Revealing biogenic sulfuric acid corrosion in sludge digesters: detection of sulfur-oxidizing bacteria within full-scale digesters

B. Huber, J. E. Drewes, K. C. Lin, R. König and E. Müller

ABSTRACT

Biogenic sulfuric acid corrosion (BSA) is a costly problem affecting both sewerage infrastructure and sludge handling facilities such as digesters. The aim of this study was to verify BSA in full-scale digesters by identifying the microorganisms involved in the concrete corrosion process, that is, sulfate-reducing (SRB) and sulfur-oxidizing bacteria (SOB). To investigate the SRB and SOB communities, digester sludge and biofilm samples were collected. SRB diversity within digester sludge was studied by applying polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) targeting the *dsrB*-gene (dissimilatory sulfite reductase beta subunit). To reveal SOB diversity, cultivation dependent and independent techniques were applied. The SRB diversity studies revealed different uncultured SRB, confirming SRB activity and H_2S production. Comparable DGGE profiles were obtained from the different sludges, demonstrating the presence of similar SRB species. By cultivation, three pure SOB strains from the digester headspace were obtained including *Acidithiobacillus thiooxidans*, *Thiomonas intermedia* and *Thiomonas perometabolis*. These organisms were also detected with PCR-DGGE in addition to two new SOB: *Thiobacillus thioparus* and *Paracoccus solventivorans*. The SRB and SOB responsible for BSA were identified within five different digesters, demonstrating that BSA is a problem occurring not only in sewer systems but also in sludge digesters. In addition, the presence of different SOB species was successfully associated with the progression of microbial corrosion.

Key words | biogenic sulfuric acid, concrete corrosion, digester, PCR-DGGE, sulfate-reducing bacteria, sulfur-oxidizing bacteria

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INTRODUCTION

Corrosion of concrete due to biogenic sulfuric acid production is a well-known problem affecting the world's sewerage infrastructure and wastewater treatment, with repair costs of several billions of dollars every year (Hewayde *et al.* 2007). In Germany alone, the estimated costs for the restoration of damaged sewer systems amount to 100 billion euros, of which 40% of the damage can be attributed to biogenic sulfuric acid corrosion (BSA) (Kaempfer & Berndt 1998). For the BSA process, a complex microbial ecosystem involving anaerobic sulfate-reducing as well as aerobic sulfur-oxidizing bacteria (SRB and SOB, respectively) is required. In a sewer pipe, where anaerobic conditions can occur due to long detention periods or the slow flow of sewage, heterotrophic SRB (e.g., *Desulfovibrio* sp.) reduce sulfur compounds with organic substances as electron donors to hydrogen sulfide (H_2S). The H_2S gas escapes into the sewer headspace and is subsequently converted abiotically to various partially reduced sulfur compounds in the condensate on the sewer crown (Roberts *et al.* 2002). In the presence of oxygen, autotrophic/mixotrophic SOB growing on the moist concrete surface (e.g., *Thiobacillus* sp.) finally oxidize these reduced sulfur compounds (e.g., $S_2O_3^-$, S^0) to sulfuric acid (Okabe *et al.* 2007). The sulfuric acid reacts with the cementitious material of concrete, and corrosion products like gypsum ($CaSO_4 \cdot 2H_2O$) and ettringite ($CaO \cdot Al_2O_3 \cdot 3CaSO_4 \cdot 32H_2O$) are formed (Bock & Sand 1986; Wiener *et al.* 2006). The formation of these expansive compounds increases the internal pressure leading to cracks and pitting of the concrete (Kaempfer & Berndt 1998; Aviam *et al.* 2004). In sewer systems corrosion rates of up to several

doi: 10.2166/wst.2014.371

millimetres per year are reported (Mori *et al.* 1991; Vincke *et al.* 2002; De Belie *et al.* 2004; Vollertsen *et al.* 2008).

While BSA is well studied in sewer pipes, a lack of understanding exists regarding corrosion processes in sludge digesters (for detailed information on anaerobic sludge digestion see Appels *et al.* (2008)) and in particular the role of BSA. The predominant anaerobic conditions in a digester would suggest SRB activity in the presence of sulfur compounds, but no sulfuric acid production by SOB, which require oxygen for growth. However, characteristic BSA damage patterns were also observed in different full-scale digesters in Germany (Figure 1). This might suggest that oxygen is locally available in the headspace ('hot spots') enabling the development of active SOB communities on the concrete wall. Due to the old age of many digesters imperfections may occur over time and oxygen might diffuse through existing cracks. Another possibility for the oxygen entry may be the desulfurization, where oxygen/air, usually in the range of 2–6 vol%, is added to the biogas (Appels *et al.* 2008). During this process *thiobacilli*, commonly present within digester sludge, convert the H_2S to elementary sulfur and sulfates reducing the H_2S level from 3,000–50,000 ppm to 50–100 ppm (Appels *et al.* 2008).

The aim of this study was to detect and characterize both SRB and SOB communities in digester sludge and biofilms growing on concrete surfaces, respectively, in order to verify concrete corrosion by biogenic sulfuric acid production in digester systems. For this purpose, five full-scale digesters in Germany with different degrees of corrosion damage were investigated regarding the occurrence of BSA-related microorganisms. Furthermore, a correlation between the presence of different SOB species and the progression of microbial concrete corrosion was revealed.

Figure 1 | Concrete corrosion pattern observed in the headspace of digester E.

MATERIAL AND METHODS

Environmental samples

Environmental samples, including digester sludge and biofilms removed from the concrete surface, were collected from five different digesters in Germany (A–E) exhibiting characteristic BSA damage patterns (see Figure 1). Further information on the investigated sludge digesters is given in Table 1. From each digester outflow, fresh digester sludge samples comprising SRB were taken. Within the digester headspace, biofilm samples containing potential SOB were scratched off from the corroded concrete surface using a sterile spatula. Field sampling of the environmental samples was performed by Weber-Ingenieure GmbH. The obtained samples were directly transferred into sterile 50 mL tubes and stored at 4 °C. The biofilm samples were immediately inoculated in specific liquid media as described below. For subsequent molecular biological analyses, both samples were stored at −20 °C.

Enrichment and cultivation of SOB

The enrichment and cultivation was carried out using a variety of media, differing in pH and energy source. DSMZ medium 35 (pH 4.5) with elementary sulfur and DSMZ

Table 1 | Detailed information on the five sludge digesters A–F

Digester	Year of construction	PE[a]	Digester volume [m³]	SRT[b] [d]	Temperature of digester sludge [°C]	pH of digester sludge	Sampling date
A	1969	110,000	1,100	18	37.5–39.0	7.3	April 2013, February 2014
B	1974	83,000	1,150	26–30	37.5–38.0	6.9–7.1	September 2013
C	1980	83,000	1,000	26–27	38.1	7.7	September 2013
D	1990	30,000	2,000	30–40	39.0–41.0	7.5–7.6	September 2013, February 2014
E	1963	10,000	320	60	30.0–33.0	7.0	November 2013, February 2014

[a]Population equivalent.
[b]Sludge retention time.

medium 68 (pH 6 and 8) with $Na_2S_2O_2$ as sole energy source were prepared according to Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/home.html). ATCC medium #125 (pH 4.1) containing $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4$ and elementary sulfur as well as *Thiobacillus* medium (pH 4.1) with $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4$, and $Na_2S_2O_3$ as energy sources were prepared as described by Starosvetsky *et al.* (2013). Inoculation of liquid media was done under sterile conditions from 100 to 500 mg of freshly collected biofilm material that had been stored at 4 °C for no longer than 2 days. Enriched SOB cultures were incubated at 30 °C and shaken at 125 rpm. Mixed enriched SOB cultures were analyzed by polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) and phylogenetic analysis. Pure cultures were finally obtained by cultivation of SOB on the corresponding agar media and identified by colony PCR amplifying the nearly full-length 16S rRNA gene and phylogenetic sequence analysis.

Analysis of SRB and SOB diversity

DNA extraction and PCR amplification of 16S rRNA and *dsrB* genes

Genomic DNA was isolated from 2 mL of digester sludge using a standard phenol–chloroform–isopropyl alcohol (25:24:1) and CTAB-buffer extraction method. The composition of the CTAB lysis buffer was as follows: 0.25 M Tris/HCl (pH 8.0), 1.60 M NaCl, 0.03 M EDTA, 1.00% (w/v) SDS, 0.05 M ammonium acetate, and 1.60% CTAB (w/v). For the amplification of the nearly full-length 16S rRNA gene (colony PCR) the universal bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′TACGGYTACCTTGTTACGACTT-3′) were applied (Lane 1991). For DGGE of mixed SOB cultures (direct PCR), the partial 16S rRNA gene fragment (~550 bp) was amplified using bacterial primers 27f and 517r (5′-GTATTACCGCGGCTGCTGGC-3′; Furushita *et al.* 2003). The temperature program for the primer sets 27f/1492r and 27f/517r was as follows: initial denaturation step of 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 40 s, and final elongation at 72 °C for 5 min. For SRB diversity analysis (PCR of extracted genomic DNA), the *dsrB* (dissimilatory sulfite reductase beta-subunit) gene fragment (~350 bp) was amplified with primers DSRp2060F (5′-CAACATCGTYCAYACCCAGGG-3′; Geets *et al.* 2006) and DSR4R (5′-GTGTAGCAGTTACCGCA-3′; Wagner *et al.* 1998). Thermal cycling was performed as described by Geets *et al.* (2006) with an initial denaturation step of 95 °C for 2 min. In order to obtain stable melting behavior of the DNA fragments during DGGE, each forward primer contained a 40 bp GC-clamp at the 5′end (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCGCCCCCGCCCCGG-3′; Muyzer *et al.* 1993). All primers were purchased from MWG Operon (Ebersberg, Germany). PCR was performed according to the GoTaq(R) G2 Hot Start Colorless Master Mix (Promega GmbH, Mannheim, Germany) in a total reaction volume of 25 μL.

DGGE of 16S rRNA and *dsrB* gene fragments

DGGE was performed with the DCode™ Universal Mutation Detection System from Bio-Rad (Munich, Germany). Ten microlitres of the PCR product, obtained either from amplification of 16S rRNA or *dsrB* genes, was loaded onto a 6% (w/v) polyacrylamide gel. Denaturing gradients ranged from 20 to 70%, where 100% is defined as 7 M urea and 40% (v/v) formamide. Electrophoresis was carried out at a constant voltage of 60 V for 16.5 h at 55 °C. Interesting DGGE bands were excised and re-amplified using the corresponding primer sets (without GC-clamp).

Sequencing and phylogenetic analysis

Sequences of 16S rRNA and *dsrB* genes were obtained either from colony PCR or re-amplification of excised DGGE bands. The PCR products were purified with the innuPREP DOUBLEpure Kit from Analytik Jena (Jena, Germany) and sent to sequencing (MWG Operon, Ebersberg, Germany). Nucleotide sequences were analyzed with public databases using BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest phylogenetic neighbors.

RESULTS AND DISCUSSION

Characterization of SRB communities in digester sludge

The examination of SRB microbial communities within digester sludge was performed by *dsrB* gene-based PCR-DGGE. The *dsrB* gene can be used as functional marker for SRB, since it encodes the dissimilatory sulfite reductase, a key enzyme in sulfate reduction catalyzing the reduction of sulfite to sulfide (Klein *et al.* 2001). The SRB diversity studies within anaerobic digester sludge from

digesters A–D revealed that quite similar DGGE fingerprinting profiles were produced from the different sludges (Figure 2(I)), demonstrating that comparable SRB communities were present in these digesters. Sequence analysis of these DGGE bands revealed the presence of uncultured SRB (*Deltaproteobacteria*) in the digester sludges, indicating SRB activity and H_2S production. The conversion of sulfate to H_2S by SRB is an initial step within the concrete corrosion process. Since the pH of digester sludge is normally slightly acidic to neutral (pH 6–7; Derbal *et al.* 2009), H_2S is the dominant sulfide species. Due to its poor water solubility, the H_2S will partition into the headspace (based on Henry's law) and re-partition into the condensate on the concrete surface because of the alkaline pH of the condensate layer (Roberts *et al.* 2002). At basic pH values, H_2S is converted to HS^- (pH 7–9.5) or S^{2-} (pH >9.5). When oxygen is available in the digester headspace, which might enter the system through cracks or desulfurization (see 'Introduction'), the sulfide species chemically react to partially oxidized sulfur species (elementary sulfur, thiosulfate, and polysulfate species), which can be subsequently used as an energy source by SOB.

Analysis of enriched SOB cultures

Since the DNA extraction from the biofilm samples scratched off from the concrete surface resulted in insufficient mass, conventional cultivation techniques were applied. Previous cultivation studies have shown that bacteria producing sulfuric acid, which were collected from concrete sampled from a sewer pipe (Nica *et al.* 2000) and bridge support (Wei *et al.* 2010), could be successfully enriched using culture media. For the enrichment of a broad range of SOB a variety of culture media differing in pH and energy source were applied. As active SOB produce acid, pH decline was used as a positive indicator for growth. Within the mixed SOB cultures the acid-producing activity was very high, since the pH within some liquid media decreased from 4.5 to 0.5 within 7 days (e.g., digester E). Similar observations were made with concrete samples collected from a bridge support, where microbes within the culture media were able to lower the pH from 6.5 to 2.5 within 7 days (Wei *et al.* 2010). All enriched SOB liquid cultures exhibiting a pronounced pH decrease were used to cultivate and isolate pure SOB cultures on the corresponding agar media. Finally, three pure cultures, originating from the headspace of digesters A, B, D, and E, were gained and identified by colony PCR and sequence analysis (Table 2): *Acidithiobacillus thiooxidans*, *Thiomonas intermedia*, and *Thiomonas perometabolis*. The same three organisms were confirmed by PCR-DGGE and sequence analysis from enriched SOB mixed cultures. The diversity study by DGGE identified even two additional SOB species for digesters A, B, and C which could not be cultivated on agar media: *Thiobacillus thioparus* and *Paracoccus solventivorans* (Table 2). Finally, enrichment methods proved to be a good approach for cultivating and isolating different SOB species although no information on the number of SOB cells actually present in the environmental samples is provided. Nevertheless Wei *et al.* (2010), for instance, have demonstrated that *T. perometabolis*, being the dominant acid-producing bacterium within the enrichment medium, was

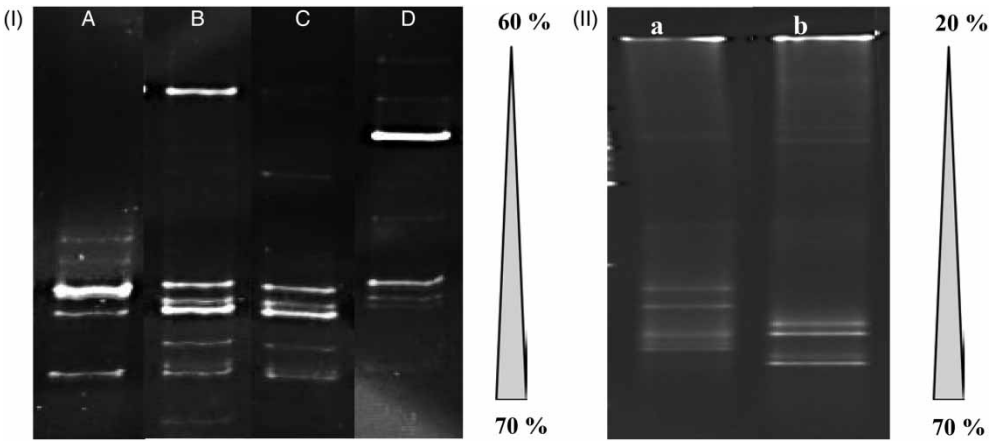

Figure 2 | DGGE fingerprinting profiles. (I) *DsrB* gene-based DGGE of SRB detected in digester sludge from digesters A–D. (II) 16S rRNA gene-based DGGE profile of the biofilm sample (digester A) inoculated in DSMZ medium 35 containing S^0 (a) and DSMZ medium 68 containing $Na_2S_2O_3$ (b) as sole energy source. 6% polyacrylamide gel with denaturing gradients ranging from 60–70% (I) and 20–70% (II).

Table 2 | List of identified SOB species relevant for the BSA process in five different digesters in Germany (A–E)

Digester	Species	Detection method	Maximum identity [%]	pH optimum	Energy source
A	*A. thiooxidans*	Pure culture: colony PCR	99	2–4	$Na_2S_2O_3$
A	*P. solventivorans*	PCR-DGGE	99	7–8	$Na_2S_2O_3$
B	*T. thioparus*	PCR-DGGE	99	6–8	$Na_2S_2O_3$
B	*T. intermedia*	PCR-DGGE	99	5.5–6	$Na_2S_2O_3$
B	*T. intermedia*	Pure culture: colony PCR	100	5.5–6	$Na_2S_2O_3$
C	*P. solventivorans*	PCR-DGGE	99	7–8	$Na_2S_2O_3$
D	*A. thiooxidans*	PCR-DGGE	99	2–4	$Na_2S_2O_3$
D	*T. intermedia*	PCR-DGGE	99	5.5–6	S^0
D	*T. intermedia*	Pure culture: colony PCR	99	5.5–6	$Na_2S_2O_3$
D	*T. perometabolis*	Pure culture: colony PCR	99	5.5–6	$Na_2S_2O_3$
E	*A. thiooxidans*	Pure culture: colony PCR	99	2–4	$Na_2S_2O_3$
E	*T. intermedia*	PCR-DGGE	99	5.5–6	$Na_2S_2O_3$; S^0
E	*T. perometabolis*	PCR-DGGE	99	5.5–6	$Na_2S_2O_3$; S^0

also the most frequently occurring one within the original corroded concrete samples. Thus, dominant SOB species identified within the enriched cultures in the present study might also be the most abundant ones growing on the concrete surface of the digester headspace.

Furthermore, DGGE analysis revealed that the supply of different energy sources (S^0 or $Na_2S_2O_3$) caused a population shift within the enriched SOB cultures, although the SOB diversity remained constant (Figure 2(II), shown for digester A). *P. solventivorans* and *T. thioparus* could only be enriched in media containing $Na_2S_2O_3$ as the only source of energy (Table 2). *T. intermedia* and *T. perometabolis* were able to use either $Na_2S_2O_3$ or S^0. However, most identified SOB were detected in liquid media containing $Na_2S_2O_3$ as sole energy source. Even when additional energy sources such as $(NH_4)_2SO_4$, $MgSO_4$, and $FeSO_4$ were provided, no other SOB species could be cultivated. These results indicate that by applying different sulfate-based compounds no growth advantages can be achieved.

Generally, the process of microbial-induced concrete corrosion in a sewer pipe proceeds in a series of stages, and a succession of microbial communities takes place (Roberts *et al.* 2002; Bielefeldt *et al.* 2010). The BSA process within sludge digesters is likely to occur in the same way. In a first step, bacteria in the digester sludge produce carbon dioxide, H_2S, and other gases with acidic properties, leading to a gradual pH decrease of the concrete surface from an initial value of approximately 12 to 9. Once these abiotic processes have lowered the pH to around 9, microbial activity can take place and neutrophilic sulfur-oxidizing microorganisms (NSOM) start colonizing the concrete surface. *T. thioparus*, *T. neapolitanus*, *T. intermedia*, and *T. perometabolis* are typically considered as NSOM in sewer systems (Vollertsen *et al.* 2008; Wei *et al.* 2014). *T. thioparus* was reported to be the first to colonize new concrete surfaces, but disappears with ongoing corrosion (Wei *et al.* 2010). The NSOM oxidize sulfide, sulfur and thiosulfate and produce polythionic acids as well as sulfuric acid, leading to a further pH decrease of the concrete matrix from approximately 9 to 3.5–5 (Bielefeldt *et al.* 2010). At pH values below 5, acidophilic sulfur-oxidizing microorganisms (ASOM), typically *A. thiooxidans*, continue sulfide oxidation by producing high amounts of H_2SO_4. The pH drops further to values of 0–1.5 (Diercks *et al.* 1991; Wei *et al.* 2014). *A. thiooxidans* is found to be the most dominant SOB in heavily corroded concrete (Diercks *et al.* 1991; Okabe *et al.* 2007). The different SOB species found in this study and their corresponding pH optima provide information on the progression of microbial corrosion. Especially in digesters A, D, and E, the detection of *A. thiooxidans* (pH optimum 2–4), the key organism in the BSA process (Diercks *et al.* 1991), provides evidence of an advanced BSA attack. In these digesters also the high acid-producing activity within the enriched SOB cultures could be positively correlated to the degree of damage (Figure 1; shown for digester E). In digesters B and C, *T. thioparus*, *T. intermedia*, and *P. solventivorans* with pH optima of 5.5–8 were identified. The occurrence of these NSOM indicates a lower extent of corrosion. Although *P. solventivorans* was not mentioned in the context of BSA before, it seems to play a role within the corrosion process as well, since it was reported to occur in

several digesters (A and C) and some *Paracoccus* species are capable of oxidizing sulfur compounds (Kelly *et al*. 2006). Finally, within this study, different active SOB were isolated from the biofilm that was taken from the headspace of different digesters. Since the SOB species found are strictly aerobic, oxygen which might come through imperfections or desulfurization must be available within the system initiating the BSA process.

However, the application of enrichment cultures and PCR-DGGE technique give no or only little information on the number of SOB cells originally present on the concrete surface of digester headspace. To avoid the enrichment step, genomic DNA needs to be isolated directly from the biofilm samples. Since the isolation of genomic DNA has not been successful so far, probably due to low biomass concentration, the optimization of extraction techniques on low DNA content is required. In a further step, the quantitative real-time PCR technique (qPCR) can be used for quantifying the different SOB species within the biofilm samples, to better correlate the dominant SOB with the degree of concrete corrosion in sludge digesters. To determine the concrete corrosion potential of NSOM and ASOM *in situ*, specific simulation chambers inoculated with digester sludge containing SRB and the three identified pure SOB cultures (*A. thiooxidans*, *T. intermedia*, and *T. perometabolis*) and concrete specimens will be carried out.

CONCLUSIONS

In this study the relevant bacteria involved in the BSA process (SRB and SOB) were consistently detected in five different digesters in Germany. The SRB diversity studies by *dsrB* gene-based DGGE revealed that different uncultured SRB (*Deltaproteobacteria*) were present in digester sludge, indicating H_2S-production potential. For the first time, SOB (*A. thiooxidans*, *T. intermedia*, *T. perometabolis*, *T. thioparus*, and *P. solventivorans*) were identified in the digesters, demonstrating that BSA not only is a problem prevalent for sewer pipes, but can also occur in sludge digesters. Conventional cultivation techniques for the enrichment and isolation of the SOB species *A. thiooxidans*, *T. intermedia*, and *T. perometabolis* proved to be very effective. With PCR-DGGE and sequence analysis the SOB communities in the mixed enriched SOB cultures could be described more accurately. In addition, the presence of SOB species characterized by an optimum growth at different pH could be correlated to the progression of microbial corrosion.

ACKNOWLEDGEMENT

We gratefully acknowledge financial support by the Bundesministerium für Wirtschaft und Technologie (BMWi); Zentrales Innovationsprogramm Mittelstand, ZIM-Kooperationsprojekt.

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First received 19 May 2014; accepted in revised form 11 August 2014. Available online 13 September 2014