Monitoring and characterisation of bacteria in corroding district heating systems using fluorescence in situ hybridisation and microautoradiography

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Abstract
Presence of biofilm and biocorrosion has been observed in Danish district heating (DH) systems despite very good water quality that was expected to prevent significant microbial growth. The microbiological water quality was investigated in order to identify the dominating bacterial groups on surfaces with corrosion problems. Water samples from 29 DH systems were investigated for the total number of bacteria and presence of sulphate reducing bacteria (SRBs). SRBs were found to be present in more than 80% of the DH systems. The microbial population in samples from 2 DH systems (biofilm from a test coupon and an in situ sample from a heat exchanger) was investigated with fluorescence in situ hybridisation, and the results showed significant differences in population composition. Betaproteobacteria was the dominant population in both samples. SRBs were present in both samples but were most numerous in the biofilm from the test coupon. Examination of functional groups based on uptake of radiolabelled acetate (microautoradiography) showed presence of both aerobic and anaerobic bacteria despite the fact that oxygen is not anticipated in DH systems.

Keywords
Biofilm, biocorrosion monitoring unit, corrosion pit characteristics, district heating systems, fluorescence in situ hybridisation, microautoradiography

Introduction
In Denmark all major cities and more than 350 county districts are supplied with district heating (DH) through a network of more than 17,000 km piping, thousands of heat exchangers and other installations. This makes DH systems important technical water systems in Denmark. Biocorrosion causes problems in several Danish DH systems even though the water quality ought to limit bacterial growth and prevent occurrence of biocorrosion. In DH systems in the major cities the water is usually treated by reverse osmosis and deaeration. The water quality can typically be characterised by anaerobic conditions, pH from 9.3 to 10.0, conductivity less than 15 µS/cm, concentration of fluoride and chloride less than 0.5 mg/l and 1.0 mg/l, respectively, and a very low content of nutrients (Frølund et al., 1999; Goeres et al., 1998).

Monitoring of biocorrosion has recently been implemented in the Danish DH industry but is currently only performed in a limited number of systems. In these systems monitoring is based on the use of biocorrosion monitoring units where test coupons of different materials are exposed to in situ conditions (Olesen et al., 2001). Monitoring is based on the determination of weight loss and pit characteristics in combination with traditional microbiological methods. In this survey the monitoring of biocorrosion will only be briefly discussed. Instead the focus of the research reported herein is on the development and use of methods to study bacterial identity and activity in DH systems. The reason for this is that knowledge about the dominating groups of bacteria in these extreme environments is very limited and the potential corroding bacteria are not yet identified.
In order to identify and to detect the activity of the dominating bacteria in corroding and non-corroding systems, this study employed molecular and isotopic methods that directly allowed detection in samples from DH systems without previous cultivation. The identification was carried out by fluorescence in situ hybridisation (FISH) using rRNA targeted fluorescently labelled oligonucleotide probes. An active uptake of radiolabelled acetate (microautoradiography, (MAR)) was used to determine the presence of active bacteria under aerobic and anaerobic conditions.

**Materials and methods**

**Water samples**

The water samples originate from 29 DH systems sampled in the return circuits. Temperature and pH at the sampling time were recorded. The content of organic matter in the water samples was measured as non-volatile organic carbon (NVOC) according to *Standard Methods*. The total number of bacteria was determined by the general bacterial stain DAPI (4′,6-diamino-2-phenylindole) and evaluation was performed by fluorescence microscopy. The presence of SRBs was evaluated by cultivation using a modified Postgate C medium (Postgate, 1984). SRBs were determined to be present when production of sulphide could be measured. Sulphide was measured colorimetrically by the methylene blue method (Cline, 1969). The media was inoculated under both mesophilic (35°C) and thermophilic conditions (55°C).

**Samples for FISH and MAR**

Samples for FISH analyses were obtained from a heat exchanger of stainless steel 316 (in situ sample) and from a test coupon of mild steel from a biocorrosion monitoring unit located at the return circuit of a DH system.

The biocorrosion monitoring unit is based on the rotortorque principle and is equipped with a number of slides with materials from DH systems (mild steel, stainless steel 316 and copper) as test coupons (Goeres *et al.*, 1998). The flow in the reactor is turbulent thereby imitating the hydraulic conditions in DH pipes.

FISH was made with 16S and 23S fluorescently labelled rRNA-targeted nucleic acid probes on biofilm samples (Amann *et al.*, 1995). The probes used were EUB338 (Amann *et al.*, 1995), Non-EUB338, ALF1b, BET42a and GAM42a (Manz *et al.*, 1992), SRB385 (Amann *et al.*, 1992), SRB385Db (Rabus *et al.*, 1996), ARCH915 (Amann *et al.*, 1990), Eelm5932 (Boetius *et al.*, 2000), CF319a (Manz *et al.*, 1996), and TM7305 (Hugenholtz *et al.*, 2001). Samples were fixed in fresh 4% paraformaldehyde, washed three times in sterile filtrated distilled water, stained with DAPI, immobilised on slides, and hybridised. Cy-3 labelled probes were found to work best for enumeration in the systems investigated. Cultivation of SRBs was performed in the same manner as with the water samples.

MAR was performed on homogenised and suspended biofilm samples removed from mild steel coupons in the biocorrosion monitoring unit. Tritium labelled acetate was used under both aerobic and anaerobic conditions. The samples were kept anaerobic in DH water from sampling to start of the experiment. The principle of the MAR method performed on suspended biofilms is illustrated in Figure 1.

The amount of tracer added to sample volumes of 3 ml was 40 µCi; the amount of non-labelled acetate was 0.1 mM; the incubation temperature was 30°C; and the incubation time was 3 days.

![Figure 1 Principles of the MAR method on suspended biofilm](https://iwaponline.com/wst/article-pdf/47/5/117/422542/117.pdf)
was 3 hours for aerobic incubations and 12 hours for anaerobic incubations. The slides were covered with a radiosensitive film emulsion (LM-1, Pharmacia Amersham) and left for exposure for 6–12 days at 5°C. After development the presence of MAR positive cells was enumerated by brightfield microscopy. The results were compared to the number of DAPI positive cells on the same glass slide. Further details about the method can be found elsewhere (Nielsen et al., 1999; Nielsen and Nielsen, 2002).

Corrosion measurements
Biocorrosion monitoring units were placed in 6 DH systems at the return circuits. Test coupons of mild steel were used for corrosion analysis. Corrosion of the test coupons was investigated by measurements of weight loss and pit formation. The purpose was to examine whether cases of corrosion were characterised by few deep pits or several minor pits. Weight loss was assessed according to ASTM-G1, 1995. Pit formation was determined for the individual coupons by visual inspection in combination with laser interferometry. Presence of sulphide associated with pits was determined according to Feigl et al. (1972).

Results
Water samples
Examination of water samples from the 29 DH systems showed major differences in the total number of bacteria and the presence of organic matter (Table 1). The total number of bacteria ranged from $10^2$ to $10^5$ cells ml$^{-1}$ while the organic matter measured as NVOC ranged from 0.4 to 2.2 mg l$^{-1}$.

The pH of the water ranged from 8.0 to 10.1 with 9.1 as the mean. SRBs were found in 23 of 29 water samples by cultivation. It was not possible to establish a correlation between the quality of water samples and reports from the specific DH systems about biofouling and corrosion.

FISH of biofilm samples
Results from investigation of biofilm samples are shown in Table 2. The number of bacteria hybridising with the general probe for Bacteria (EUB338) expressed as the ratio EUB338/DAPI varied from 31% for the heat exchanger to 68% for the test coupon. Furthermore, 9% could be determined as Archaea (ARCH915) at the test coupon. Since the detection of cells with gene probes is dependent on the presence of ribosomal RNA (rRNA) in the cells, these observations indicate that the highest fraction of metabolic active bacteria were present in the biofilm from the test coupons in the biocorrosion monitoring unit even though some Archaea could be present in the sample from the heat exchanger (not tested yet).

When the group-specific probes were tested, it was found that the Betaproteobacteria was the most dominant group in both samples. This observation corresponds with findings from many natural and man-made systems e.g. activated sludge (Witzig et al., 2002). A major difference between the samples was the presence of many sulfate reducers in the biocorrosion monitoring unit. Deltaproteobacteria (tested with the general probe SRB385)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DAPI count (cells ml$^{-1}$)</th>
<th>NVOC (mg l$^{-1}$)</th>
<th>pH</th>
<th>SRB cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>$1.2 \pm 2.2 \times 10^4$</td>
<td>$0.9 \pm 0.4$</td>
<td>$9.1 \pm 0.4$</td>
<td>23 positive/29</td>
</tr>
<tr>
<td>Minimum</td>
<td>$1 \times 10^2$</td>
<td>0.4</td>
<td>8.0</td>
<td>–</td>
</tr>
<tr>
<td>Maximum</td>
<td>$1 \times 10^5$</td>
<td>2.2</td>
<td>10.1</td>
<td>–</td>
</tr>
</tbody>
</table>
constituted about 14% of the bacterial population in the biocorrosion monitoring unit while it was less than 1% in the heat exchanger. In addition, the occurrence of another group of sulfate reducers, *Desulfobacteriaceae*, showed a difference between the two samples. Cultivation did not show the presence of SRBs in any of the samples. In the biocorrosion monitoring unit, the presence of bacteria belonging to *Archaea* was observed. Whether it was methanogens, certain sulphate reducers or others remains to be investigated.

The reason for the difference in the population composition in the two systems remains unknown. The water quality is very similar, so the difference may be due to the two surfaces, stainless steel (heat exchanger) versus mild steel (biocorrosion monitoring unit).

**MAR**

The determination of two overall functional groups, the aerobic and anaerobic bacteria, in the DH systems was performed on a homogenised biofilm sample from the test coupons by the use of tritium labelled acetate. The results are shown in Table 3. Both aerobic and anaerobic bacteria able to utilise acetate were present in the biofilm. The FISH experiment indicated that 77% could be hybridised with the Bacterial or Archaea domain probes (see Table 2) and approximately one third of these cells were able to take up acetate under aerobic conditions. This high number is surprising due to the fact that DH systems are kept anaerobic to avoid oxygen induced corrosion, and may be attributed to the occurrence of local micro-aerophilic environments in the DH systems due to leaks or insufficient deaeration of the supply water. Most DH systems have a threshold limit for oxygen at 50 ppb, which is in the micro-aerophilic range. Another reason could be that they are mainly facultative anaerobic bacteria that can grow anaerobically or aerobically. Some anaerobic bacteria, however, may also be active.

### Table 2 Presence of bacterial groups in samples from two DH systems. The percentage of DAPI positive cells is shown

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity/target organism</th>
<th>Heat exchanger</th>
<th>Test coupon</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB-338</td>
<td>Domain <em>Bacteria</em></td>
<td>31</td>
<td>68</td>
</tr>
<tr>
<td>Non-EUB338</td>
<td>Control for non-specific binding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALF1b</td>
<td><em>Alphaproteobacteria</em> and some other bacteria</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>BET42a</td>
<td><em>Betaproteobacteria</em></td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>GAM42a</td>
<td><em>Gammaproteobacteria</em></td>
<td>&lt;1</td>
<td>12</td>
</tr>
<tr>
<td>SRB385</td>
<td>Sulfate reducing bacteria in <em>Deltaproteobacteria</em></td>
<td>&lt;1</td>
<td>14</td>
</tr>
<tr>
<td>SRB385Db</td>
<td><em>Desulfbacteriaceae</em></td>
<td>Not tested yet</td>
<td>4</td>
</tr>
<tr>
<td>ARCH 915</td>
<td>Domain <em>Archaea</em>, control for cells not detectable with EUB338</td>
<td>Not tested yet</td>
<td>9</td>
</tr>
<tr>
<td>Eelm5932</td>
<td><em>Archaea</em></td>
<td>Not tested yet</td>
<td>0</td>
</tr>
<tr>
<td>CF319a</td>
<td>Members of the <em>Cytophaga-Flavobacterium</em> cluster</td>
<td>0</td>
<td>Not tested yet</td>
</tr>
<tr>
<td>TM7305</td>
<td>Uncultured TM7 group</td>
<td>0</td>
<td>Not tested yet</td>
</tr>
</tbody>
</table>

a in situ sample from a heat exchanger (stainless steel)
b biofilm sample from biocorrosion monitoring unit (mild steel)

### Table 3 Enumeration of functional groups by uptake of tritium labelled acetate in a sample from test coupons of mild steel from a biocorrosion monitoring unit

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Percentage of DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic bacteria</td>
<td>28 (± 6)</td>
</tr>
<tr>
<td>Anaerobic bacteria</td>
<td>21 (± 9)</td>
</tr>
</tbody>
</table>
Furthermore, some sulfate reducers have been shown to be able to respire under aerobic conditions although they are not able to grow (Sass et al., 1997).

The number of anaerobic bacteria able to consume acetate was relatively high. Acetate is a common end product for many anaerobic processes, so the results indicate that the anaerobic bacteria could be iron(III) reducers, sulfate reducers and methanogens. The more precise population composition will be investigated in future studies.

The results of MAR support the results of FISH showing a relatively high level of bacterial activity in the biofilm samples from the biocorrosion monitoring unit. Use of other substrates will most likely increase the number of active bacteria, as not all anaerobic bacteria can use acetate.

Corrosion measurements
Monitoring of corrosion by use of biocorrosion monitoring units in the 6 examined DH systems revealed differences in the corrosion patterns of mild steel (data not shown). Some of the DH systems showed significant pitting corrosion while others had very little corrosion mainly due to general corrosion most likely induced by micro levels of oxygen.

Monitoring of corrosion characteristics of mild steel coupons in the biocorrosion monitoring unit also used for FISH and MAR in this study showed significant pitting. An example of one of these pits from a mild steel coupon is shown in Figure 2.

Corrosion measurements on mild steel coupons showed formation of relatively deep pits with an average depth of 220 µm and a width of 600 µm, respectively (data not shown). The pits were associated with deposits of sulphide and a relatively thick biofilm with an estimated thickness of 100–200 µm, which taken together indicate the presence of biocorrosion.

Conclusions
The examination of water samples from 29 DH systems showed variations in the water quality determined by the total number of bacteria, content of organic matter, pH and presence of SRB by cultivation. However, none of the results were directly correlated to observations of biofouling and corrosion in the systems.

A detailed analysis of the microbial population in the two DH systems investigated by FISH analysis showed significant differences in populations.

Determination of functional groups using MAR by uptake of radiolabelled acetate showed the presence of bacteria active under both aerobic and anaerobic conditions in the investigated DH system. The high proportion of aerobic bacteria was not expected given the fact that DH systems are anaerobic due to avoidance of oxygen induced corrosion.
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