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Microbial diversity in biofilms from corroding heating systems

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Abstract
Culture-independent investigations of the bacterial diversity and activity in district heating systems with and without corrosion did not make it possible to relate one group of microorganisms with the observed corrosion. Fluorescence *in situ* hybridization by oligonucleotide probes revealed the dominance of β-proteobacteria, sulphate reducing prokaryotes and α-proteobacteria. Analysis of a clone library from one Danish heating (DH) system showed that the most sequences formed two clusters within the α-proteobacteria affiliated to the families Rhizobiaceae and Acetobacteraceae and two clusters within the β-proteobacteria belonging to the family Comamonadaceae. Functional groups were determined by microautoradiography showing aerobic and anaerobic bacteria (sulphate reducing and methanogenic bacteria). The corrosion study showed that pitting corrosion rates were five to ten times higher than the general corrosion rates, suggesting the presence of biocorrosion. The results indicate that several bacterial groups could be involved in corrosion of DH system piping including sulphate reducing prokaryotes, *Acidovorax* (within the β-proteobacteria), methanogenic bacteria and others.

Key words: *Fluorescence* in situ *hybridization (FISH), microautoradiography (MAR), biocorrosion, phylogenetic analysis, cultivation independent techniques, heating systems*

Introduction

Danish heating (DH) systems have been shown to suffer from biofouling and biocorrosion causing reduced lifetime and increased operational costs (Kjellerup et al. 2003). These problems occur despite adherence to strict requirements regarding water quality for the largest DH systems to prevent most microbial growth. The water is treated by reverse osmosis, deaerated and thus anaerobic, nutrient poor and highly alkaline (pH 9.5 – 10) due to addition of sodium hydroxide. Until recently, water chemistry has been implicated as the sole cause for corrosion in DH systems. New studies focusing on biofilm formation and possible biocorrosion were recently conducted in a number of DH systems. These studies showed that microorganisms were present in high numbers in biofilms and that many of these were sulphate reducing prokaryotes (SRP) (Goeres et al. 1998; Kjellerup et al. 2004) that can be corrosive or form corrosive sulphide species (Lee et al. 1995; Hamilton, 2000).

The microbial diversity in DH systems may be expected to be limited due to the special water characteristics regarding the microbially important parameters such as pH, conductivity and oxygen concentration. Therefore, it might be possible to find microorganisms related to biocorrosion. If so, monitoring and control strategies may be improved enabling intervention strategies at an earlier stage. One group of important microorganisms belongs to the SRP. Beech et al. (1994) showed that some SRP species originating from corroded systems differed in their aggressiveness towards mild steel, despite identical growth conditions (Beech et al. 1994).

Other studies have suggested that certain bacterial groups could be related to the presence of biocorrosion for example *Sphingomonas* species were related to copper corrosion (Arens et al. 1995) and *Desulfotomaculum, Sulfobacillus* and *Thermoproteus* species were isolated from corroding pipes of the Moscowian heating system (Golovacheva et al. 1986). Corrosion processes on mild steel plates showed that SRP and iron oxidizing/reducing bacteria together with sulphur oxidizing bacteria were

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likely involved in corrosion (Rozanova *et al*. 2003b). However, other microorganisms may also cause biocorrosion including methanogenic bacteria causing cathodic depolarization due to their utilization of hydrogen, acid producing bacteria, sulphur oxidizing bacteria and manganese oxidizing bacteria (Lee et al. 1995; Dickinson & Lewandowski, 1996; Domingo et al. 1998; Dexter et al. 2003; Hamilton, 2003; Rozanova et al. 2003a; Zhang et al. 2003). It is presently unknown whether any of these groups may be present or be corrosive in DH systems.

It may be difficult to identify the particular microorganisms responsible for corrosion in DH systems. Cultivation of bacteria from such extreme environments can be extremely difficult, and this can lead to underestimation of the real number of active bacteria, isolation and identification of non-dominant bacteria, and perhaps implementation of insufficient control strategies (Wilderer et al. 2002). It is therefore critical to apply culture-independent techniques for identification of the microorganisms, such as fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR), denaturant gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) or clone library analysis (Muyzer et al. 1993; Amann, 1995; Wilderer et al. 2002; Wagner et al. 2003). Furthermore, it is important to establish a link between the identity and the activity of important functional groups by microautoradiography (MAR) where the key physiological features of dominant microbial groups can be quantified (Nielsen et al. 2002; Nielsen & Nielsen, 2002b).

In this study the microbial diversity was determined by culture-independent techniques in a number of DH systems in order to investigate the identity of dominant microbial groups. A more detailed investigation of selected microbial groups was also carried out by use of corrosion monitoring units (CMUs) placed on side streams of selected DH systems. Analysis of biofilm development, microbial diversity and activity was monitored and compared to measurements of corrosion of mild steel in order to search for dominant bacteria involved in biocorrosion.

Materials and methods

Sampling

CMUs based on the rotortorque principle (Griebe & Flemming, 2000) were installed on a side stream of the return circuit in several DH systems in order to monitor biocorrosion. Screening by FISH was performed in five DH systems and CMUs (see Table I). The water quality varied among the screened DH systems, but it generally complied with the DH guidelines (Danish Council of District Heating, 1999).

Test coupons of mild steel (SAE 1015) with a surface area of 14.4 cm^2 (2.4 x 6.0 cm, width x height) and a thickness of 1.1 mm were coated on the backside with a chemically and electrochemically inert material (Carbon-like-Diamond, Danish Technological Institute, Aarhus, Denmark). The coupons were cleaned according to ASTM standard G1-90 (ASTM, 1999), weighed prior to exposure and sampled for analysis after exposure for one, two and three months. Coupons used for corrosion measurements were dried on site by the use of oxygen-free N_2. The water flow through the CMUs was approximately 2 l h^{-1} (hydraulic resistance time of approximately 30 min) with turbulent conditions (surface velocity at approximately 2 m s^{-1}) simulating the conditions of a DH pipe. The CMUs were run at mesophilic conditions (35 – 40°C). In one case molybdate was continuously dosed to a CMU (final concentration was 20 mg l^{-1} molybdate added as sodium molybdate) in order to test the effect of inhibition of SRP.

Biofilm was scraped off the coupons using sterile cell scrapers (Orange Scientific, Braine-'l'Alleud,

Table I. Sampling locations and characteristics for the screening of DH systems (Danish Council of District Heating, 1999).

DH System	DH1	DH2	DH3	DH4	DH5
Sample type	*In situ*	CMU	CMU	CMU	*In situ*
Sampling point	Filter unit	Mild steel	SS316	Mild steel	Filter unit
Corrosion	No	No	No: SS316 Yes: MS)	Yes	No
pH	9.8 ± 0.2	9.8 ± 0.2	9.8 ± 0.2	9.8 ± 0.2	9.8 ± 0.2
Conductivity (μS cm^{-1})	< 1,500	< 50	< 500	< 15	< 500
Oxygen (ppb)	< 20	< 10	< 20	< 20	< 20
Chloride (mg l^{-1})	< 300	< 3	< 50	< 1	< 50
Sulphate (mg l^{-1})	—	< 2	—	< 1	—
Org. matter (mg l^{-1})	—	—	—	< 2	—
Total iron (μg l^{-1})	< 100	< 50	< 100	< 50	< 100

cmu = corrosion monitoring unit; — = no water quality demand exists.

Belgium), stored in 0.2 μm filtered (Millipore, Glostrup, Denmark) DH water and fixed with formaldehyde (2% final concentration, v/v) for total bacterial enumeration. For FISH and MAR experiments the biofilm samples were transported anaerobically in DH water containing 5 mg l^{-1} dithionite to the laboratory. The coupons were evaluated with DAPI after scraping in order to test the efficiency of the removal and only up to 10^4 cells cm^{-2} were left at the surface. Biofilm used for FISH, MAR and DNA extraction was sampled after three months' exposure.

Analytical methods

Total bacterial number in biofilm. Determination of total number of bacteria was performed in biofilm samples by the general bacterial stain DAPI (4',6-diamidino-2-phenyl indole) and use of epifluorescence microscopy (Standard Methods, 1995). An Axioscop II (Zeiss, Jena, Germany) epifluorescence microscope with the filter set 01 (Zeiss, Jena, Germany) and 100 × magnification oil immersion objective was used. Counting was performed on black polycarbonate filters (Osmonics Inc., Herentals, Belgium) where ten randomly chosen fields (100 μm x 100 μm) were selected. Within each of those fields 20 – 200 cells were counted, with a minimum of 400 cells in total per filter.

Fluorescence in situ *hybridisation.* Fluorescence *in situ* hybridisation (FISH) was performed with 16S and 23S fluorescently labelled rRNA-targeted nucleic acid probes (Thermo Hybaid, Ulm, Germany) on biofilm samples (Amann, 1995). Samples were fixed in fresh 4% (w/v) paraformaldehyde/PBS or 50% ethanol (v/v), washed three times in filtered distilled water, stained with DAPI (1 mg ml^{-1}), immobilised on slides (Marienfeld, Lauda-Koenigshofen, Germany), and hybridised. Filter sets 09 and 10 (Zeiss, Jena, Germany) were used with a 100 × magnification oil immersion objective for microscopic examination. Cy3 labelled specific probes were used for enumeration (Bouvier & Del Giorgio, 2003). Total bacterial numbers in the range of 20 – 200 were counted with DAPI at each spot and at the same spots the number of FISH-positive cells was determined. A minimum of 400 DAPI stained cells were counted for each hybridisation (performed in triplicate). The following probes were used: ARCH915 (Archaea), Eelm5932 (Archaea), EURY498 (Euryarchaeota within Archaea), CREN499 (Crenarchaeota within Archaea), EUB338-I + II + III, the same as EUB-mix (Bacteria), NONEUB (Nonsense probe), ALF968 (α-proteobacteria), SPH120 (*Sphingomonas*), BET42a (β-proteobacteria), the two sub-groups BONE23a (Beta-1) and BTWO23a (Beta-2), Cte (mainly *Acidovorax*), GAM42a (γ-proteobacteria), SRB385 (δ-proteobacteria), SRB385Db (Desulfobacteriaceae within the δ-proteobacteria), PLA46 (*Planctomycetes*). Specific probes used for identification of SRB were DSB129 (*Desulfobacter*), DSS658 (*Desulfosarcina*), DBB660 (*Desulfobulbus*), DSV687 (*Desulfovibrio*), DSV698 (*Desulfovibrio*), Dsb804 (several species of *Desulfobacter* and *Desulfosarcina*), Dscoc814 (*Desulfococcus, Desulfosarcina, Desulfobulbus*) and DSB985 (*Desulfobacter*).

The following probes targeting Gram-positive bacteria were also tested: HGC69a (Actinobacteria), LGC354 A + B + C (Firmicutes), CF319 a + b (Cytophagales) and Dtm229 (*Desulfotomaculum*). Other details about the probes and the hybridisation conditions can be found in probeBase (Loy et al. 2003). Non-specific binding to the samples was tested by the Cy3 labelled nonsense probe (NONEUB), and no signals could be seen. As positive controls, a number of pure cultures (*E. coli, Desulfovibrio desulfuricans*) and enrichment cultures from DH systems (SRP) were applied, as well as a number of activated sludge samples where the examined genus or species were known to be present. In particular anaerobically treated activated sludge was used as a positive control for Archaea (methanogens) and SRP.

PCR, DGGE and Sequencing. Nucleic acids were extracted using FastDNA spin kit for soil (Qbiogene, Carlsbad, CA, USA), according to the manufacturer's instructions. Fresh PCR products generated using the primers 26F-1392R were purified using a QIAquick PCR Purification Kit (Qiagen, Albertslund, Denmark). The products were inserted into a pCR® 4-TOPO® vector and transformed into ONE SHOT *E. coli* cells following the manufacturer's directions (Invitrogen, Taastrup, Denmark). Plasmids of 100 randomly selected clones were then recovered using a QIAprep spin miniprep kit (Qiagen). PCR products were first obtained from the selected plasmids using the primers 341F-GC (Muyzer et al. 1993) and 907R (Lane, 1991). Clones were subsequently screened by DGGE using the D-Gene System (BioRad, Herlev, Denmark) according to the manufacturer's guidelines using polyacrylamide gels with a denaturing gradient of 30 – 70%. Gels were run for 15 h at 100 V in 1 × TAE buffer at 60°C, and were stained in 100 ng μl^{-1} SYBR Gold (Molecular Probes, Leiden, The Netherlands). Plasmids showing different band patterns were selected for sequencing and full-length PCR products were made.

The purified PCR products (4 – 7 μl) were sequenced using 0.5 μl primers (10 pmol μl^{-1}) and 5 μl DYEnamic ET dye terminator sequencing kit (Amersham Biosciences, Hillerød, Denmark) per

reaction. The same primers as described by Blackall (1994) were used, and the sequencing reactions were separated and read on a MegaBACE DNA sequencer (Amersham Biosciences). Checks for chimeric sequences were conducted using the CHECK_CHIMERA program of Ribosomal Database Project (http://rdp.cme.msu.edu) and the program BELLEROPHON developed by Hugenholtz and Huber (2003). Nearly complete 16S rRNA gene sequences were compiled and then aligned using the automatic nucleic acid aligner in the ARB software package (http://www.arb-home.de). Alignments were then refined manually. A total of 1374 unambiguously aligned nucleotides from position 26–1400 were used for calculation of trees by distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software.

Nucleotide accession numbers. The Genbank accession numbers for the 16S rRNA sequences reported in this paper are AY682678 – AY682684.

Microautoradiography

Microautoradiography (MAR) was performed using [^{3}H]-acetate and [^{14}C]-bicarbonate (Amersham Biosciences, Hillerød, Denmark) under aerobic and anaerobic conditions. Anaerobic incubations were performed directly, while samples for aerobic incubations were aerated for 30 min before addition of tracer to ensure aerobic conditions. Tracers were added to sample volumes of 2 ml ensuring a specific activity of 40 μCi. The final concentration of acetate (labelled and non-labelled) was 0.1 mM, and the final concentration of bicarbonate was 0.2 mM (labelled and non-labelled), adding the non-labelled bicarbonate as a buffer at pH 8.5. The concentration of sulphate was 0.1 mM. For incubations with labelled bicarbonate, 10% of the headspace volume was exchanged with H_2. The incubation temperature was 30°C, and the incubation time for aerobic and anaerobic incubations was 3 h and 12 h, respectively. Molybdate (1 mM) for SRP and bromoethane-sulfonic acid (BES) (1 mM) for methanogenic bacteria were used as inhibitors to determine bacterial functional groups (Nollet et al. 1997; Nielsen & Nielsen, 2002a). The incubations were terminated by the addition of 2 ml of 8% freshly prepared PFA/PBS solution (to a final concentration of 4%), which was removed after 2 h fixation by washing three times in filtered (0.2 μm) tap water. Incubations with bicarbonate were washed with a glycine buffer (pH 3) for 1 h to remove precipitated carbonate. The samples were stained with DAPI, and 50–100 μl were spread on cover slides in triplicate. The slides were covered with a radiosensitive film emulsion (LM-1, Amersham Biosciences, Hillerød, Denmark) and exposed for 12 days at 5°C. After development, the total number of DAPI positive (DAPI-pos) cells were enumerated in ten randomly chosen microscopic fields, and the number of MAR positive (MAR-pos) cells were enumerated by brightfield microscopy in the same spots. DAPI-pos cells covered with five or more silver grains were evaluated to be positive. A minimum of 250 DAPI-pos cells was counted on each slide. Further details about the method, including controls, can be found elsewhere (Nielsen & Nielsen, 2002b; Kjellerup et al. 2003).

Corrosion

Corrosion of the mild steel coupons was evaluated through determination of weight loss and pit formation. Weight loss was assessed according to G1-90 (ASTM, 1999). Pit formation was determined for the individual coupons by visual inspection in combination with laser interferometry. Five to ten individual pits were identified by visual inspection at each coupon according to G46-94 (ASTM, 1994). The width and the depth of each pit were measured with laser interferometry (Kjellerup et al. 2003; UBM, 2004). Sulphide in the biofilm was determined qualitatively with a method based on catalytic formation of nitrogen bubbles due to an azide-sulphide reaction (Feigl et al. 1972) as observed in a light microscope (Leica, Hvidovre, Denmark).

Results

Screening of corrosion and microbial diversity in DH systems

Observations and measurements of corrosion were performed in five DH systems (DH1–DH5) and compared to the results of the screening of the microbial diversity conducted by FISH. The samples were collected from different locations in the DH systems including filtration units, heat exchangers and coupons from CMUs (see Table I). Corrosion was observed on test coupons of mild steel from DH3 and DH4. In DH3 corrosion was observed on mild steel coupons but not on stainless steel 316 coupons. The FISH examination was performed on the stainless steel coupons due to difficulties in performing FISH on mild steel biofilm (see Table II). In the remaining DH systems corrosion was not observed *in situ* or on the test coupons.

The FISH screening showed that the proportion of total bacteria stained with DAPI that could be hybridized with the general probe for Bacteria varied from 28% (DH2) to 55% (DH4), while Archaea was found only in DH4 (11%). Whether the remaining micro-organisms were dead, inactive or impermeable

Table II. Screening of bacterial diversity of DH systems by FISH analysis (% of DAPI counts).

Probe	Probe	DH1	DH2	DH3	DH4	DH5
Nonsense probe	NONEUB	0	0	0	0	0
Archaea	ARCH915	0	nt	nt	11 ± 1	0
Bacteria	EUB-mix	42 ± 4	28 ± 5	45 ± 3	55 ± 10	47 ± 14
α-proteobacteria	ALF968	4 ± 2	2 ± 1	15 ± 7	12 ± 8	5 ± 1
β-proteobacteria	BETA42	27 ± 10	16 ± 2	23 ± 7	33 ± 10	25 ± 12
- Betaone	BTWO23a	< 1	nt	12 ± 4	16 ± 5	1 ± 2
- Acidovorax	Cte	nt	nt	nt	0	0
- Betatwo	BTWO23a	22 ± 5	nt	11 ± 2	1 ± 1	16 ± < 0.1
γ-proteobacteria	GAM42a	< 1	2 ± 1	15 ± 9	6 ± 5	18 ± 4
δ-proteobacteria	SRB385	5 ± 1	3 ± 1	19 ± 8	15 ± 6	11 ± < 0.1
Desulfobacteriaceae	SRB385Db	0	nt	nt	7 ± 5	3 ± 4
Actinobacteria	HGC69a	nt	nt	0	0	0
Firmicutes	LGC354a + b + c	nt	nt	0	0	0
Desulfotomaculum	Dtm229	nt	nt	0	< 1	3 ± 2
Cythophagales	CF319a + b	nt	4 ± 1	nt	0	nt

Average ± SD is shown for triplicate analysis; nt = not tested.

to the probe is not known. Both of these samples originated from mild steel test coupons. Almost all microbial groups tested were found in the samples, however varying in abundance. β-proteobacteria was the most abundant group in all samples and constituted 16 – 33 % of the total number of bacteria (DH2, DH4), corresponding to more than half of all bacteria (positive with EUB-mix) in all samples. SRP were found in all systems to a varying extent from 3 – 20% of the total number of bacteria.

Comparison of the corrosion characteristics from the sampling sites with the microbial diversity as investigated by FISH applying mainly group-specific probes did not show any clear correlation. Thus, it was not possible to identify any indicator organisms or group of microorganisms that were specifically related to the corroded DH systems. Therefore, a more detailed investigation of the microbial diversity was carried out at DH4 due to significant corrosion at this location.

Detailed investigation of DH4

In order to investigate whether the SRP were actively involved in the corrosion at DH4, two CMUs were installed on a side stream of DH4. Molybdate was added in one CMU in order to selectively inhibit sulphate reduction in the biofilm on mild steel and thereby eliminate corrosion caused by SRP. Another CMU served as a control only with DH water. Biofilm developed on mild steel coupons in both the control and the molybdate CMU. The number of bacteria (DAPI count) reached a constant level of $0.5 - 1.0 \times 10^8$ cells cm^{-2} after approximately one month (see Table III).

Among the total microorganisms counted by DAPI in the control reactor 75% were FISH-positive (see Table IV), and of these, 85% were bacteria while the remaining 15% were Archaea. The dominant groups of bacteria belonged to the β-proteobacteria, δ-proteobacteria (many SRP) and α-proteobacteria. Among the β-proteobacteria, the sub-group Betaone accounted for 16%. Further tests with specific SRP probes showed presence of *Desulfovibrio* and *Desulfobacter*, but the majority (85% of SRP) were not identified with the SRP probes tested, indicating the presence of unidentified SRP, or that some non-SRP were targeted by the applied general SRP probe (SRB385).

Table III. Enumeration of the total number of bacteria in biofilms on mild steel coupons (DAPI count).

	Total number of bacteria ($x10^6$) (Cells cm^{-2})		
	Exposure time		
CMU	1 month	2 months	3 months
Control	35 ± 14	54 ± 39	22 ± 5
Molybdate	31 ± 13	54 ± 23	35 ± 8

Average ± SD is shown for triplicate analysis.

In the molybdate treated CMU 77% of the total number of the bacteria were identified either as Bacteria (68%) or Archaea (9%), and the dominant groups were β-proteobacteria (mainly Betaone) and α-proteobacteria, very similar to the results from the control CMU (see Table IV). However, no SRP were detected when molybdate was dosed, and the number of γ-proteobacteria was significantly below the control (1% compared to 6%, respectively). The results showed that only 73% and 47% of the bacteria in the control and molybdate CMUs, respectively, were identified leaving a large proportion unidentified. While the presence of Gram-positive bacteria could make up some of this remaining population Firmicutes and Actinobacteria were not identified in

Table IV. Identification of bacterial diversity in biofilm samples from mild steel coupons in CMUs by use of FISH analysis for triplicate analysis (% of DAPI counts).

Bacterial target	Probe	Control	Molybdate
Archaea			
Archaebacteria	ARCH915	11 ± 1	9 ± 1
- Crenarchaeota	CREN499	0	0
- Euryarchaeota	EURY498	0	0
- Archaebacteria	Eelm5932	0	0
Bacteria	EUB-mix	64 ± 2	68 ± 7
Proteobacteria			
α-proteobacteria	ALF968	14 ± 4	11 ± 3
- *Sphingomonas*	SPH120	4 ± 1	2 ± 2
β-proteobacteria	BETA42a	33 ± 1	35 ± 3
- Betaone	BONE23a	16	15
- Betatwo	BTWO23a	4 ± 3	2 ± 1
γ-proteobacteria	Gam42a	6	< 1
δ-proteobacteria	SRB385	15 ± 1	< 1
- Desulfovibrionaceae			
- *Desulfovibrio*	DSV687	< 1	0
- *Desulfovibrio*	DSV698	2	0
- Desulfobulbus	SBB660	0	0
- Desulfobacteriaceae	SRB385Db	5 ± 3	< 1
- *Desulfobacter*	SRB129	< 1	0
- *Desulfosarcina*	DSS658	0	0
- *D.fococcus-D.sarcina-D.fobotulus*	Dsb804	0	0
Planctomycetes	PLA46	< 1	< 1
Cytophagales	CF319a	0	nt
Firmicutes	LGC a + b + c	0	nt
- *Desulfotomaculum*	Dtm229	< 1	nt
Actinobacteria	HGC69a	0	nt
Nonsense probe[I]	NONEUB	0	0

nt = not tested; [I] = negative control with no target to known sequences.

the later biofilm sample, and *Desulfotomaculum* was only present in a low number (< 1%).

Phylogenetic analysis of clone library

DNA was extracted from the biofilm from mild steel coupons from the control CMU, amplified and cloned, resulting in a clone library consisting of 100 colonies. The colonies were screened using DGGE and were grouped into five different clusters from which 25 selected clones representing the five clusters were sequenced. Phylogenetic trees based on aligned 16S rRNA gene data were estimated by using distance, parsimony and maximum likelihood algorithms. The applied methods generated congruent tree topologies, and in Figure 1, a maximum likelihood tree shows the relationships between the selected clones and the closest relatives.

The results of the clone library showed that almost all clones belonged to β-proteobacteria (59%) and α-proteobacteria (40%). Five phylogenetic clusters were found (Accession numbers listed). Cluster 1a (AY682682): most clones (30%) were closely related to *Acidovorax* sp. (AF235010, similarity > 99%) within the β-proteobacteria. Cluster 1b (AY682678, AY682681): 29% of the clones were affiliated with an *Acidovorax* sp. (Y18617, similarity between 96–99%). Cluster 2a (AY682684, AY682683): 15% of the clones were related to *Agrobacterium tumefaciens* (D13294, similarity between 94–98%) within the α-proteobacteria. Cluster 2b (AY682679): 25% of the clones affiliated with other α-proteobacteria, an uncultured Antarctic bacterium and *Roseococcus thiosulfatophilus* (AF173825 and X72908 respectively, similarity between 95–98%). Cluster 3 (AY682680): one clone affiliated distantly with *Flavobacterium ferrugineum* (M28237, similarity 90%).

Quantification of functional groups with MAR

In order to determine dominant physiological groups of microorganisms in the DH system, MAR was applied. When biofilm from the control CMU was exposed to [^{3}H]-acetate under aerobic conditions, 28% of the total DAPI-count took up labelled substrate as quantified by counting all cells that were MAR-positive (see Table V).

Under anaerobic conditions 21% were active. A more detailed study of the anaerobic population, using the specific inhibitors: molybdate against SRP and BES against methanogenic bacteria, showed that 5% of the total DAPI count were SRP that were able to assimilate [^{3}H]-acetate only in absence of molybdate. Methanogenic bacteria constituted 2% of the total DAPI-count (being active in absence of BES), while the remaining 14% were unidentified anaerobic micro-organisms. In biofilm from the molybdate treated CMU, 16% of the total number of bacteria took up [^{3}H]-acetate under anaerobic conditions, which was slightly lower than the control. No bacteria seemed to be SRP in this biofilm, since the numbers of active bacteria as determined by MAR were similar with and without addition of molybdate in the laboratory experiment. The difference in the number of anaerobic bacteria between the control and the molybdate CMUs constituted 5% of the total DAPI count, and they were most likely SRP since molybdate added to the CMU inhibited the activity of this fraction. The presence of autotrophic bacteria was also studied in the MAR experiments with uptake of labelled bicarbonate together with hydrogen. Under both aerobic and anaerobic conditions uptake of labelled bicarbonate was observed in biofilm from the control, but only in very low numbers (< 1% of the total number of bacteria).

Corrosion

Corrosion was observed on mild steel coupons from both CMUs. Moreover, it appeared as both general

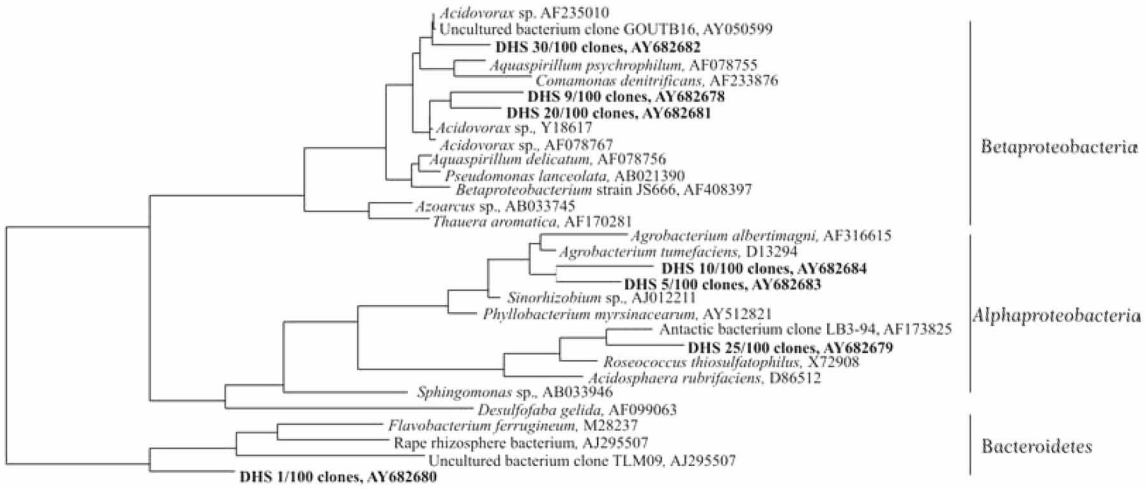

Figure 1. Phylogenetic tree based on sequencing of 100 clones from a biofilm sample originating from mild steel coupons in the control CMU.

Table V. Determination of bacterial functional groups with MAR analysis using ^{3}[H]-acetate for triplicate analysis (% of total DAPI counts).

Bacterial functional group	MAR-pos *vs.* DAPI-pos (%)
Aerobic bacteria	28 ± 5
Anaerobic bacteria	21 ± 9
- Sulphate reducing bacteria	5 ± 2
- Methanogenic bacteria	2 ± 2
- Other anaerobic bacteria	14 ± 1

and pitting corrosion. The general corrosion rates in the two CMUs were at the same level for the first month of exposure (approx 30 μm year^{-1}). For the second and third samplings the corrosion rates for the control decreased to 5 and 10 μm year^{-1}, respectively, while the corrosion rates for the molybdate treated CMU increased to 56 and 60 μm year^{-1}, respectively (see Table VI). The rates were the highest in the molybdate-treated CMU showing that treatment with molybdate did not inhibit the corrosion.

Pitting characteristics were studied in order to determine whether corrosion was mainly caused by general corrosion or pitting (localized) corrosion. By differentiating general and localized corrosion rates, the involvement of bacteria may be inferred, since pitting corrosion has long been used as an indicator of the presence of corrosive bacteria in cases where only general corrosion was predicted (Little et al. 1992). The pit characteristics showed that the depth of the pits ranged from 30 – 220 μm while the width of the pits varied from 220 – 1040 μm (data not shown). The deepest pits were measured on the coupons from the molybdate-treated CMU after three months. Localized corrosion rates were in the range of 300 – 900 μm year^{-1} with the lowest rates in the control CMU at 300 – 400 μm year^{-1}. Local corrosion was most pronounced in the molybdate CMU with rates in the range of 400 – 900 μm year^{-1}, thus showing that molybdate did not reduce the incidence of pitting corrosion. Furthermore, sulphide was present on the surface of all mild steel coupons from both CMUs at all sampling times. This sulphide was possibly due to transport of sulphide from the overall DH system into both CMUs, since SRP were not detected by either FISH or MAR in the molybdate CMU.

Discussion

The microbial diversity in the DH systems investigated was high as demonstrated by the FISH screening. Both Archaea and Bacteria were present with the latter being the most abundant. Among the Bacteria almost all probes tested hybridized with some cells indicating a high bacterial diversity. The FISH analysis also showed bacteria belonging to a few dominant groups including the β-proteobacteria and to a lesser extent the α- and δ-proteobacteria (many SPR). Based on this general survey it was not possible to find a relationship between the water quality of the DH systems, the presence of corrosion and the presence of one or two dominant groups related to corrosion. In the overall examination of the two CMUs at DH4 a similar pattern was observed with high bacterial diversity based on FISH results and with only a few dominant groups of bacteria present. β-proteobacteria was the most abundant group as determined by both FISH and in the clone library, followed by α-proteobacteria. This agrees with other complex microbial systems such as activated sludge, groundwater, drinking water systems and oligotrophic aquatic biofilms where it has also been shown that β-proteobacteria was the

Table VI. General corrosion and pitting corrosion rates for mild steel coupons.

Reactor	General corrosion rate (μm year^{-1})			Pitting corrosion rate (μm year^{-1})		
Exposure time	1 month	2 months	3 months	1 month	2 months	3 months
Control	30 ± 2	6 ± 0.1	10 ± 0.5	386 ± 84	284 ± 69	383 ± 58
Molybdate	30 ± 12	56 ± 5.0	64 ± 20	608 ± 290	296 ± 70	872 ± 225

Average ± SD is shown for triplicate analysis.

predominant group (Wagner et al. 1994; Kalmbach et al. 1997; Eshenhagen et al. 2003).

Due to the fact that the water qualities of the DH systems evaluated in this survey were very much alike regarding important growth parameters including pH, conductivity and oxygen concentration, it was speculated that differences in biocorrosion rates were due to differences in bacterial populations. However, this was not the case since the tested DH systems were treated by the use of different chemicals such as phosphates or slowly degradable organic substances. In addition, the FISH screening was performed on both *in situ* and biofilm samples due to difficulties in obtaining samples from the DH systems. This difference might affect the quantitative results. However, the bacterial diversity would still be reflected.

Surprisingly, the MAR investigation of DH4 showed presence of many bacteria that were able to assimilate labelled acetate under aerobic conditions. This was not expected, as anaerobic conditions are assumed to prevail in the DH systems in order to reduce oxygen induced corrosion. Whether the organisms were facultative aerobes is not known, but since the fraction was higher than the fraction able to consume acetate under anaerobic conditions, some may have been obligate aerobes. Some of the anaerobes might also be oxygen-tolerant SRP that can consume oxygen, and probably also organic compounds, under aerobic conditions (Dilling & Cypionka, 1990). The MAR results also revealed presence of SRP in agreement with the FISH results and methanogenic bacteria in the biofilm. Once again the presence of these different physiological groups supports the finding of a high microbial diversity in the DH system.

As determined by FISH, the β-proteobacteria were the most abundant group in the two DH systems with corrosion, and most of them belonged to the sub-group Betaone. This finding is in accordance with the analysis of the clone library where more than half of the sequences belonged to two clusters in the genus *Acidovorax* within the Betaone sub-group. However, FISH analysis with the more specific oligonucleotide probe, Cte, that should have hybridised with most members of the *Acidovorax* group (Schleifer et al. 1992) did not confirm their presence (data not shown). The reason could be that the probe was tested on a biofilm sample obtained one year later from the same CMU. The possibility that *Acidovorax* were responsible for at least part of the corrosion seen in the DH systems has been demonstrated in studies of copper plumbing (Critchley et al. 2003). In addition, members of the genus *Acidovorax* have been found in a variety of potentially corrosive environments including aerobic, iron-reducing and anaerobic environments (Johnson et al. 2001; Röling et al. 2001; Mailloux & Fuller, 2003). *Acidovorax* have also been shown to be very efficient in degrading complex organic substances such as PCBs (poly chlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons) (Zhao & Ward, 1999; Eriksson et al. 2003), nitrobenzene (Etchebere et al. 2001), plastics (Uchida et al. 2000; Wang & Lee, 2001), phenols (Watanabe et al. 2002) or chloroanilines (Boon et al. 2003). This physiology fits well with the nutrient poor conditions present in the DH systems and it is possible that a fraction of aerobic bacteria as determined by MAR may have been *Acidovorax*. Whether there is a direct correlation between this group and biocorrosion in this DH system remains to be seen.

The α-proteobacteria also were abundant as determined both by FISH and clone library analysis. Bacteria related to the families Rhizobiaceae and Acetobacteriaceae (including the genus *Sphingomonas*) have been identified in a wide variety of environments including terrestrial and rhizosphere soil, marine (oligotrophic) and fresh waters and sediments (Leys et al. 2004). The locations have often been exposed to pollution, and it has been shown that *Sphingomonas* has the ability to degrade PAHs (Johnson & Hill, 2002; Johnson & Karlson, 2004), pesticides such as carbofuran (Kim et al. 2004) and the nonylphenols with oestrogenic effects (Ushiba et al. 2003). The specific probe for *Sphingomonas* (SPH120) (Eilers et al. 2000)) was applied, but *Sphingomonas* were found in very low numbers indicating that they were apparently not abundant in the DH system investigated (data not shown).

SRP are often known to cause corrosion in industrial systems (Englert & Müller, 1996; Almeida & Franca, 1999; Rao et al. 2000). Two mechanisms

are generally used to explain the observations. First, corrosion is directly caused by SRP through cathodic depolarisation (Hamilton, 2000). Second, the corrosion is caused by the presence of corrosive sulphide species, which due to their electron conductivity and noble electrode potential, can cause corrosion Lee et al. 1995; Hamilton, 2000). In this study SRP were most likely present in all samples in the survey, as well as in the control reactor in DH4 as shown by FISH and MAR. SRP were not found in the clone library, which may be due to the relatively small number of obtained clones. This discrepancy between techniques has also been observed in other environmental ecological studies, where the dominant groups of bacteria often vary depending on the applied technique (Wagner & Amann, 1997; Eshenhagen et al. 2003). However, the results show a lack of correlation between corrosion occurring with and without SRP present ($\pm$ molybdate). This could indicate that microorganisms other than SRP caused the corrosion, or that corrosive sulphide species were transported with the water to the CMUs. The presence of sulphide in the biofilms supported the latter hypothesis (data not shown), whereas MAR results and the presence of *Acidovorax* supported the presence of other potential corrosive bacterial groups, such as methanogenic bacteria that oxidize hydrogen and reduce carbonate. The presence of methanogenic bacteria was shown by MAR and supported by FISH because Archaea (which include methanogenic bacteria and many other organisms) were present in relatively high numbers (11%). Studies of biocorrosion in marine environments have shown that methanogenic bacteria together with SRP were capable of scavenging hydrogen, causing cathodic depolarization, and thereby increasing the corrosion rate on mild steel (Zhang & Fang, 2001; Zhang et al. 2003). Another explanation could be presence of acid- producing bacteria that might be favoured in the absence of SRP when molybdate was added. Preliminary results using microelectrodes for pH measurement supported this by showing a decrease from 9.5 in the bulk water to approximately 5 inside a corrosion pit (Lorenzen et al. 2004).

The large microbial diversity and the presence of aerobic and anaerobic conditions suggest that several types and mechanisms of respiration exist. Thus, corrosion is a very complex phenomenon in the DH system investigated, and many different corrosion mechanisms may be involved. Corrosion caused by oxygen is often experienced as general corrosion with low corrosion rates over a large surface area (Lee et al. 1995). The presence of biofilm and biological processes usually change the electrochemical properties at the surface, and local areas with anodes and cathodes are developed (Little et al. 1992). Pitting corrosion rates were five to ten times higher than the general corrosion rates in this study, indicating that the corrosion was most likely biologically induced (Franklin et al. 1991). Several mechanisms could be involved in the initiation of localized corrosion, such as intermittent surface coverage by a spotty biofilm producing differential concentration cells (Dexter et al. 2003), or different microbiological processes producing corrosive metabolites, such as sulphides (Lee et al. 1995) or changes in chemical properties within the biofilm/substratum (Little et al. 1992).

In order to reduce the potential of pitting corrosion, it seems essential to avoid any oxygen penetration into the DH system. Oxygen is responsible for the presence of a number of microbiological and chemical reactions that can produce corrosive species (e.g. elemental sulphur). In addition, it may prevent or disrupt formation of a uniform and homogenous layer of sulphide, which would otherwise passivate the metal surface and protect it from corrosion. Similar complex corrosion mechanisms have been used to explain biocorrosion observed in a heating system in Moscow where the presence of SRP, iron reducing and oxidizing bacteria together with sulphur oxidizing bacteria was found using cultivation methods (Rozanova et al. 2003b). Despite the differences in water quality between the Moscow system and the DH systems investigated here, the functional diversity in the Moscow system showed a similar composition and complex coupling of biocorrosion mechanisms.

Conclusion

FISH analysis of the microbial population in five DH systems with and without corrosion showed a high diversity although the systems were dominated by α- and β-proteobacteria and SRP. A more detailed analysis of a clone library from the biofilm in a corroding DH system revealed that most retrieved sequences formed two clusters within the α-proteobacteria consisting of the families Rhizobiaceae and Acetobacteriaceae, and two clusters within the β-proteobacteria, consisting of the family Comamonadaceae including the genus *Acidovorax*. However, it was not possible to conclusively correlate the distribution of organisms with the corrosion observed. Also, although SRP were present in the biofilm, it was not possible to relate this presence to biocorrosion since it also occurred in the absence of SRP when molybdate was added to the CMU to inhibit their activity. Furthermore, MAR experiments revealed an unexpectedly high presence of both aerobic and anaerobic bacteria, indicating the presence of a very complex microbial community with several respiration types and several corrosion mechanisms possible. Therefore, corrosion may have been due to other bacterial species than SRP or to

transport of sulphide present in combination with oxygen in the DH system. However, future studies involving microaerophilic conditions in relation to pitting corrosion on mild steel may further clarify the underlying chemical conditions, as well as the bacterial species responsible for corrosion within Danish DH systems.

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