The ISME Journal (2008) 2, 1231–1242
© 2008 International Society for Microbial Ecology All rights reserved 1751-7362/08 $32.00
www.nature.com/ismej

## ORIGINAL ARTICLE

# Bacteria associated with iron seeps in a sulfur-rich, neutral pH, freshwater ecosystem

Suzanne CM Haaijer[1], Harry R Harhangi[1], Bas B Meijerink[1], Marc Strous[1], Arjan Pol[1], Alfons JP Smolders[2], Karin Verwegen[1], Mike SM Jetten[1] and Huub JM Op den Camp[1]

[1]*Department of Microbiology, IWWR, Radboud University Nijmegen, Toernooiveld 1, Nijmegen, The Netherlands and* [2]*Department of Aquatic Ecology and Environmental Biology, IWWR, Radboud University Nijmegen, Toernooiveld 1, Nijmegen, The Netherlands*

**The freshwater nature reserve De Bruuk is an iron- and sulfur-rich minerotrophic peatland containing many iron seeps and forms a suitable habitat for iron and sulfur cycle bacteria. Analysis of 16S rRNA gene-based clone libraries showed a striking correlation of the bacterial population of samples from this freshwater ecosystem with the processes of iron reduction (genus *Geobacter*), iron oxidation (genera *Leptothrix* and *Gallionella*) and sulfur oxidation (genus *Sulfuricurvum*). Results from fluorescence *in situ* hybridization analyses with a probe specific for the beta-1 subgroup of *Proteobacteria*, to which the genera *Leptothrix* and *Gallionella* belong, and newly developed probes specific for the genera *Geobacter* and *Sulfuricurvum*, supported the clone library data. Molecular data suggested members of the epsilonproteobacterial genus *Sulfuricurvum* as contributors to the oxidation of reduced sulfur compounds in the iron seeps of De Bruuk. In an evaluation of anaerobic dimethyl sulfide (DMS)-degrading activity of sediment, incubations with the electron acceptors sulfate, ferric iron and nitrate were performed. The fastest conversion of DMS was observed with nitrate. Further, a DMS-oxidizing, nitrate-reducing enrichment culture was established with sediment material from De Bruuk. This culture was dominated by dimorphic, prosthecate bacteria, and the 16S rRNA gene sequence obtained from this enrichment was closely affiliated with *Hyphomicrobium facile*, which indicates that the *Hyphomicrobium* species are capable of both aerobic and nitrate-driven DMS degradation.**
*The ISME Journal* (2008) **2**, 1231–1242; doi:10.1038/ismej.2008.75; published online 28 August 2008
**Subject Category:** microbial ecology and functional diversity of natural habitats
**Keywords:** bacterial community composition; DMS degradation; iron bacteria; nitrate reduction; sulfur bacteria

## Introduction

De Bruuk (5°45′N; 5°58′E) is a small freshwater nature reserve comprising swamp and wet grass lands with silt/loamy sediment situated near the village of Groesbeek in the eastern part of The Netherlands. Owing to its unique hydrochemistry, it harbors a vast variety of protected flora and fauna, hence the status of nature reserve. Situated at a lower level than the surrounding areas, De Bruuk is a minerotrophic peatland receiving the major input of minerals from seepage and groundwater rather than from deposition of rainwater. In this ecosystem, previous research indicated active and interrelated iron-, sulfur- and nitrogen cycling (Smolders *et al.*, 1995; Lomans *et al.*, 1997, 1999a, 1999b).

In addition to the variation in landscape, vegetation and flora, one of the striking features of this wetland is the presence of many ferrous iron-rich and sulfate-rich, neutrophilic ditches and puddles. These ditches and puddles have a blackish-to-gray sediment covered with large masses of ochre-colored flocculent material overlain by a layer of clear water, with an iridescent film at the water surface. This phenomenon has been described in other freshwater wetlands and bogs (Emerson and Revsbech, 1994; Emerson and Weiss, 2004). The ochre-colored masses indicate the presence of natural iron seeps and result from precipitation of large quantities of iron hydroxide by ‘iron bacteria’ in places where ferrous iron-rich anoxic water reaches oxygenated zones (Emerson and Revsbech, 1994).

Correspondence: HJM Op den Camp, Department of Microbiology, IWWR, Radboud University Nijmegen, Toernooiveld 1, NL-6525 ED, The Netherlands.
E-mail: H.opdenCamp@science.ru.nl
Received 15 May 2008; revised 3 July 2008; accepted 4 July 2008; published online 28 August 2008

Microorganisms that are most commonly associated with these specific neutrophilic, iron-rich habitats are *Gallionella ferruginae* and *Leptothrix* species (Emerson and Revsbech, 1994; Carlile and Dudeney, 2000; Emerson and Weiss, 2004). The presence of these two types of organisms in natural environments is often based only on observation of their typical morphological features. *Leptothrix* species are chemoorganotrophic, aerobic, sheath-forming filamentous organisms, which deposit iron or ferromanganese oxides on their sheaths. These organisms benefit from the encrustation that forms a barrier between the cells and the environment (which often contains high amounts of soluble iron). The use of ferrous iron as an electron donor in lithoautotrophic metabolism has not been shown for pure cultures of *Leptothrix* species. Most organisms belonging to the *Leptothrix* species are restricted to natural unpolluted environments with low concentrations of easily degradable material (Mulder, 1989; Spring, 2002). *Leptothrix ochraceae* is the type species of the genus; however, there is no pure culture or 16S rRNA gene sequence available for this species. *Gallionella ferruginae* is an iron-oxidizing, aerobic microorganism that can be easily distinguished in natural systems because it secretes colloidal ferric hydroxide forming a characteristic stalk (Hanert, 1999). However, the stalk is not present under all conditions (Hallbeck *et al*., 1993). This microorganism has the ability to obtain carbon from both organic compounds and through $CO_2$ fixation (Hallbeck and Pedersen, 1991).

In addition to the morphologically distinct bacteria mentioned above, less-distinct iron-oxidizing and reducing bacteria are also expected to play a major role in the iron-seep-influenced ditches and puddles of the De Bruuk wetland. With regard to iron oxidation, next to microaerobic iron oxidation, bacteria have been found to perform anoxygenic photosynthesis with ferrous iron as electron donor (Ehrenreich and Widdel, 1994), to use nitrate as electron acceptor for ferrous iron oxidation under anaerobic conditions (Straub *et al*., 2004) and even, in a form of stationary phase metabolism, couple perchlorate reduction to ferrous iron oxidation (Chaudhuri *et al*., 2001). Dissimilatory iron reduction is also widespread among microorganisms (Nealson and Saffarini, 1994; Lovley *et al*., 2004; Weber *et al*., 2006), including *Geobacter* species, *Desulfovibrio* species and *Shewanella putrefaciens*. These organisms couple the oxidation of organic compounds to the reduction of ferric iron.

In addition to iron, pore water from De Bruuk sediments contains high amounts of sulfate and nitrate (about 0.7 mM sulfate, up to 1 mM nitrate; Smolders *et al*., 1995; Lomans *et al*., 1999b). This indicates that, together with iron cycle bacteria, sulfur cycle bacteria could find a suitable niche in De Bruuk. Previous research (Lomans *et al*., 1999a) already showed that sediment slurries from De Bruuk incubated under aerobic and anaerobic conditions degraded dimethyl sulfide (DMS). Dimethyl sulfide is an important volatile organic sulfur compound produced by algae, animals, microorganisms and plants by various mechanisms (Bentley and Chasteen, 2004). Volatile organic sulfur compounds, such as DMS, play an important role in the global sulfur cycle, in affecting the global climate and in acid precipitation (Lomans *et al*., 2002).

This study describes the bacterial population of iron seep areas of De Bruuk, analyzed using molecular tools (fluorescence *in situ* hybridization (FISH) and 16S rRNA gene analysis). Furthermore, a nitrate-reducing, DMS-degrading *Hyphomicrobium* enrichment culture is described.

## Materials and methods

*Sampling procedure*

In September 2002, two samples were collected at an iron seep area in De Bruuk. The first sample (BW) was taken from a ditch and consisted of ochre-colored flocculent material. As a reference, a sample of blackish-gray sediment (BS) was collected from the same ditch. Reduced conditions were expected to prevail in the sediment. The ochre-colored material was more exposed to air and therefore expected to receive a larger oxygen input. For an initial screening of the bacterial community composition, clone libraries (16S rRNA gene-based, see below) of these samples were constructed. In March 2006, three different iron seep samples were collected from De Bruuk. A sample of the top water column (top 10 cm) was taken from a ditch (depth 50 cm) that did not contain any apparent ochre-colored flocculent material, but did have an iridescent film on top of the water surface indicative of the presence of elevated iron concentrations (DI). From a similar ditch (also 50 cm deep), a mixed sample of the water phase above the sediment was taken; however, this ditch did contain visible amounts of ochre-colored flocculent material (DO). Another mixed sample containing ochre-colored flocculent material was taken from a puddle that was 10 cm deep (PO). The pH and concentrations of sulfate and total iron of these samples were determined (Table 1). Nitrate concentrations in the groundwater samples varied between 0 and 1 mM. Portions of the samples were fixed with paraformaldehyde for FISH analysis (as described below) to compare their microbial community compositions. Furthermore, samples of sediment were collected from other

**Table 1** Samples 2006

| *Sample* | *pH* | *Sulfate (mM)* | *Ferric iron (mM)* | *Ferrous iron (mM)* |
|---|---|---|---|---|
| DI | 7.1 | 0.04 | 0.1 | 0.03 |
| DO | 6.9 | 0.02 | 1.5 | 0.06 |
| PO | 6.9 | 0.01 | 11 | 0.3 |

ditches (no iron seep) in De Bruuk, and portions of the sediment (25 ml each) transferred to 60 ml serum bottles immediately upon arrival at the lab, closed with butyl rubber stoppers and aluminium crimp seals and incubated with DMS and different electron acceptors (see below) under a gas atmosphere of $N_2/CO_2$ (80%/20%).

*Chemical analyses*

All samples were centrifuged for 5 min at 13 000 r.p.m. and the resulting supernatant was used for analysis unless specified otherwise. For determination of thiosulfate, a cyanolysis-based protocol (Kelly *et al.*, 1969) was used. In brief, a mixture of 690 μl sample, 240 μl of a 0.2 M $NaH_2PO_4 \cdot NaOH$ buffer (pH 7.4) and 300 μl of a 0.1 M KCN solution was incubated for 20–30 min at 4 °C. After this, 90 μl of 0.1 M $CuSO_4$ and 180 μl of 1.5 M $Fe(NO_3)_3$ in 4 M $HClO_4$ solution were added and the resulting solution measured at 460 nm. Nitrate, nitrite and sulfate analyses were performed as described previously (Haaijer *et al.*, 2006). The ferrous iron contents of the 2006 samples taken from De Bruuk were determined by an adapted ferrozine assay (Stookey, 1970): a mix of 50 μl of sample (not centrifuged) and 150 μl of 1 M HCl was incubated for 1 h at room temperature. After this, 20 μl of this mix was added to 200 μl of ferrozine, vortexed, 1 ml of demineralized water added and measured at 562 nm. For determination of total iron, the 1 M HCl was replaced with a saturated hydroxylamine solution in 1 M HCl. Methanethiol DMS and hydrogen sulfide concentrations (detection limit 0.1 nmol $ml^{-1}$) in the headspace of DMS-fed incubations were determined using a Packard 438A gas chromatograph equipped with a Carbopack B HT100 column (40/60 mesh) and a flame photometric detector (Derikx *et al.*, 1990).

*Paraformaldehyde fixation*

Samples taken from De Bruuk were immediately fixed upon arrival at the lab. For each sample, 0.2 g (wet weight) was suspended in 0.9 ml of fixative, kept on ice for 2 h and centrifuged (5 min, 13 000 r.p.m.), after which the resulting pellet was washed with 1 ml phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2), by suspending and centrifuging. Finally, the fixed material was suspended in 1 ml of a phosphate-buffered saline and 100% ethanol mixture (1:1) and stored at 20 °C until use. The fixative consisted of 4% paraformaldehyde in phosphate-buffered saline (pH 7.2).

*16S rRNA gene sequence analyses*

High-molecular-weight DNA was extracted by standard procedures. Hot-start polymerase chain reactions (PCR) were performed in a Tgradient PCR apparatus (Whatman Biometra, Göttingen, Germany). The general bacterial 16S rRNA gene PCR primers 616F and 630R (see Haaijer *et al.*, 2006) resulted in 1500 bp products. PCR products were purified prior to cloning (QIAEX II gel extraction kit; Qiagen Benelux B.V., Venlo, The Netherlands). The TOPO TA cloning kit was used according to the instructions supplied by the manufacturer (Invitrogen, Groningen, The Netherlands). Isolation of plasmid DNA was performed with the FlexiPrep kit (Amersham Biosciences, Roosendaal, The Netherlands). Clones were checked by restriction analysis of plasmid DNA (*Eco*R1; Fermentas UAB, Vilnius, Lithuania). For the clone libraries of samples BW and BS, partial sequencing of clones, resulting in 510–800 bp fragments, was performed using primer M13F. Clone 16S rRNA gene sequences were compared with their closest relatives in the GenBank database by BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Using the RDP classifier tool (http://rdp.cme.msu.edu/classifier/; Cole *et al.*, 2005), clones were assigned to the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology, release 6.0 (http://www.bergeysoutline.com). Further phylogenetic and molecular evolutionary analyses were performed with the MEGA 3.1 program (Kumar *et al.*, 2004).

A selection of clones, based on phylogenetic positions and/or affiliation with functional groups of interest, was sequenced using primers M13F, M13R, 610IIF and 1390R (see Haaijer *et al.*, 2006 for primer specifications). This resulted in almost full-length 16S rRNA gene sequences. These sequence data have been submitted to the GenBank database under accession numbers EF079080–EF079087.

*FISH and microscopic analyses*

Probe design, FISH analyses and microscopic inspections were performed as described previously (Haaijer *et al.*, 2006). Vectashield (Vector Laboratories, Peterborough, England) mounting medium with 4,6-diamidino-2-phenylindole (DAPI) was used to enhance the fluorescent signal and stain all DNA. Specifications and details of probes used in this study are presented in Table 2. Probes EUB 338, EUB II and EUB III were used in combination (bacterial probe mix) to hybridize all bacteria. To minimize the necessary amount of hybridizations, probes were used in suitable combinations. A stringency was chosen for each hybridization, which facilitated binding of all probes. This turned out to be 25% for all hybridizations. The total cell number (based on DAPI staining) of each fixed sample of De Bruuk was determined by analysis of 50 images. To quantify specific probe signals, 10 images of each fixed sample were analyzed for each probe. Background fluorescence was determined for each sample through analysis of 10 images of DAPI-stained sample without probes. Specific probe

**Table 2** Probe specifications

| Name | Sequence (5′–3′) of probe | Target molecule, position[a] | Specificity | Reference |
|---|---|---|---|---|
| EUB 338 | GCT GCC TCC CGT AGG AGT | 16S rRNA, 338–355 | *Bacteria* | Daims (1999) |
| EUB 338 II | GCA GCC ACC CGT AGG TGT | 16S rRNA, 338–355 | *Planctomycetales* | Daims *et al.* (1999) |
| EUB 338 III | GCT GCC ACC CGT AGG TGT | 16S rRNA, 338–355 | *Verrucomicobiales* | Daims *et al.* (1999) |
| BET 42a | GCC TTC CCA CTT CGT TT | 23S rRNA, 1027–1043 | *Betaproteobacteria* | Manz *et al.* (1992) |
| GAM 42a | GCC TTC CCA CAT CGT TT | 23S rRNA, 1027–1043 | *Gamma-proteobacteria* | Manz *et al.* (1992) |
| BONE 23a | GAA TTC CAT CCC CCT CT | 16S rRNA, 663–679 | Beta1 group of *Betaproteobacteria* | Amann *et al.* (1996) |
| BTWO 23a | GAA TTC CAC CCC CCT CT | 16S rRNA, 663–679 | Competitor for BONE 23A | Amann *et al.* (1996) |
| Alf 986 | GGT AAG GTT CTG CGC GTT | 16S rRNA, 968–985 | *Alpha-proteobacteria*, except *Rickettsiales* | Neef (1997) |
| Arch 915 | GTG CTC CCC CGC CAA TTC CT | 16S rRNA, 915–934 | *Archea* | Stahl and Amann (1991) |
| Pla 46 | GAC TTG CAT GCC TAA TCC | 16S rRNA, 46–63 | *Planctomycetales* | Neef *et al.* (1998) |
| EPS 681 | ACG GAT TTT ACC CCT ACA CCA | 16S rRNA, 681–701 | *Epsilonproteo-bacteria* | This study |
| EpsC 1 | ACG GAT TTC ACC CCT ACA CCK | 16S rRNA, 681–701 | Competitor for EPS 681 | This study |
| EpsC 2 | ACA GAT TTC ACC CCT ACA CCA | 16S rRNA, 681–701 | Competitor for EPS 681 | This study |
| EpsC 3 | ACG GAT TTC ACC CCT ACA CCA | 16S rRNA, 681–701 | Competitor for EPS 681 | This study |
| SUCL 1431 | CAG TTT GGC ATC CCG ATT TCG | 16S rRNA, 1431–1451 | Genus *Sulfuricurvum* | This study |
| Geo 1423 | TCA CGC ACT TCG TCG GGA CCA | 16S rRNA, 1423–1443 | *Geobacter* species | This study |

[a]*Escherichia coli* numbering.

signals were regarded as significant only when they exceeded the background fluorescence.

*Culturing conditions and media*
All liquid cultures were incubated under a gas atmosphere of $N_2/CO_2$ (80%/20%) at 30 °C and 150 r.p.m. A mineral medium (composition: Haaijer *et al.*, 2006) was used. Ferric iron, sulfate, nitrate and DMS were supplied from sterile stock solutions. Incubations of sediment (in duplicate) with DMS (50 μM end concentration) contained ferric iron, sulfate or nitrate as electron acceptor (25 mM end concentration). The pH of the mineral medium was 7. Solid mineral media plates were prepared by adding 15 g l$^{-1}$ agarose as a solidifying agent.

*Enrichment of DMS-degrading bacteria*
Ten additions of DMS (50 μM) to the initial incubations with DMS and nitrate were performed. Hereafter, enrichment cultures of DMS-degrading, nitrate-reducing bacteria were established by 20-fold dilution into fresh mineral media containing DMS and nitrate (50 μM, 25 mM end concentrations, respectively). After 4 weeks of incubation, 100 μl of these cultures was plated onto solid media and incubated for 2 weeks. Single colonies were subsequently transferred to mineral medium and incubated for 2 weeks, after which 2 ml was sampled for microscopic inspection and high-molecular-weight DNA extraction followed by cloning and sequencing of the 16S rRNA gene.

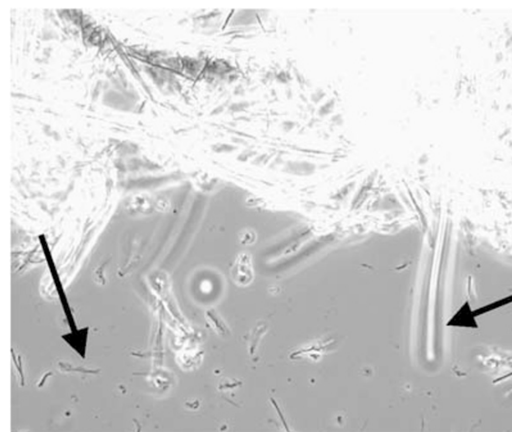

**Figure 1** Phase-contrast picture of iron seep material from De Bruuk. The arrow on the right indicates one of the visible *Leptothrix* sheaths; the arrow on the left indicates one of the visible *Gallionella* stalks.

## Results

*Sample description*
Figure 1 shows a phase-contrast picture of the morphologically distinct *Leptothrix* and *Gallionella*

species visible in iron seep material from De Bruuk. Based on phase-contrast microscopy, both types of organisms were present in the ochre-colored flocculent material taken from a ditch in De Bruuk in 2002 (BW). In contrast, no morphotypes indicating the presence of these microorganisms could be distinguished in the sediment sample (BS). Morphotypes indicating the presence of *Leptothrix* and *Gallionella* species were present in all iron seep samples taken in 2006, even in the sample that did not contain any visible ochre-colored material (DI).

*Initial screening of the bacterial community composition: 16S rRNA clone libraries*

Bacterial diversity was surveyed by generating two separate 16S rRNA gene sequence-based clone libraries from the material collected in 2002. The clone library of the ochre-colored flocculent material (BW) and the sediment (BS) consisted of 58 and 52 clones, respectively. In Figures 2 and 3, an overview of phylum distribution within Bacteria and order distribution within *Proteobacteria* is given for the BW and BS clone libraries, respectively. Assignment to genus level was possible for 34 clones of the BW clone library and 21 clones of the BS clone library. An overview of the genera present in the clone libraries is given in Table 3. The presence of functional groups of interest for this study was most prominent in the clone library of the ochre-colored flocculent material (BW). In this clone library, a total of 11% of all clones could be assigned to genera associated with iron reduction (*Geobacter* and *Rhodoferax*). Nine percent of all clones could be assigned to the genus *Gallionella* and 3% to the genus *Leptothrix*, which are the genera associated with iron oxidation. Furthermore, 21% of all clones could be assigned to the epsilonproteobacterial genus *Sulfuricurvum*, which is associated with oxidation of reduced sulfur compounds.

Six BW clones were selected for full sequencing (1300–1500 bp). Phylogenetic positions of these clones are shown in Figure 4. The highest blast hit for the alfaproteobacterial clone 208 was 95% sequence identity with the methane-oxidizing, dinitrogen-fixing bacterium *Methylocapsa acidiphila* (AJ278726; Dedysh *et al.*, 2002). Clone 202's highest blast hit was 96% sequence identity with the hydrogen-oxidizing betaproteobacterium *Variovorax paradoxus* (DQ256485). Clone 187, a deltaproteobacterium, could be assigned to the dissimilatory, iron-reducing genus *Geobacter*. However, the highest sequence identity to a previously described *Geobacter* species was only 94% (Y19191; Cummings *et al.*, 1999). Clone 221, a possible iron oxidizer, had a highest blast hit of 97% sequence identity with an uncultured bacterium from a reactor system treating monochlorobenzene-contaminated groundwater (AY050584; Alfreider *et al.*, 2002). Clone 221's highest sequence identity to a

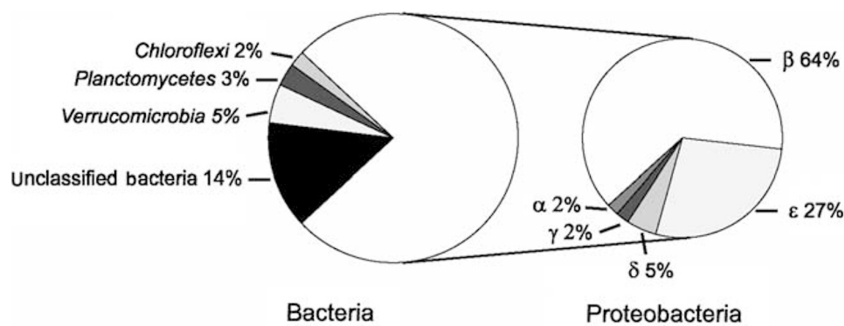


**Figure 2** Overview of the bacterial community composition of the 16S rRNA gene sequence-based clone library generated from the ochre-colored material collected from a ditch in De Bruuk in 2002 (BW, 58 clones).

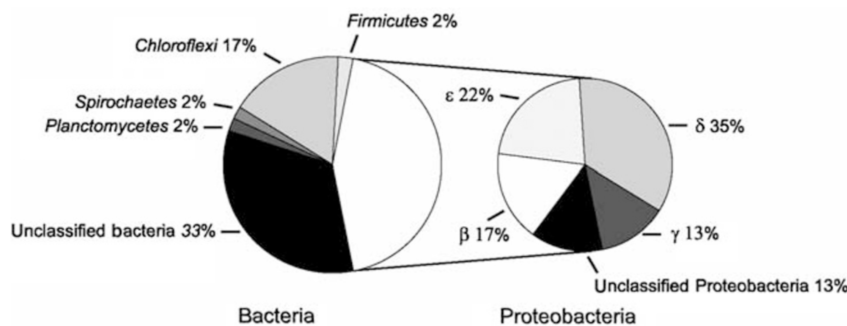


**Figure 3** Overview of the bacterial community composition of the 16S rRNA gene sequence-based clone library generated from the sediment material collected from a ditch in De Bruuk in 2002 (BS, 52 clones).

**Table 3** Overview genera present in the clone libraries

| *Genus* | *Phylum* | *Representative of the genus*[a] | *Association representative* | *BW*[b] *clones (%)* | *BS*[b] *clones (%)* | *Reference representative genus* |
|---|---|---|---|---|---|---|
| *Odysella* | *Alphaproteobacteria* | *O. thessalonicensis* (AF069496) | Obligate intracellular parasite | 2 | 0 | Birtles *et al.* (2000) |
| *Rhodoferax* | *Betaproteobacteria* | *R. ferrireducens* (AF435948) | Iron reduction | 2 | 2 | Finneran *et al.* (2003) |
| *Leptothrix* | *Betaproteobacteria* | *L. mobilis* (X97071) | 'Passive' iron oxidation aerobic heterotrophy | 3 | 0 | Spring *et al.* (1996) |
| *Ferribacterium* | *Betaproteobacteria* | *F. limneticum* (Y17060) | Iron reduction | 0 | 2 | Cummings *et al.* (1999) |
| *Acidovorax* | *Betaproteobacteria* | *Acidovorax sp* KSP2 (AB076843) | Heterotrophic denitrification | 7 | 0 | Khan *et al.* (2002) |
| *Gallionella* | *Betaproteobacteria* | *G. ferruginae* (L07897) | Microaerobic iron oxidation | 9 | 0 | Hallbeck *et al.* (1993) |
| *Smithella* | *Deltaproteobacteria* | *S. propionica* (AF126282) | Syntrophic propionate fermentation | 0 | 4 | Liu *et al.* (1999) |
| *Syntrophus* | *Deltaproteobacteria* | *S. gentianeae* (X85132) | Syntrophic benzoate fermentation | 0 | 4 | Wallrabenstein *et al.* (1995) |
| *Desulfocapsa* | *Deltaproteobacteria* | *D. thiozymogenes* (X95181) | Sulfate reduction | 0 | 2 | Janssen *et al.* (1996) |
| *Geobacter* | *Deltaproteobacteria* | *Geobacter* sp CdA-3 (Y19191) | Iron reduction | 9 | 0 | Cummings *et al.* (2000) |
| *Thiomicrospira denitrificans* | *Epsilonproteobacteria* | *Th. denitrificans* (L40808) | Anaerobic oxidation reduced sulfur compounds | 0 | 2 | Muyzer *et al.* (1995) |
| *Sulfurovum* | *Epsilonproteobacteria* | *S. lithotrophicum* (AB091292) | Aerobic/anaerobic oxidation reduced sulfur compounds | 0 | 4 | Inagaki *et al.* (2004) |
| *Sulfuricurvum* | *Epsilonproteobacteria* | *S. kuijense* YK-2 (AB080643) | Aerobic/anaerobic oxidation reduced sulfur compounds | 21 | 4 | Kodama and Watanabe (2003) |
| *Anaerolinae* | *Chloroflexi* | Filamentous bacterium YMTK-2 (AB109438) | Anaerobic carbohydrate degradation | 2 | 17 | Yamada *et al.* (2005) |
| *Opitutus* | *Verrucomicrobiae* | *O. terrae* (AJ229235) | Carbohydrate fermentation | 2 | 0 | Chin *et al.* (2001) |
| *Verrucomicrobium* | *Verrucomicrobiae* | *V. spinosum* (X90515) | Carbohydrate fermentation | 3 | 0 | Schlesner (1987) |

[a]Accession numbers within parentheses.
[b]Percentage of the total amount of clones in the library.

cultured species was 93% to *Gallionella ferruginae* (L07897; Hallbeck and Pedersen, 1991; Hallbeck *et al*., 1993). Clone 212 could be assigned to the family Incertae cedis 5, but not to the genus *Leptothrix*, although it had 95% sequence identity to *L. discophora* strain-1 (L33975; Siering and Ghiorse, 1996). Clone 181 could be assigned to the epsilonproteobacterial, sulfur-oxidizing and nitrate-reducing genus *Sulfuricurvum*, and had a 96% sequence identity to *Sulfuricurvum kuijense* strains YK-2 and YK-4 (Kodama and Watanabe, 2003).

### *Design of new probes for FISH*

Thus far, no probes were available for all *Epsilonproteobacteria*, the genus *Sulfuricurvum* and the genus *Geobacter*. Therefore, three new probes were designed to facilitate a thorough FISH-based determination of the composition of the microbial community. The first was probe EPS 681, which targeted almost all *Epsilonproteobacteria* present in the ARB database. Some target organisms, however, had a mismatch to the probe (for example, members of the genus *Wollinella*). Additionally, non-target organisms showing only minor mismatches to the probe, which could result in nonspecific probe binding in hybridization analyses, were also present in the database. An overview of the respective mismatches is shown in Figure 5. The second probe was SUCL 1431, specific to the genus *Sulfuricurvum*, designed on the basis of clone sequences obtained in this study and previously published sequences. A mix of probes EPS 681 and SUCL 1431 was used for hybridization analyses. Probe Geo 1423 was designed for the genus

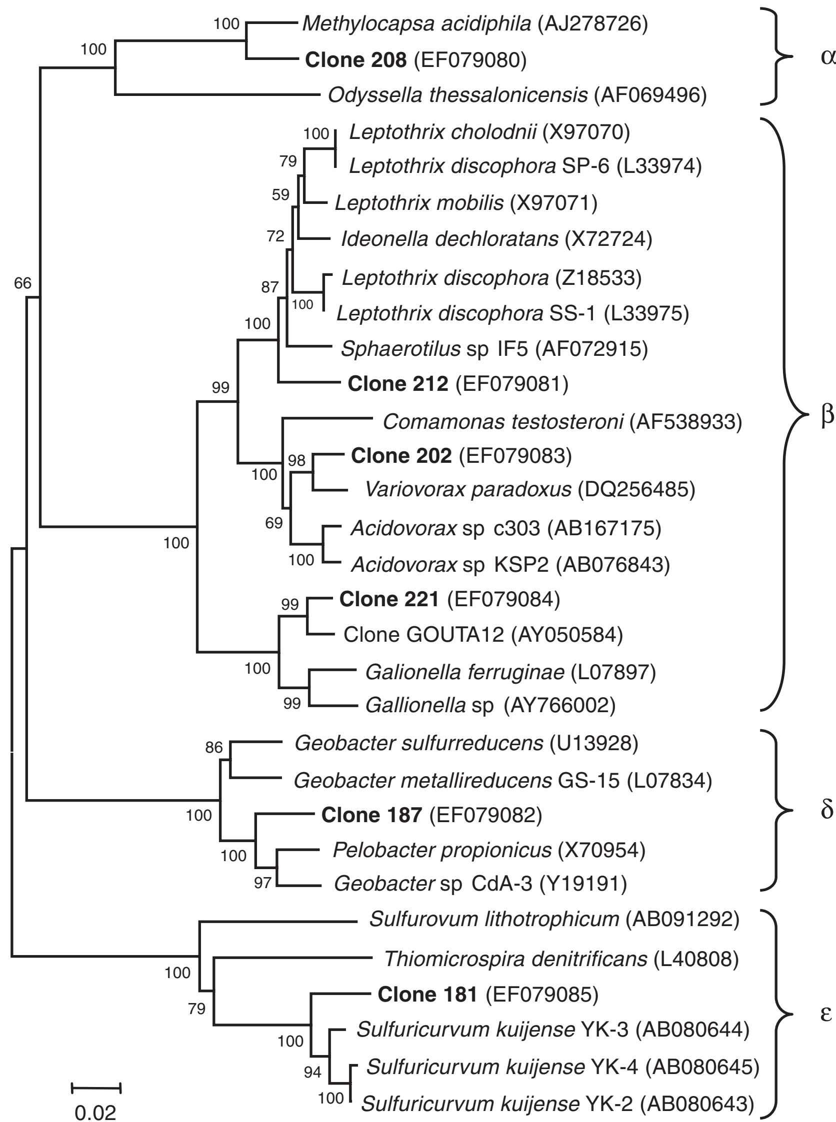


**Figure 4** Phylogenetic tree showing the positions of the fully sequenced 16S rRNA clones. Clones originated from the library generated with material from the ochre-colored material collected from a ditch in De Bruuk in 2002 (BW). Unrooted bootstrap consensus tree. Bootstrap values (1000 replicates) higher than 50% are shown. Calculation of the tree was performed using the MEGA program (Kumar *et al*., 2004) and the distance method of Jukes and Cantor (1969). A total of 1400 nucleotide positions were considered in the alignment. The scale bar indicates 2 base substitutions per 100 homologous sequence positions.

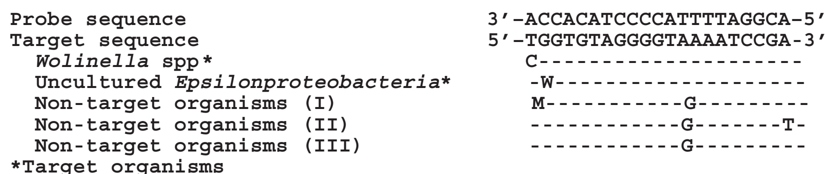


**Figure 5** Difference alignment for probe EPS 681.

*Geobacter* and had at least four mismatches to all non-target organisms.

The optimal stringency for hybridization analyses of 25% formamide adopted in this study was regarded suitable for analyses with the new probes considering the strength and position of the mismatches of probe EPS 681 to target and non-target organisms and the high specificity of the probe Geo 1423.

### *FISH analyses of microbial community compositions*

A common problem in FISH analysis of environmental samples is the discrepancy between DAPI

counts (DNA stain) and counts with fluorescently labeled probes (ribosomal stain). In theory, combining the counts of the general archaeal probe and the bacterial probe mix would yield the same number as the DAPI count. In practice, these numbers differ considerably and their ratio differs with the environment. Combination of the bacterial probe mix and archaeal counts resulted in numbers between 47% and 78% of the total DAPI count (Figure 6). A likely cause is the physiological state of cells in environmental samples; low rRNA content leads to defective detection of cells (Bouvier and del Giorgio, 2003). To circumvent this problem, the specific probe counts were related to the detectable microbial population (the sum of the bacterial probe mix count and general archaeal probe count) instead of to the DAPI count. The 2006 samples from the iron seep areas of De Bruuk were dominated by bacteria. FISH analysis resulted in a significant number of archaea only for the PO sample (5% of the total microbial population). No significant counts were obtained with the *Planctomycetes*- and *Gammaproteobacteria*-specific probes. All samples contained members of the beta-1 subgroup of *Proteobacteria*, to which the iron-oxidizing genera *Leptothrix* and *Gallionella* belong, members of the reduced sulfur compound-oxidizing genus *Sulfuricurvum* and members of the iron-reducing genus *Geobacter*. Figure 6 shows the respective microbial community compositions of samples DI, DO and PO resulting from the FISH analyses. All *Epsilonproteobacteria* detected belonged to the genus *Sulfuricurvum* (all cells that hybridized with probe EPS 681 also hybridized with probe SUCL 1431).

### *DMS activity tests and enrichments with material from De Bruuk*

Under both oxic and anoxic standard conditions, DMS is chemically stable, but based on earlier results with material from De Bruuk (Lomans *et al.*, 1999a), biological DMS degradation under anaerobic conditions was expected. All incubations of sediment from De Bruuk with DMS along with nitrate, ferric iron or sulfate as the electron acceptor showed DMS-degrading activity (Figure 7). DMS degradation rates were as follows: 1.3 μM day$^{-1}$ with nitrate, 0.9 μM day$^{-1}$ with sulfate and 0.6 μM day$^{-1}$ with ferric iron as the electron acceptor. The rate of DMS degradation in the culture with nitrate increased from 1.3 to 6.7 μM day$^{-1}$ upon a second addition of DMS (Figure 7). Based on these results, an enrichment culture was established with nitrate as the electron acceptor. No intermediates (methanethiol, hydrogen sulfide or nitrite) were detected. Microscopic inspection of the obtained enrichment

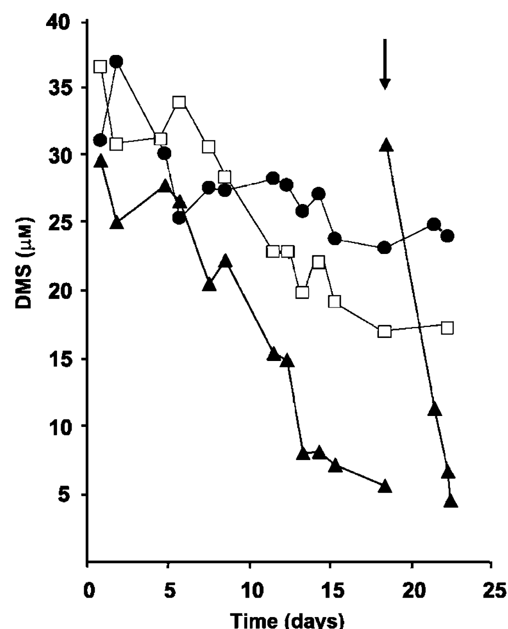


**Figure 7** Anaerobic dimethyl sulfide (DMS) degradation in sediment from De Bruuk amended with different electron acceptors. □, sulfate; ●, ferric iron; ▲, nitrate. The DMS concentration in the liquid is depicted. The arrow indicates a second DMS addition to the nitrate incubation.

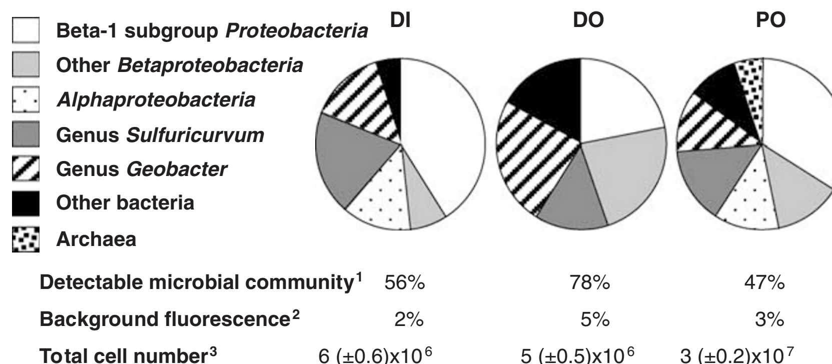


**Figure 6** Fluorescence *in situ* hybridization analyses of the samples collected in 2006. DI, material from the ditch with an iridescent film without ochre-colored material; DO, material from the ditch with ochre-colored material; PO, material from the puddle with ochre-colored material. The pie charts are oriented clockwise and represent the total detectable microbial community for each sample. 1, sum of the bacterial probe mix and archaeal count expressed as a percentage of the 4,6-diamidino-2-phenylindole (DAPI) count; 2, expressed as a percentage of the DAPI count; 3, DAPI count per gram wet weight; $n = 50$; s.e. within parentheses.

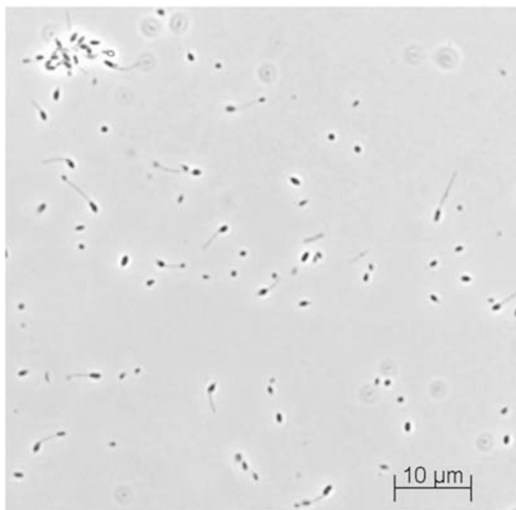


**Figure 8** Phase contrast microscopy pictures of the dimorphic prosthecate bacteria in the DMS and nitrate enrichment.

culture showed dominance of dimorphic prosthecate bacteria (Figure 8). The 1425 bp 16S rRNA gene sequence obtained from the enrichment showed a high sequence identity (99.7%) to the 16S rRNA gene sequence of *Hyphomicrobium facile* (Y14311; Rainey *et al.*, 1998).

## Discussion

*Iron oxidation and reduction*

The iron seep areas of De Bruuk possess an interesting and quite uniform microbial population dominated by bacteria (Figure 6). Analysis of 16S rRNA clone libraries immediately revealed a relation of the bacterial population to the processes of iron reduction and iron oxidation (Table 3). The clone library data suggested that bacteria belonging to the genus *Geobacter* could be involved in iron reduction in the more oxidized compartment of the iron seep areas in De Bruuk. This finding was supported by FISH analysis with the newly developed *Geobacter*-specific probe (14%, 24% and 11% of the microbial community of samples DI, DO and PO, respectively, Figure 6).

The clone library data implied a role for the genera *Gallionella* and *Leptothrix* in iron oxidation in De Bruuk. The full sequences obtained for the iron-oxidation-associated (*Gallionella*-like and *Leptothrix*-like) sequences, however, did not exhibit sufficient sequence identities to previously described species to enable identification to genus level. FISH analysis showed that the beta-1 subgroup of the *Betaproteobacteria*, to which the genera *Gallionella* and *Leptothrix* belong, constituted a major portion of the microbial communities of samples DI, DO and PO (41%, 22% and 34%, respectively). The process of iron oxidation was therefore likely to be mediated by a yet unknown *Gallionella*- and *Leptothrix*-related species in De Bruuk.

The full-length sequences obtained in this study exhibited sequence identities lower than 95% to known sequences of iron-reducing and iron-oxidizing species. To exclude the possibility that the sequences in this study represent bacteria possessing metabolic functionalities other than, respectively, iron reduction and iron oxidation, further physiological characterization of the bacteria in the iron seep areas of De Bruuk would be necessary.

*Oxidation of reduced sulfur compounds*

The incubations of De Bruuk sediment with DMS and several different electron acceptors showed most rapid DMS conversion when nitrate was used as the electron acceptor. The conversion of DMS and nitrate in the *Hyphomicrobium facile*-dominated enrichment culture seemed to proceed completely to sulfate and dinitrogen as no intermediates were detected. Both aerobic and anaerobic DMS-degrading microorganisms have been previously described. Aerobic DMS-degrading microorganisms that have been previously isolated from sewage treatment plants, marine sediments, soil and biofilters include *Thiobacilli* (De Zwart and Kuenen, 1992; Visscher and Taylor, 1993), *Hyphomicrobia* (Suylen and Kuenen, 1986; Pol *et al.*, 1994) and *Methylophaga* (De Zwart *et al.*, 1996). Anaerobic DMS-degrading bacteria previously described are methanogens (Kiene *et al.*, 1986; Lomans *et al.*, 1999b), anoxygenic phototrophs (Widdel and Pfennig, 1981; Zeyer *et al*, 1987), sulfate-reducing bacteria (Tanimoto and Bak, 1994) and denitrifying bacteria (Visscher and Taylor 1993b). Our findings suggest that in addition to aerobic DMS conversion, *Hyphomicrobium* species are capable of using DMS under nitrate-reducing conditions.

The clone library data furthermore suggested members of the genus *Sulfuricurvum* as possible players in the oxidation of reduced sulfur compounds in iron seep material. Type species of the genus *Sulfuricurvum* is *Sulfuricurvum kuijense*, a sulfur-oxidizing, facultative anaerobic (nitrate-reducing) chemolithotroph, which was isolated from a crude oil storage cavity (Watanabe *et al.*, 2000; Kodama and Watanabe, 2004). FISH analysis supported the clone library data; 21%, 14% and 15% of the microbial communities of sample DI, DO and PO, respectively, were shown to belong to the genus *Sulfuricurvum*. Owing to double-hybridization of probes EPS 681 and SUCL 1431, there was no need to include competitor probes to exclude nonspecific binding of probe EPS 681 in the hybridization analyses. Under other circumstances, the competitor probes EPSC 1, EPSC 2 and EPSC 3 (Table 2) are proposed for use in combination with probe EPS 681. Despite the expected abundance of *Sulfuricurvum*-like microorganisms based on FISH and 16S rRNA gene sequence analysis, attempts to enrich these

bacteria in mineral media supplemented with thiosulfate and nitrate proved unsuccessful (data not shown). High abundance of members of the genus *Sulfuricurvum* indicates that, in addition to iron-cycling, chemolithotrophic sulfur oxidation at the expense of nitrate or oxygen is an important process in the iron seep areas of De Bruuk.

*Microbial community compositions*

The microbial community compositions as determined with FISH analyses reflected the physiochemical properties of the different samples. The DI sample was a sulfur-rich, iron-poor system in comparison with the other samples (Table 1). This, in combination with the depth of the system (limited availability of oxygen), explains why this sample contained the highest amount of bacteria belonging to the anaerobic-to-microaerobic, sulfur-oxidizing genus *Sulfuricurvum*. The contribution of the genus *Geobacter* to the total proteobacterial population was highest in the DO sample. This can be explained by the high iron content in combination with restricted $O_2$ diffusion to the sediment because of the 50-cm-deep water column (Table 1). Both conditions favor strict anaerobic iron reduction. The limited availability of oxygen restricts the proliferation of the aerobic and microaerobic beta-1 subgroup genera *Leptothrix* and *Gallionella*, which explains the relatively low fraction of beta-1 subgroup bacteria in the DO sample. Although characteristics of the PO sample (shallow system, high iron concentrations; Table 1) were expected to favor the aerobic and microaerobic, iron-oxidizing *Leptothrix* and *Gallionella* species, the beta-1 subgroup, to which these species belong, was not more dominant in the PO sample than in the DI sample (Figure 6). This can be explained by the higher total cell number and higher microbial diversity of sample PO (Figure 6), which suggest that competition between different microbial trophic groups could be more severe in this sample.

## Conclusions

The results obtained on DMS degradation with samples from De Bruuk provide strong evidence for nitrate-reducing DMS degradation mediated by *Hyphomicrobium* species, in addition to the known aerobic DMS conversion by these microorganisms. The combination of an initial screening of the bacterial community (construction of 16S rRNA gene sequence-based clone libraries) followed by an elaborate FISH analysis proved invaluable in describing the bacterial population of the iron seep areas of De Bruuk. This approach showed the presence and abundance of several interesting functional groups involved in iron cycling and sulfur oxidation in De Bruuk. Our data suggest that *Geobacter* species are involved in the reduction of iron in more oxidized compartments of the iron seep areas in De Bruuk, and that members of the epsilonproteobacterial, sulfur-oxidizing genus *Sulfuricurvum* are involved in sulfur oxidation. *Epsilonproteobacteria* are recognized as globally ubiquitous key players in sulfidic habitats (Campbell *et al*., 2006), but only a few species have been cultured and characterized. Furthermore, we found indications that iron oxidation in the iron seep areas of De Bruuk is mediated by a yet unknown *Gallionella*- and *Leptothrix*-related species. A similar lack of cultured species, as in the case of the sulfur-oxidizing *Epsilonproteobacteria*, exists for the genus *Leptothrix*, although significant progress has been made during the last decade in the description of isolated species and their phylogeny (Siering and Ghiorse, 1996; Spring *et al*., 1996). *Gallionella*-like sequences have recently been found in an acidic environment (Hallberg *et al*., 2006), indicating that there is still much to learn about the physiology of these microorganisms. Our study shows that iron seep areas such as De Bruuk are promising environments to study these types of bacteria.

## Acknowledgements

We thank Jack van de Vossenberg and Marcus Schmid for practical assistance, advice and discussion.

## References

Alfreider A, Vogt C, Babel W. (2002). Microbial diversity in an *in situ* reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis. *Syst Appl Microbiol* **25**: 232–240.

Amann R, Snaidr J, Wagner M, Ludwig W, Schleifer KH. (1996). *In situ* visualization of high genetic diversity in a natural microbial community. *J Bacteriol* **178**: 3496–3500.

Bentley R, Chasteen TG. (2004). Environmental VOSCs—formation and degradation of dimethyl sulfide, methanethiol and related materials. *Chemosphere* **55**: 291–317.

Birtles RJ, Rowbotham TJ, Michel R, Pitcher DJ, Lascola B, Alexiou-Daniel S *et al*. (2000). *Candidatus Odysella thessalonicensis* gen. nov., sp. nov., an obligate intracellular parasite of *Acanthamoeba* species. *Int J Syst Evol Microbiol* **50**: 63–72.

Bouvier T, del Giorgio PA. (2003). Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): a quantitative review of published reports. *FEMS Microbiol Ecol* **44**: 3–15.

Campbell BJ, Summers Engel A, Porter ML, Takai K. (2006). The versatile ε-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol* **4**: 458–468.

Carlile MJ, Dudeney AWL. (2000). A microbial mat composed of iron bacteria. *Microbiology (UK)* **146**: 2092–2093.

Chaudhuri SK, Lack JG, Coates JD. (2001). Biogenic magnetite formation through anaerobic biooxidation of Fe(II). *Appl Environ Microbiol* **67**: 2844–2848.

Chin KJ, Liesack W, Janssen PH. (2001). *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division '*Verrucomicrobia*' isolated from rice paddy soil. *Int J Syst Evol Microbiol* **51**: 1965–1968.
Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM *et al.* (2005). The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* **33**(Database Issue): D294–D296.
Cummings DE, Caccavo F, Spring S, Rosenzweig RF. (1999). *Ferribacterium limneticum* gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch Microbiol* **171**: 183–188.
Cummings D, March A, Bostick B, Spring S, Caccavo F, Fendorf S *et al.* (2000). Evidence for microbial Fe(III) reduction in anoxic, mining-impacted lake sediments (Lake Coeur d'Alene, Idaho). *Appl Environ Microbiol* **66**: 154–162.
Daims H, Bruhl A, Amann RI, Schleifer KH, Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.
Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W *et al.* (2002). *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int J Syst Evol Microbiol* **52**: 251–261.
Derikx PJL, Op den Camp HMM, van der Drift C, van Griensven JLD, Vogels GD. (1990). Odorous sulfur compounds emitted during production of compost used as a substrate in mushroom cultivation. *Appl Environ Microbiol* **56**: 176–180.
De Zwart JMM, Kuenen JG. (1992). $C_1$-cycle of sulfur compounds. *Biodegradation* **3**: 37–59.
De Zwart JMM, Nelisse PN, Kuenen JG. (1996). Isolation and characterization of *Methylophaga sulfidovorans* sp. nov.: an obligately methylotrophic, aerobic, dimethylsulfide oxidizing bacterium from a microbial mat. *FEMS Microbiol Ecol* **20**: 261–270.
Ehrenreich A, Widdel F. (1994). Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Appl Environ Microbiol* **60**: 4517–4526.
Emerson D, Revsbech NP. (1994). Investigation of an iron-oxidizing microbial mat community located near Aarhus, Denmark: field studies. *Appl Environ Microbiol* **60**: 4022–4031.
Emerson D, Weiss J. (2004). Bacterial iron oxidation in circumneutral freshwater habitats: findings from the field and the laboratory. *Geomicrobiol J* **21**: 405–414.
Finneran KT, Johnsen CV, Lovley DR. (2003). *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *Int J Syst Evol Microbiol* **53**: 669–673.
Haaijer SCM, van der Welle MEW, Schmid MC, Lamers LPM, Jetten MSM, Op den Camp HJM. (2006). Evidence for the involvement of betaproteobacterial *Thiobacilli* in the nitrate-dependent oxidation of iron sulfide minerals. *FEMS Microbiol Ecol* **58**: 439–448.
Hallbeck L, Pedersen K. (1991). Autotrophic and mixotrophic growth of *Gallionella ferruginea*. *J Gen Microbiol* **137**: 1531–1535.
Hallbeck L, Stahl F, Pedersen K. (1993). Phylogeny and phenotypic characterization of the stalk-forming and iron-oxidizing bacterium *Gallionella ferruginea*. *J Gen Microbiol* **139**: 1531–1535.
Hallberg KB, Coupland K, Kimura S, Johnson DB. (2006). Macroscopic growths in acidic, metal-rich mine waters in North Wales consist of novel and remarkably simple bacterial communities. *Appl Environ Microbiol* **72**: 2022–2030.
Hanert HH. (1999). *The Genus Gallionella. The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*, 2nd ed Springer-Verlag: New York,, http://www.link.springer-ny.com/link/service/books/10125.
Inagaki F, Takai K, Nealson KH, Horikoshi K. (2004). *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the epsilon-*Proteobacteria* isolated from Okinawa trough hydrothermal sediments. *Int J Syst Evol Microbiol* **54**: 1477–1482.
Janssen PH, Schuhmann A, Bak F, Liesack W. (1996). Disproportionation of inorganic sulfur compounds by the sulfate-reducing bacterium *Desulfocapsa thiozymogenes* gen. nov., sp. nov. *Arch Microbiol* **166**: 184–192.
Jukes TH, Cantor CR. (1969). Evolution of protein molecules. In: Munro HN (ed). *Mammalian Protein Metabolism*. Academic Press: New York, pp 21–132.
Kelly DP, Chambers LA, Trudinger PA. (1969). Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. *Anal Chem* **41**: 898–902.
Khan ST, Horiba Y, Yamamoto M, Hiraishi A. (2002). Members of the family *Comamonadaceae* as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. *Appl Environ Microbiol* **68**: 3206–3214.
Kiene RP, Oremland RS, Catena A, Miller LG, Capone DG. (1986). Metabolism of reduced methylated sulfur compounds in anaerobic sediments and by a pure culture of an estuarine methanogen. *Appl Environ Microbiol* **52**: 1037–1045.
Kodama Y, Watanabe K. (2003). Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. *Appl Environ Microbiol* **69**: 107–112.
Kodama Y, Watanabe K. (2004). *Sulfuricurvum kujiense* gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. *Int J Syst Evol Microbiol* **54**: 2297–2300.
Kumar S, Tamura K, Nei M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.
Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR. (1999). Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. *Int J Syst Bacteriol* **49**: 545–556.
Lomans BP, Smolders AJP, Intven L, Pol A, Op den Camp HJM, Vogels GD. (1997). Formation of dimethyl sulfide and methanethiol in anoxic freshwater sediments. *Appl Environ Microbiol* **63**: 4741–4747.
Lomans BP, Op den Camp HJM, Pol A, Vogels GD. (1999a). Anaerobic versus aerobic degradation of dimethyl

sulfide and methanethiol in anoxic freshwater sediments. *Appl Environ Microbiol* **65**: 438–443.

Lomans BP, Maas R, Luderer R, Op den Camp HJM, Pol A, Van der Drift C *et al.* (1999b). Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Appl Environ Microbiol* **65**: 3641–3650.

Lomans BP, van der Drift C, Pol A, Op den Camp HJM. (2002). Microbial cycling of volatile organic sulfur compounds. *Cell Mol Life Sci* **59**: 575–588.

Lovley DR, Holmes DE, Nevin KP. (2004). Dissimilatory Fe(III) and Mn(IV) reduction. *Adv Microb Physiol* **49**: 219–286.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *System Appl Microbiol* **15**: 593–600.

Mulder EG. (1989). *Leptothrix*. In: Staley MP, Bryant MP, Pfennig N, Holtz (eds). *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins Co: Baltimore, pp 1998–2003.

Muyzer G, Teske A, Wirsen CO, Jannasch HW. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165–172.

Nealson KH, Saffarini D. (1994). Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. *Annu Rev Microbiol* **48**: 311–343.

Neef A. (1997). Anwendung der *in situ* einzelzell-identifizierung von bakterien zur populationsanalyse in komplexen mikrobiellen biozönosen. PhD thesis. Technische Universität München.

Neef A, Amann R, Schlesner H, Schleifer K-H. (1998). Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology (UK)* **144**: 3257–3266.

Pol A, Op den Camp HJM, Mees SG, Kersten MA, van der Drift C. (1994). Isolation of a dimethylsulfide-utilizing *Hyphomicrobium* species and its application in biofiltration of polluted air. *Biodegradation* **5**: 105–112.

Rainey FA, Ward-Rainey N, Gliesche CG, Stackebrandt E. (1998). Phylogenetic analysis and intrageneric structure of the genus *Hyphomicrobium* and the related genus *Filomicrobium*. *Int J Syst Bacteriol* **48**: 635–639.

Schlesner H. (1987). *Verrucomicrobium spinosum* gen. nov., sp. nov.: a fimbriated prosthecate bacterium. *Syst Appl Microbiol* **10**: 54–56.

Siering PL, Ghiorse WC. (1996). Phylogeny of the *Sphaerotilus–Leptothrix* group inferred from morphological comparisons, genomic fingerprinting, and 16S ribosomal DNA sequence analyses. *Int J Syst Bacteriol* **46**: 173–182.

Smolders AJP, Nijboer RC, Roelofs JGM. (1995). Prevention of sulphide accumulation and phosphate mobilization by the addition of iron(II)chloride to a reduced sediment: an enclosure experiment. *Freshw Biol* **34**: 559–568.

Spring S, Kaempfer P, Ludwig W, Schleifer KH. (1996). Polyphasic characterization of the genus *Leptothrix*: new descriptions of *Leptothrix mobilis* sp. nov. and *Leptothrix discophora* sp. nov. nom. rev. and ammended description of *Leptothrix cholodnii* emend. *Syst Appl Microbiol* **19**: 634–643.

Spring S. (2002). The genera *Leptothrix and Sphaerotilus*. *The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*. 3rd ed Springer-Verlag: New York, http://www.link.springer-ny.com/link/service/books/10125.

Stahl DA, Amann R. (1991). Development and application of nucleic acid probes. In: Stackebrandt E, Goodfellow M (eds). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons Ltd: Chichester (UK), pp 205–248.

Stookey LL. (1970). Ferrozine—a new spectrofotometric reagent for iron. *Anal Chem* **42**: 779–781.

Straub KL, Schönhuber WA, Buchholz-Cleven BEE, Schink B. (2004). Diversity of ferrous iron-oxidizing, nitrate-reducing bacteria and their involvement in oxygen-independent iron cycling. *Geomicrobiol J* **21**: 371–378.

Suylen GMH, Kuenen JG. (1986). Chemostat enrichment and isolation of *Hyphomicrobium* EG. A dimethyl-sulphide oxidizing methylotroph and reevaluation of *Thiobacillus* MS 1. *Antonie Leeuwenhoek* **52**: 281–293.

Tanimoto Y, Bak F. (1994). Anaerobic degradation of methylmercaptan and dimethyl sulfide by newly isolated thermophilic sulfate-reducing bacteria. *Appl Envir Microbiol* **60**: 2450–2455.

Visscher PT, Taylor BF. (1993). Aerobic and anaerobic degradation of a range of alkyl sulfides by a denitrifying marine bacterium. *Appl Environ Microbiol* **59**: 4083–4089.

Wallrabenstein C, Gorny N, Springer N, Ludwig W, Schink B. (1995). Pure culture of *Syntrophus buswellii*, definition of its phylogenetic status, and description of *Syntrophus gentianae* sp. nov. *Syst Appl Microbiol* **18**: 62–66.

Watanabe K, Kodama Y, Syutsubo Y, Harayama S. (2000). Molecular characterization of bacterial populations in petroleum-contaminated groundwater discharged from underground crude oil storage cavities. *Appl Environ Microbiol* **66**: 4803–4809.

Weber KA, Achenbach LA, Coates JD. (2006). Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nat Microb Rev* **4**: 752–764.

Widdel F, Pfennig N. (1981). Photoautotrophic growth of *Thiocapsa roseopersicina* on dimethyl sulfide. *FEMS Microbiol Lett* **81**: 247–250.

Yamada T, Sekiguchi Y, Imachi H, Kamagata Y, Ohashi A, Harada H. (2005). Diversity, localization, and physiological properties of filamentous microbes belonging to *Chloroflexi* subphylum I in mesophilic and thermophilic methanogenic sludge granules. *Appl Environ Microbiol* **71**: 7493–7503.

Zeyer J, Eicher P, Wakeham SG, Schwarzenbach RP. (1987). Oxidation of dimethyl sulfide to dimethyl sulfoxide by phototrophic purple bacteria. *Appl Environ Microbiol* **53**: 2026–2032.