate-reducing activity in the deeper parts of the middle biofilm was

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

Corroded materials.

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

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

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

5. Conclusions

The microbial community structures and the in situ H_2S production and consumption rates in biofilms and corroded materials developed on mortar specimens in a corroded sewer

system was investigated by 16S rRNA gene-cloning analysis, FISH and microsensor measurements. The results in this study indicated that T-H_2S was provided from the biofilms in the bottom part of the manhole. The T-H_2S derived from the sludge settling tank was also the source of T-H_2S. Then the dissolved H_2S was emitted to the sewer atmosphere and oxidized to corrosive compounds on the mortar surface in the upper and middle parts of the manhole. However, only the upper part of the mortar specimens was corroded. In the biofilm developed around the air-water interface (i.e., the middle biofilm), the corrosive compounds generated in the upper filamentous biofilm were reduced in the deeper gel-like biofilm. This was the reason why the mortar surface in the middle part of the manhole was preserved from corrosive attack, and concrete corrosion was restricted to the upper parts in the sewer system (e.g., the crown of sewer pipes). In situ analyses of microbial community and their activities involved in the MICC process would provide us with valuable information to efficiently control, prevent, and/or predict MICC process in sewer systems.

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

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

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

[a] Accession numbers are from the EMBL/GenBank/DDBJ databases.