RESEARCH ARTICLE

Microbial diversity of an oil–water processing site and its associated oil field: the possible role of microorganisms as information carriers from oil-associated environments

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DGGE; MEOR; petroleum microbiology; subsurface microbiology.

Abstract

The phylogenetic diversity of Bacteria and Archaea in water retrieved from a Dutch oil field and units of the associated oil–water separation site were determined using two culture-independent methods. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments was used to scan the microbial diversity in (1) the oil–water emulsion produced, (2) two different oil–water separator tanks, (3) a wash tank and (4) a water injector. Longer 16S rRNA gene fragments were amplified, cloned and sequenced to determine the diversity in more detail. One of the questions addressed was whether the detected microorganisms could serve as indicators for the environments from which they were retrieved. It was observed that the community found in the production water resembled those reported previously in oil reservoirs, indicating that these ecosystems harbor specific microbial communities. It was shown that changes, like a decrease in temperature, cause a distinctive shift in these communities. The addition of SO_3^{2-} to the wash tank as ammonium bisulphite, used in the oil industry to scavenge oxygen, resulted in a complete community change, giving rise to an unwanted sulphate-reducing community. The fact that these changes in the community can be linked to changes in their environment might indicate that these tools can be used for the monitoring of changing conditions in oil reservoirs upon, for example, water flooding.

Introduction

It is well recognized that microorganisms thrive in oil fields and industrial oil-associated processing sites. Activities of microorganisms in oil–water environments, such as oil reservoirs, have been reported frequently. Examples of this activity are the occurrence of heavy oil (Head *et al.*, 2003), and the internal corrosion of oil pipelines (Neria-Gonzalez *et al.*, 2006). Since the discovery of aerobic and anaerobic microorganisms associated with oil–water systems, many species have been detected with the help of molecular techniques. A variety of production waters from different oil fields have been studied (Dahle *et al.*, 2008; Grabowski *et al.*, 2005; Nazina *et al.*, 2007). These studies yielded a large variety of microbial communities. Many new microorganisms have been isolated from these environments, belonging to diverse groups including sulphate reducers, fermenting bacteria, iron reducers, acetogens and methanogens (Magot *et al.*, 2000).

Changes in a reservoir are difficult to assess. Because microorganisms are directly influenced by their environment, they might be used as indicators for changes in reservoir conditions. The presence of specific communities could provide information on the characteristics of the oil reservoir itself (e.g. temperature, acidity, salt content, redox, etc.). Changes in the microbial community could also be used to indicate changes caused by human activity, such as water flooding. In this way, the community composition could be used as a biomonitoring tool, providing information about the conditions and processes that occur downhole. This information could be a welcome contribution to a better oil recovery in so-called 'smart well' applications. Because microorganisms also thrive in surface facilities,

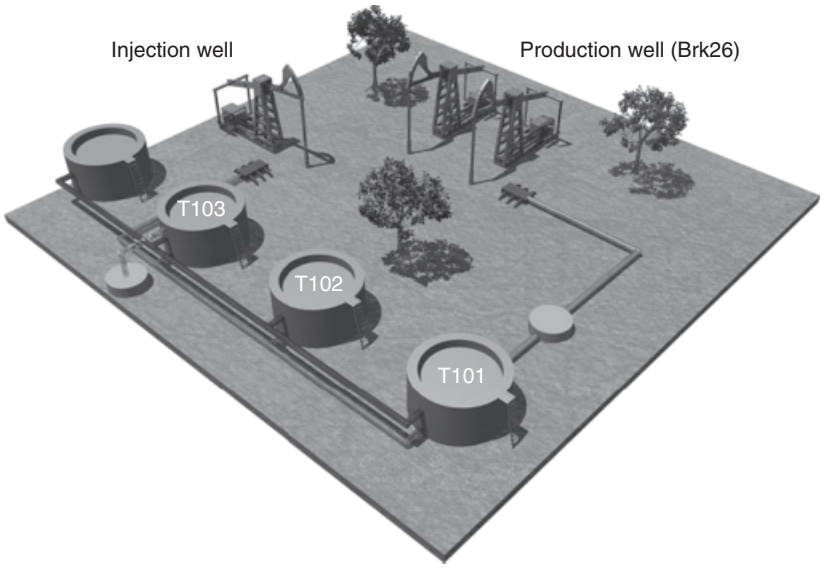

Fig. 1. Overview of the sampling sites. Samples were taken from the oil well Brk26, the first oil–water separator T101, the second oil–water separator T102, the wash tank T103 and the injection well.

where the oil is separated from the water, such a location was picked as a case-study environment to investigate the sensitivity of the communities found towards changes in the environmental characteristics such as heating and the addition of an external electron acceptor. The different units might provide an insight into the microbial processes in the oil field, when temperature and chemistry are similar between oil reservoirs and units.

The aim of this paper was to take a step towards the possible application of community composition as an information source in oil–water systems. Here, we performed a comprehensive study of the bacterial and archaeal communities present in an oil production well and its associated oil–water separation facility that are located in the western part of the Netherlands. The collected samples were first analysed by PCR-denaturing gradient gel electrophoresis (DGGE) in an attempt to obtain a rapid overview of the complexity of the bacterial and archaeal communities and to assess the (dominant) species present in the oil–water emulsion. Subsequently, samples were analysed in more detail by comparative analysis of 16S rRNA gene sequences obtained by cloning.

Materials and methods

Description of the oil field and the oil–water processing site

The studied Berkel (designated later as Brk) oil field is located in the western part of the Netherlands and holds multiple oil production wells and an oil emulsion separation facility, in which the crude oil phase is separated from the water phase (see Fig. 1 for an overview). The field is young in geological terms. The origin of its deposition is estimated to be lower-Cretaceous. It is a shallow marine unconsolidated sandstone deposit found at a depth of approximately 1250 m. The reservoir contains medium heavy crude oil, holding an API gravity of 20°. The porosity of the field is on average 25%; the permeability is between 750 and 1800 mD. The reservoir holds an anticline structure. With respect to oil production, the field is mature. The exploitation of the field started in 1977. The field has a small gas cap. The produced oil on average has a viscosity of 29 cp under reservoir conditions. The produced oil–water emulsion is pumped up by several nodding donkeys (Beam pumps) and is collected at a central point from where it is pumped to a primary oil–water separation tank located at the separation site. The travel time of the liquid from the reservoir to the surface takes 2.5 h, and from the collector to the first separator tank it takes an additional 2.5 h. In the first oil–water separator tank (T101, the 'cold primary' separator), the emulsion is separated based on physical properties only; the bulk of the water is being removed here (the retention time of the water phase is 1 day). After this procedure, the remainder of the oil-rich emulsion is pumped towards a second oil–water separator tank (T102, the 'hot secondary' separator), where it is heated to a temperature of approximately 50 °C to achieve a better separation between the oil and the water, which is removed (the retention time of the water is 3.5 days). The last traces of water are removed in a wash tank (T103). The salinity is reduced through the addition of tap water (the retention time of the water is 6 days). Ammonium bisulphite (NH_4HSO_3) is added to the tap water to scavenge and remove oxygen, because tap water is O_2 saturated. The remaining oil fraction is separated by flotation. The oil fraction is sent from this tank to the refinery. The water from all industrial units is collected at a central water

collection point, from where it is disposed in a deep subsurface aquifer, beneath the depth from which the oil is retrieved. Therefore, it is designated as a water disposal process. The distance between the production wells, the injection point and the surface facility location (oil–water separator systems) is about half a kilometre. Samples for molecular analysis were taken from the oil–water emulsion produced, the water phase from both oil–water separator tanks, the wash tank and the injection water. Data on the chemical composition of the produced water have been provided by Shell International Exploration and Production.

Sampling procedure and preparation

From each site, 10-L samples were taken in sterile jerry cans. The jerry cans were completely filled and sealed directly with screw caps to avoid oxygen intrusion. The samples were immediately taken to the laboratory (the time between the sampling and the filtration procedure was approximately 30 min). Water samples from the different tanks were taken close to the oil–water interface. In the laboratory, the water samples were filtered using 0.2-μm hollow fibre filters (Spectrumlabs, mediakap-5 hollow fibre filter), in order to concentrate the biomass present in the water. The filtered volume of every sample was 4 L (performed in duplicate). After the filtration procedure, the filters were stored at − 20 °C for further analysis. During filtration, attempts were made to avoid filtering the oil phase. Oil blocked the filters and had a negative influence on the later DNA extraction.

DNA extraction

One filter of each sampled environment was thawed on ice, filter lamella were washed with buffer and approximately 3 mL of the cell suspension released from the filter was collected in sterile Eppendorf tubes and centrifuged for 1 min at 16 100 ***g***. Ninety per cent of the supernatant was removed, thereby achieving a 10-fold concentration of the biomass and the removal of most potential PCR inhibitors (e.g. residual oil molecules). The pellet was resuspended into the rest of the supernatant. This suspension was subjected to DNA extraction using the Soil DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad) according to the manufacturer's protocol. DNA extraction was also performed on the remaining filter pieces. Subsequently, the DNA amount from all extractions was quantified using a Nanodrop 1000 Spectrophotometer (Thermoscientific, the Netherlands). The obtained DNA was used for further PCR amplification. To check for cell lysis after the freeze–thaw cycle also the supernatant was subjected to DNA extraction, but DNA levels were below the detection limit, and no PCR product could be obtained using this as a template, indicating that the vast majority of the DNA was present in the pellet. Also, the DNA extraction on the filtered pieces yielded no significant amounts of DNA and no PCR result was obtained.

16S rRNA gene amplification

Partial 16S rRNA gene sequences were amplified from the genomic DNA retrieved from the different Berkel field industrial units, the production water and the injection water. All sampled environments were tested for the presence of bacterial and archaeal 16S rRNA genes. To obtain partial bacterial 16S rRNA gene sequences, the primer pair 341F+GC and 907R (Schäfer & Muyzer, 2001) was used. In the bacterial PCR, a TOUCHDOWN program was implemented. To obtain partial archaeal 16S rRNA gene sequences, an additional nested approach was used complementary to the direct PCR. In previous studies, it was observed that the direct use of the archaeal primer pair Parc519F and Arc915+GC also yielded bacterial sequences (Vissers *et al.*, 2009). Therefore, before this PCR amplification, we performed an amplification of the nearly complete archaeal 16S rRNA gene using the primer pair SD-arch-0025-a-S17 and S-*-Univ-1517-a-A-21. Dilutions up to 1/10 000 were made from all the PCR products obtained, which served as templates for PCR amplification with the primer pair Parc519F–Arc915+GC. The partial archaeal 16S rRNA genes were amplified as described by Coolen *et al.* (2004). Full (by approximation 1500 bp) 16S rRNA gene PCR fragments were used as a template. The amplification of these fragments was modified after Wilms *et al.* (2006). In brief: A first DNA denaturation step of 5 min at 95 °C was followed by 34 cycles of 30 s denaturation at 95 °C, 40-s annealing at 58 °C and 90-s elongation at 72 °C. Finally, an extra 10-min elongation at 72 °C was added to the end of the PCR. The bacterial 16S rRNA gene fragments for clone library construction were obtained by amplification using the universal bacterial primer pair GM3–GM4 as described by Muyzer *et al.* (1995). For the cloning of the full archaeal 16S rRNA genes, the full PCR products obtained during the first amplification of the nested PCR approach have been used as inserts. All PCR amplification reactions were performed in a T1 Thermocycler (Biometra, Goettingen, Germany).

DGGE and clone library construction

DGGE was performed according to the method reported by Schäfer & Muyzer (2001). In brief: 1-mm-thick 6% acrylamide gels with a 20–80% urea-formamide gradient were applied for the separation of the partial bacterial 16s rRNA gene fragments. Gels (30–70%) were used for the separation of archaeal 16S rRNA gene fragments. Gels loaded with bacterial PCR products were run at 100 V for 18 h. The gels loaded with archaeal PCR products were run at 200 V for

5 h. The bands obtained were placed in 15 μL, 10 mM Tris buffer, pH 8.5 and maintained at 4 °C for 24 h. The solution obtained was used as template DNA for reamplification according to the protocol mentioned above. Clone libraries were constructed using a commercial pCR®4-TOPO cloning kit (Invitrogen) according to the standard manufacturer's protocol. The vectors obtained were placed into chemically competent *Escherichia coli* cells by transformation (delivered with the kit). They were grown on agar plates containing kanamycin. From each environment, 96 clones were selected. Their colonies were picked and reamplified using the MF–MR primer pair provided with the kit according to the manufacturer's instructions.

Cluster analysis of DGGE results

Cluster analyses were performed on all the DGGE gels, using the software package GELCOMPAR2, v5.0 (Applied Maths, Belgium). Gel images were loaded into the software, gel strips were identified and bands were determined. The gel images were normalized accordingly using internal standards. Cluster analysis was performed using the Pearson correlation. Dendrograms were created using maximum parsimony clustering. The intensity of the bands was not taken into account.

PCR product purification and sequencing

Twenty-five microlitre PCR products obtained from reamplification of the clones or DGGE bands were placed in separate, sterile vials. A 1.6-μL ExoSap-IT enzyme solution (USB Europe) was added to remove the single-stranded primers and the rest of the nucleotides. Purification was performed according to the manufacturer's protocol. The purified PCR products were diluted accordingly to obtain a 50 ng μL^{-1} PCR products solution; subsequently, they were sequenced by a commercial company (Macrogen, Seoul, Korea).

Comparative sequence analysis

To obtain a first indication, the 16S rRNA gene sequences obtained (partial) were compared with sequences stored in the GenBank nucleotide database by applying the BLAST algorithm (Altschul *et al.*, 1990). Sequences were then imported into the ARB SSU rRNA database (Ludwig *et al.*, 2004) see also http://www.arb-home.de). They were aligned using the automatic alignment tool in the software package and subsequently checked manually for errors. Phylogenetic trees were generated by application of the maximum likelihood (ML) algorithm, FastDNA ML. First, the sequences from the clone library were imported into the ARB database and an ML tree was created. Later, the DGGE band sequences were added to this tree. The lengths of all the clones holding sequences with high quality were around 700–800 bp, all the bacterial DGGE bands were around 500 bp, and the archaeal DGGE bands were around 400 bp. All the 16S rRNA gene sequences obtained (partial) were deposited in the GenBank database. Bacterial DGGE bands, accession numbers (FJ941796–FJ941826). Archaeal DGGE bands, accession numbers (FJ941438–FJ941470). Bacterial clones, accession numbers (FJ941471–FJ941795). Archaeal clones accession numbers (FJ941116–FJ941437).

Statistical analysis of the clone libraries

The similarity between the clone libraries was assessed by applying the available WEBLIBSHUFF tool. This tool estimates the similarity of two sets of sequences within a 95% confidence limit (Henriksen, 2004). All clone libraries were compared pair wise.

Results

Chemical composition of the production water

The water produced at the oil wells and the water from the first two oil–water separator tanks is hypersaline. It has a NaCl concentration of around 73 g L^{-1} (1.25 mol L^{-1}), which is over two times the average salt content of seawater. The ions K^+, Ca^{2+} and Mg^{2+} are present as minor cations, respectively, 0.35, 3.41 and 1.06 g L^{-1}. Ba, Sr and Fe are present in microquantities. Inorganic carbon is present as CO_2 0.67 g L^{-1}, and bicarbonate HCO_3^- 0.18 g L^{-1}. The produced water is low in SO_4^{2-} (below the detection limit of 0.1 mg L^{-1}). The field is not seawater flooded. It is, however, asserted that minute quantities of SO_4^{2-} are present because small quantities of H_2S are detected in the off-gas from the wells (this might also arise from organo-sulphur oil component degradation). The water that ends up in the wash tank is diluted with 15–20% tap water. NH_4HSO_3 is added up to a concentration of 15 p.p.m. The pH of the produced water on the surface is 6.4. The pH formation water in the field is lower, typically around 5.5., because the water contains dissolved CO_2.

DGGE and clone library analyses

To assess the overall diversity present in all the sampled environments, DGGE was used as a first screening method (Fig. 2). The DGGE analysis targeting the 16S rRNA genes of bacteria yielded a total of 38 distinct bands from which 31 bands yielded sequences of satisfactory quality: 10 were from the production water (Brk26), five from the primary (cold) separator tank (T101), seven from the secondary (hot) separator tank (T102) and nine from the wash tank (T103) (Figs 1 and 2a). The injection water displayed DGGE patterns similar to the primary separator tank. The largest

part of the water that is produced at the well site is removed in the first oil–water separator tank and consequently also represents the bulk of the injection water (see results cluster analysis, Fig. 2a and b). Therefore, it was decided to exclude the injection water environment from clone library construction. Also, the archaeal community has been analysed with DGGE. For this, we used both a direct and a nested PCR approach, because direct amplification of Archaea also yielded deep-branching bacterial 16S rRNA genes. The nested DGGE analysis targeting the 16S rRNA genes of Archaea yielded 18 bands of good sequence quality out of 21: three from the produced water (Brk26), seven from the primary cold oil–water separator tank (T101), six from the secondary oil–water separator tank (T102) and two from the wash tank (T103). The direct DGGE analysis yielded 15 bands with good sequences out of 22 cut bands: two from the produced water, seven from the primary oil–water separator tank, six from the secondary oil–water separator tank and four from the wash tank (Figs 1 and 2b). Clone libraries of the production water and the different tanks (T101, T02 and T103) provided a more detailed view of the communities (Fig. 3). The results were in line with those found using the DGGE screening method. From each environment, 96 bacterial and 96 archaeal clones were picked. Between 85% and 90% of the bacterial clones and between 70% and 96% of the achaeal clones yielded high-quality sequences (see Supporting Information, Table S1). As a quick indication for the coverage of all the clone libraries, the method of Good (1953) was used. This method takes the ratio of unique clones ('singletons') into account compared with the number of total investigated clones. The coverage percentage for the bacterial clone libraries varied between 85% and 95%; the coverage of the archaeal clone libraries varied between 96% and 99%. In addition to the differences in diversity, the clone library compositions were compared with each other using the WEBLIBSHUFF tool. This analysis showed that all environments were significantly different from each other with respect to both the bacterial and the archaeal communities within a 95% confidence interval. The clone libraries were compared pair wise. A

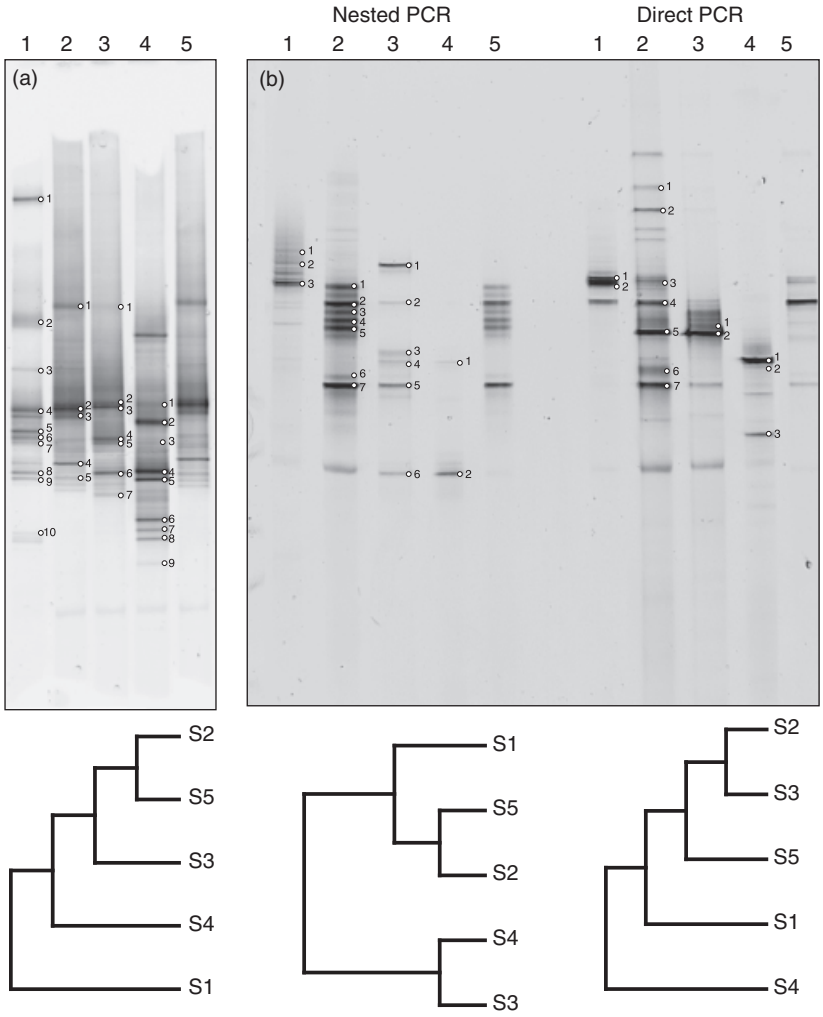

Fig. 2. DGGE analysis of Bacteria (a) and Archaea (b). The numbers above the lanes refer to the different sample sites: 1, Brk26; 2, T101; 3, T102; 4, T103; 5, injector well. Cluster analyses of the different DGGE profiles are below the denaturing gels. Bands indicated by a number were sequenced successfully and used for phylogenetic analysis (see Figs 4 and 5).

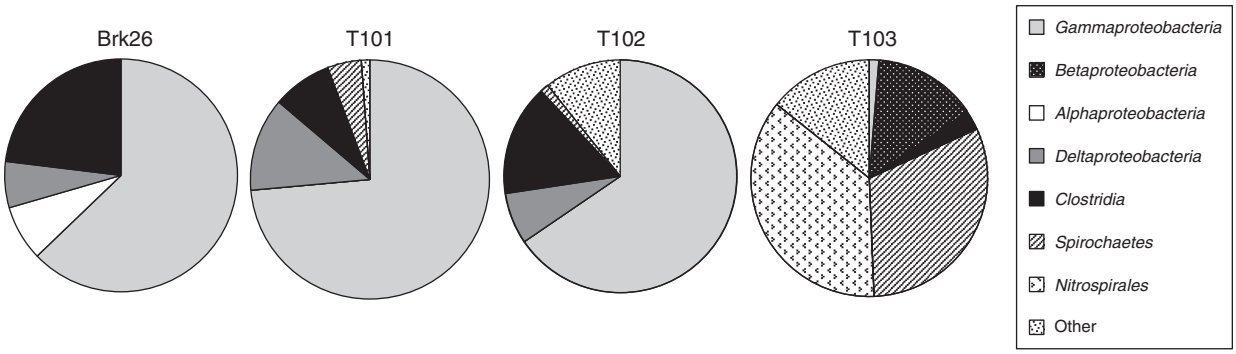

Fig. 3. Phylogenetic distribution of the 16S rRNA gene sequences obtained from the clone libraries of Bacteria.

P-value lower than 0.001 indicates a significant difference between the compared environments. All pairwise comparisons of bacterial clone libraries gave *P*-values below 0.001. Pairwise comparisons of the archaeal clone libraries yielded *P*-values below 0.001; with the exception of the archaeal communities found in the produced water and the primary oil–water separator tank, the XY comparison yielded a *P*-value of 0.61. The YX *P*-value, however, was 0.001. X and Y are, in this case, assigned designations for both clone libraries compared in each pairwise comparison.

Identification of microorganisms in the production water

Detailed phylogenetic analysis of the sequences retrieved by DGGE and clone library construction yielded a large variety of Bacteria and Archaea (Fig. 4). Sequences retrieved from the production water samples displayed a similarity to those found in other production water (wellhead) samples taken from oil fields in similar studies (examples below). DGGE bands were associated with the *Clostridia*, *Flavobacteria* and *Alphaproteobacteria*. The sequence Brk26_2 (Fig. 2a) shared a 99% sequence identity with the extremely halophilic *Halanaerobium congolense* (Ravot *et al.*, 1997) that was isolated from an off-shore Congolese oil field. It was also picked up in the clone library from the production water. The sequence Brk26_10 (Fig. 2a) was 99% related to the species *Anaerobaculum thermoterrenum* (Rees *et al.*, 1997), which was also isolated from the production water of a petroleum reservoir. This sequence was also picked up in the production water clone library. The sequences from Brk26_8 and numerous Brk26 clones were affiliated to the genus *Thermovirga* and displayed relatedness to the sequences found in water produced from a high-temperature North Sea oil field (Dahle *et al.*, 2008). The sequences from the production water clones revealed a wider diversity than the DGGE bands only. The bacterial community in the produced water was dominated by the *Gammaproteobacteria* (63%, Fig. 3). This division accommodates most of the known halophilic bacteria. The two other major groups in these environments are the *Deltaproteobacteria* and the *Clostridia*. Sequences related to the sulphur-cycle bacteria, such as the halophilic SRB species *Desulfohalobium utahense* (Jakobsen *et al.*, 2006) (Great Salt Lake), *Pelobacter carbinolicus* (Lovley *et al.*, 1995) and *Thiomicrospira thermophila* (Takai *et al.*, 2004) (hydrothermal fumarole), were identified. Members of the genus *Marinobacter* were extensively present in the clone library of the production water. The *Marinobacter* sequences were affiliated mostly to sequences found in studies investigating oil-contaminated environments (see e.g. the sequence with accession number EU328021.1). The closest described relatives were *Marinobacter lipolyticus* (Martin *et al.*, 2003) and *Marinobacter hydrocarbonoclasticus* (Gauthier *et al.*, 1992). This last species is well known for its capability to degrade a substantial variety of hydrocarbons at elevated salinity. It was isolated from oil-polluted seawater and is an obligate aerobe. Members of the genera *Halomonas* and *Idiomarina*, known as versatile heterotrophs developing within a very broad range of salinity, were also found. Some clones showed a 99% match to sequences retrieved from an Alaskan mesothermic petroleum reservoir (Pham *et al.*, 2009). Many of the sequences matched strongly with the sequences obtained from oil- and salt-associated environments, such as salt wells and production well brines, around the world. Examples are an offshore Brazilian basin (Sette *et al.*, 2007), Colombian oil fields and North Sea oil fields such as the Ekofisk Oil Reservoir (published only in GenBank).

With respect to the Archaea, the production water displayed the lowest diversity in the DGGE analysis of PCR products obtained with the direct and the nested PCR approach. All DGGE band sequences were affiliated with sequences found in archaeal communities from hydrothermal fluids at the Yonaguni Knoll IV hydrothermal field (Nunoura & Takai, 2009) (Fig. 2b). The bands from the nested DGGE showed an affiliation to the same study. These sequences were not present in the archaeal clone library. The direct DGGE displayed an additional band that had a 100% similarity to a sequence of *Methanocalculus halotolerans* (Fig. 2b) isolated from oil field production water (Ollivier *et al.*, 1998). With both PCR approaches, it was shown that

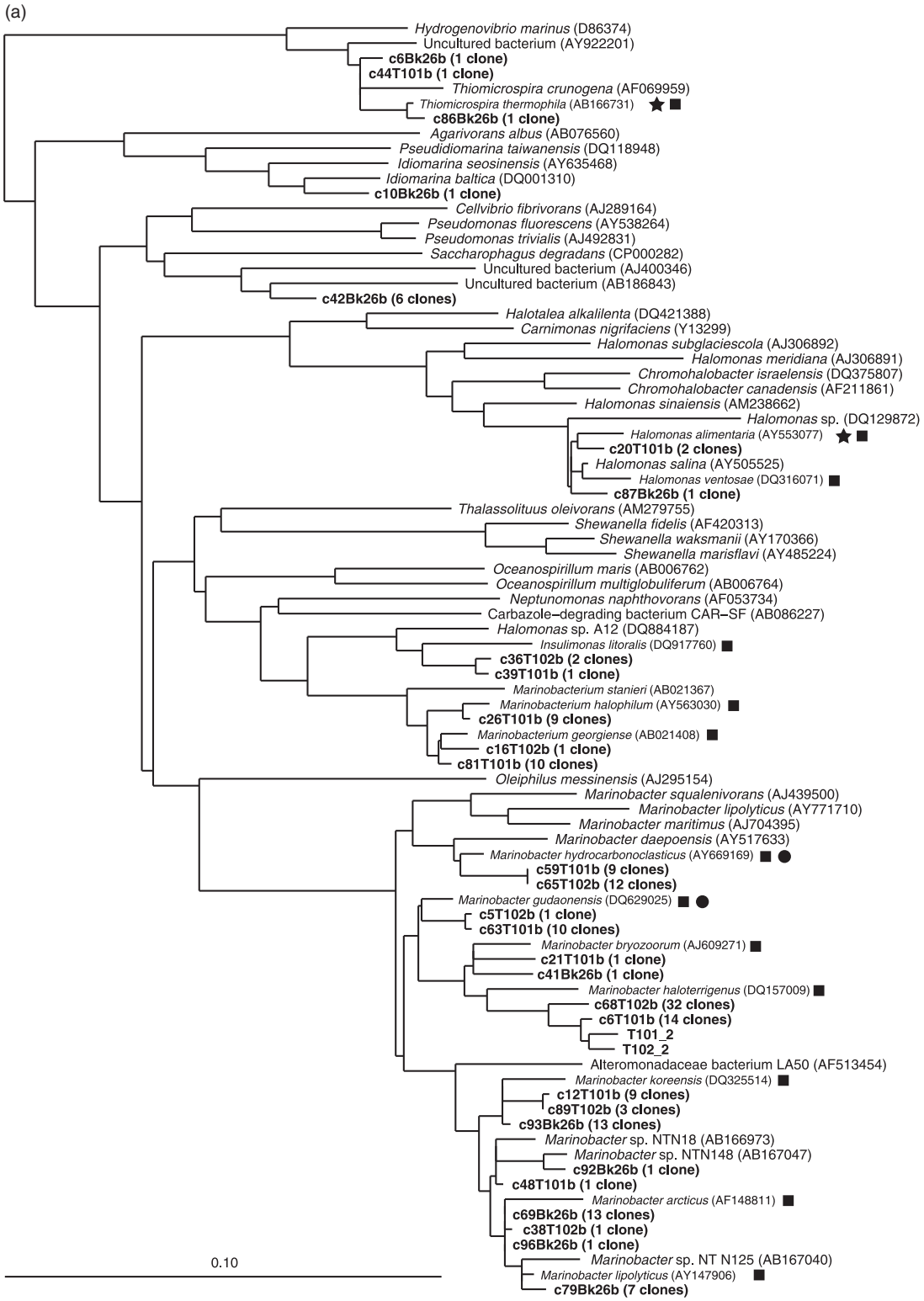

Fig. 4. Phylogenetic analysis of the bacterial 16S rRNA gene sequences: (a) *Gammaproteobacteria*, (b) *Deltaproteobacteria* and (c) *Clostridia*. Sequences determined in this study are printed in bold. Names indicate sequences obtained from the clone library (starting with a 'c') or from DGGE, and from which sample site (i.e., Brk26, T101, T102 and T103). The number of clones with the same sequence is written between parentheses. The bar indicates 10% sequence difference. A star behind the sequence name indicates thermophily; a square indicates salt-loving; a dot indicates the isolation from an oil-associated environment.

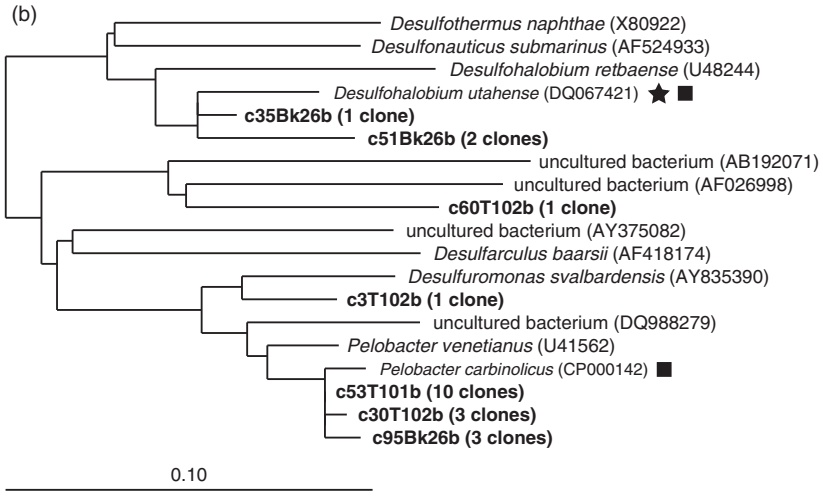

Fig. 4. Continued

this species was not only present in the production water but also in the two oil–water separator tanks. The DGGE bands T101_2n-a, T102_2n-a and T101_4d-a (Fig. 2b) and one of the clones from the secondary oil–water separator tank also belonged to the same species. The archaeal clone library from the water produced contained many clones affiliated with sequences found in the hydrothermal sediments of the Guaymas Basin (Dhillon *et al.*, 2003). These sequences were also detected in the oil–water separator tanks. Several clones in the production water matched 99% with the described species *Methanohalophilus euhalobius* (*Methanococcoides euhalobius*) isolated from oil field production waters (Davidova *et al.*, 1997). This halophilic methanogen was also present in the primary oil–water separator tank, but was not detected in the other tanks. The sequence was also found on DGGE, band T101_1n-a (Fig. 2b). In addition, members of the *Methanomicrobiales* were present in the production water clone library (Fig. 5).

Identification of microorganisms in the oil–water separator tanks

The clone and DGGE band sequences from the primary separator tank overlapped partly with the found sequences in the production water; this can also be deduced from the bacterial cluster analysis (Fig. 2). An example is that the sequence of a DGGE band from the primary separator tank is T101_5, which resembles the sequence of Brk26_8 from the production water (Fig. 2a). Like the production water clone library, the primary separator tank bacterial clone library was dominated by *Gammaproteobacteria* (74%, Fig. 3) and contained sequences related to *Marinobacter*, specifically to *M. hydrocarbonoclasticus*, *Marinobacter bryozoorum* (Romanenko *et al.*, 2005), *Marinobacter koreensis* (Kim *et al.*, 2006) and *Marinobacter gudaonensis* (Gu *et al.*, 2007). *Marinobacter bryozoorum* is a polyaromatic hydrocarbon degrader isolated from the deep sea; *M. gudaonensis* was isolated from oil-polluted saline soil in a Chinese oil field. Sequences related to the genus *Marinobacter* also shared a close association with sequences found in a similar study on the Chinese Qinghai oil field. DGGE band T101_2 also showed a strong similarity to these sequences. Band T101_3 was affiliated to the genus *Thermotoga* (Fig. 2a); it was not picked up in the primary separator tank clone library. Clones related to members of the genus *Halomonas* were also detected. Similar to the production water clone library, sequences were found related to the species *P. carbinolicus*, *H. congolense* and several *Thermovirga* members.

In the secondary separator tank, the bacterial sequences detected by DGGE and in clone libraries displayed an overlap with those found in the production water. It included moderately halophilic genera *Marinobacter* and *Halomonas*. Sequences related to two unique species were found that were not detected in the two previous environments: *Desulfotomaculum geothermicum* (100%), isolated previously from geothermal groundwater (Daumas *et al.*, 1988), and members of the genus *Flexistipes*, although the sequence identity was only 94% with the described species *Flexistipes sinusarabici* (Fiala *et al.*, 1990). The appearance of sequences related to the genus *Marinobacterium* was of special interest. Members of this genus were not detected in the production water. The observed sequences were related to the species *Marinobacterium halophilum* (Chang *et al.*, 2007) isolated from the Yellow Sea, *Marinobacterium georgiense* (Gonzalez *et al.*, 1997), isolated from a marine enrichment, and *Marinobacterium stanieri* (Satomi *et al.*, 2002) and *Marinobacterium litorale* (*Insulimonas litoralis*), also retrieved from the Yellow Sea (Kim *et al.*, 2007), which have been described as strictly aerobic. The appearance of *Spirochaeta* members is also observed. A notable difference is the absence of *Alphaproteobacteria* in both separator tanks, since they were detected in the production water.

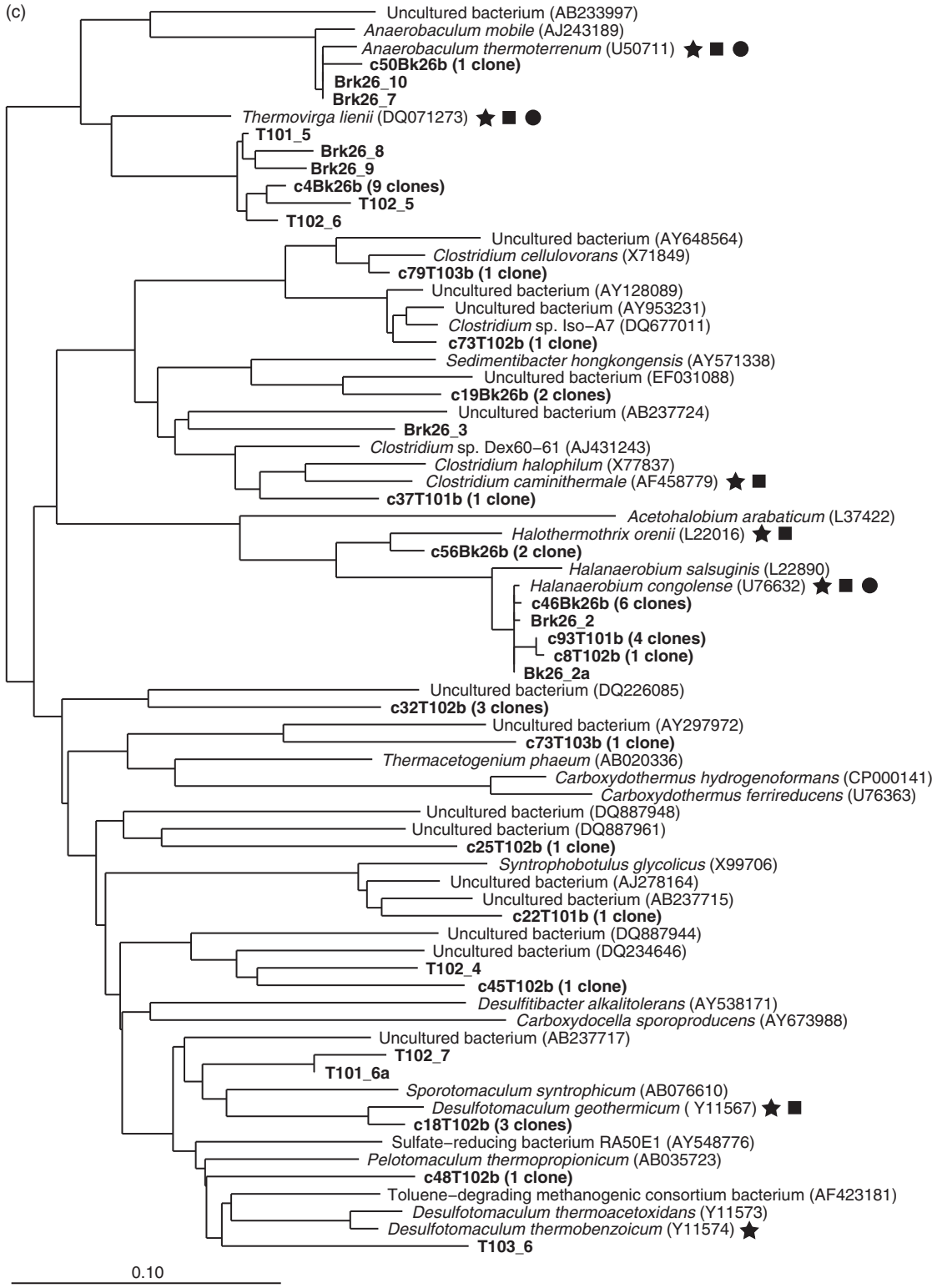

Fig. 4. Continued

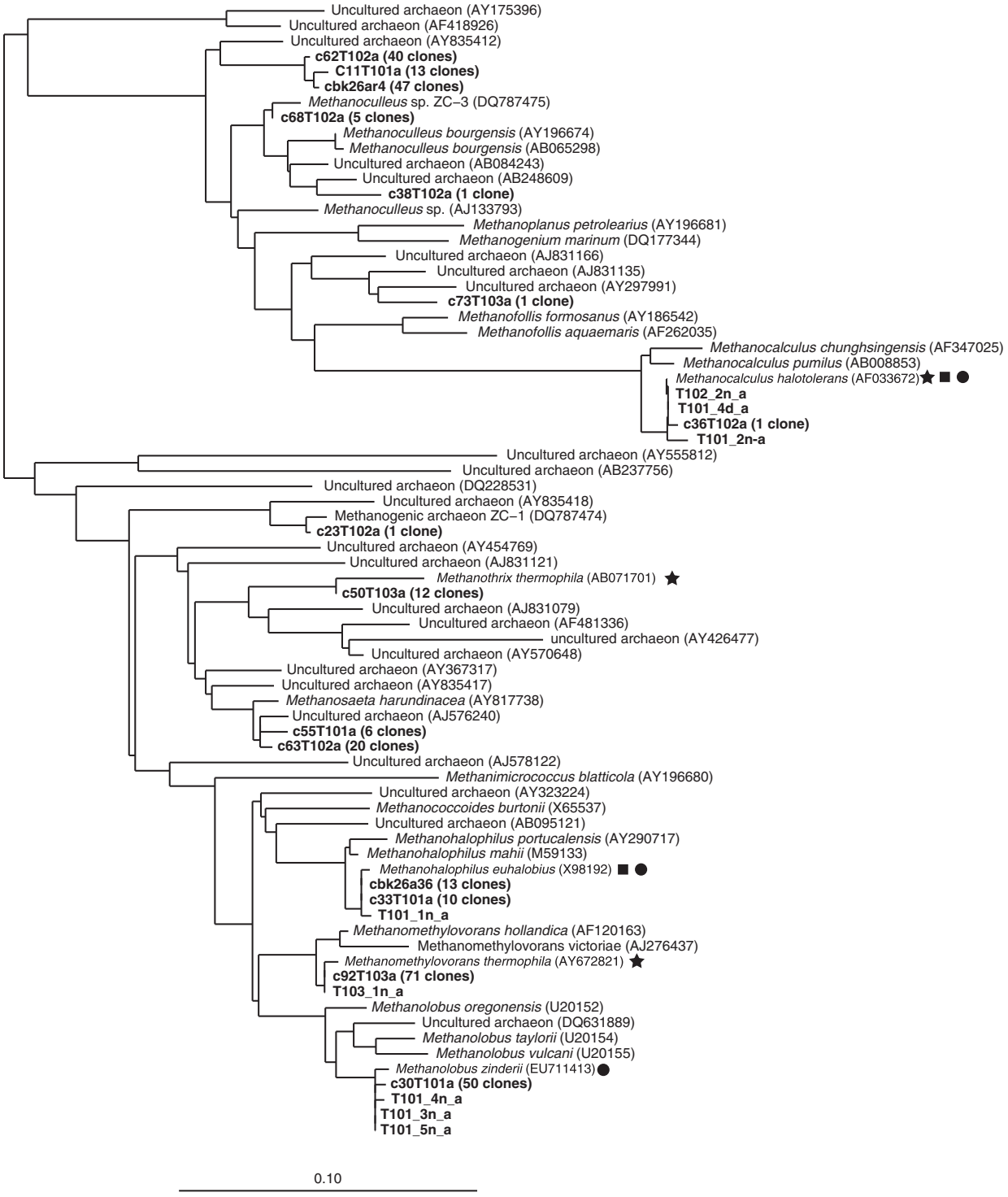

Fig. 5. Phylogenetic analysis of the archaeal 16S rRNA gene sequences. Sequences determined in this study are printed in bold. Names indicate sequences obtained from clone library (starting with a 'c') or from DGGE, and from which sample site (i.e., Brk26, T101, T102 and T103). The number of clones with the same sequence is written between parentheses. The bar indicates 10% sequence difference. A star behind the sequence name indicates thermophily; a square indicates salt-loving; a dot indicates the isolation from an oil-associated environment.

The archaeal diversity in the primary oil–water separator tank was higher than that in the production water. Many of the sequences found in the production water were also found in the primary oil–water separator tank. As mentioned earlier, dominant bands from the direct and nested DGGE matched with *M. halotolerans*. The sequences from a second dominant band found using both DGGE techniques in the oil–water separator were closely related (99%) to an uncultured archaeon from a deep subsurface shale (GenBank EF117481). This archaeon was also detected in the secondary oil–water separator tank. The clone library from the primary oil–water separator tank was dominated by members of the genus *Methanolobus*. The sequences shared a 99% sequence similarity to sequences retrieved from the Qinghai oil field. The bacterial community also holds sequences associated with this habitat. The closest described archaeon (97%) is *Methanolobus zinderii*, isolated from a deep subsurface coal seam (GenBank EU711413). The same sequences were also obtained using the nested DGGE approach (bands T101_3n-a and T101_4n-a). This dominant archaeon was not present in the production water. As mentioned earlier, the clone library from the primary oil–water separator tank also contained sequences closely related to the species *M. euhalobius*. A few sequences were found that were related to sequences from tar sand. The closest related described species was *Methanosaeta harundinacea*.

The clone library from the secondary oil–water separator tank resembled the clone library from the primary oil–water separator tank, except that sequences related to *M. euhalobius* could not be detected in the secondary separator. The cluster analysis of the nested DGGE in this case does not show a good grouping, because one environment (S4) displays only one band (Fig. 2b). The sequence from the DGGE band T102_6n-a showed a 99% match to an uncultured sequence found in the Ekofisk Oil Reservoir, which also contained sequences found in the bacterial community studied in the present work. It was 93% related to the described species *Ferroglobus placidus* (Hafenbradl *et al.*, 1996). This species was not detected in the clone library.

Identification of microorganisms in the wash tank

The bacterial community in the wash tank is dominated by *Thermodesulfovibrio yellowstonii*, which belongs to the phylum *Nitrospirales*. It was isolated from thermal vent water in Yellowstone National Park (Henry *et al.*, 1994) and it was by far the most dominant sequence in the clone library (36% from the total). It is known for its sulphate-reducing capabilities at high temperatures. DGGE band T103_3 shares a 99% sequence identity with this species (Fig. 2a). Also, a substantial increase of the *Spirochaeta* (31%) was observed. Furthermore, members of the *Betaproteobacteria* (14%) were detected only in the wash tank. The *Gammaproteobacteria* displayed a nearly complete disappearance. Members of the *Deltaproteobacteria* were no longer detectable (Fig. 3). DGGE band T103_6 matches (95%) with a described isolate from a study towards marine sulphate-reducing bacteria able to oxidize short-chain alkanes anaerobically (Kniemeyer *et al.*, 2007); it was not detected in the clone library. DGGE band T103_1 (Fig. 2a) and the wash tank clone library also detected members from the phylum *Aquificales* accommodating a deep lineage of extremely thermophilic bacteria. The sequences found had up to a 98% sequence identity to sequences retrieved from hot spring microbial mats (Skirnisdottir *et al.*, 2000). DGGE band T103_2 was affiliated to an unknown member of the *Spirochaetales* and was also dominant in the wash tank clone library (Fig. 2a). It shared 98% identity to the sequences obtained from an anaerobic toluene-degrading aquifer microbial community in a tar oil-contaminated plume (Winderl *et al.*, 2008). DGGE band T103_9 (Fig. 2a) sequence was 98% similar to sequences of thermophiles in deep subsurface geothermal environments (Kimura *et al.*, 2006). It was not detected in the clone library. On the other hand, the wash tank clones, however, did identify members from the *Bacteroidetes* and *Thermotogales* families.

On the nested archaeal DGGE, only two bands were distinctly visible (T103_1n-a and T103_2n-a, Fig. 2b). T103_1n-a was similar to the most dominant sequence present in the clone library and shared a 100% sequence match with the methylotrophic methanogen *Methanomethylovorans thermophila* isolated from a methanol-fed thermophilic bioreactor (Jiang *et al.*, 2005). T103_2n-a clustered with an uncultured archaeon and was similar to the sequence from the band T102_6n-a (Fig. 2b). Only a single additional sequence was detected in the clone library, which displayed 99% sequence similarity to the acetoclastic methanogen *Methanothrix thermophila* (*Methanosaeta thermophila*) isolated form a thermopillic anaerobic digester (Kamagata *et al.*, 1992). The sequences found were also affiliated to the sequences retrieved from a high-temperature gas field in Japan (Mochimaru *et al.*, 2007).

Discussion

The presence of similar microorganisms in production water from oil fields

Analysis of the microbial diversity from DGGE and clone libraries yielded several sequences commonly found in oil field environments (Dahle *et al.*, 2008; Pham *et al.*, 2009). Their frequent occurrence indicates that they are part of specific communities associated with these environments, and at least some of these species can therefore be seen as indicators for an ecosystem, which contain fossil

hydrocarbons and high amounts of salt. Phylogenetic grouping of our sequences with sequences of described organisms isolated from oil fields substantiates this and indicates similar physiologies. Because the investigated oil field in this study is not extensively water flooded, it is to be expected that at least part of the observed microorganisms are indigenous to the field. There is also no sulphate introduced into the field, which is the case in many studied oil fields. Detected were sequences related to the moderately thermophilic and halophilic citrate-fermenting *A. thermoterrenum*, and the extremely halophilic fermentative bacterium *H. congolense*. Both these species are isolated from oil field production waters and share a profile that fits the field characteristics with respect to salt concentration, pH and temperature optimum. *Halanaerobium congolense* belongs to a specific group of obligately anaerobic halophilic bacteria that have a salt-in strategy (similar to Haloarchaea) using inorganic K^+ ions to maintain their osmotic balance, which is quite uncommon because only two groups of bacteria have this feature. It has fermentative capabilities, but is also known to reduce thiosulphate and elemental sulphur, which could indicate the presence of low quantities of sulphur compounds in the habitat. This is reinforced by the detection of sequences related to the halophillic Deltaproteobacterium *D. utahense*, which is a known sulphate/thiosulphate reducer. With respect to the detected archaeal species, sequences were found related to *M. halotolerans* (dominant in the DGGE profile, Fig. 2b) and *M. euhalobius* (dominant in the clone library). These Archaea also share properties that match their environmental conditions. Both Archaea have been isolated from oil field production waters and both are halophillic. *Methanocalculus halotolerans* requires acetate for growth when growing with H_2+CO_2 and formate. *Methanohalophilus euhalobius* is a methylotrophic methanogen. It also has a calcium requirement. Sequences related to another archaeon extensively present in the clone library was the filamentous organism *M. harundinacea*, which is specialized on acetoclastic methanogenesis (Ma *et al.*, 2006). All the above-mentioned species have characteristics that are complementary to the environmental conditions of the investigated oil field. The detected clones match with species isolated from oil field production waters, which is an indication that these clones have a similar physiology.

The presence of members from the genus *Marinobacter* distinctively indicates the presence of species not directly linked to the oil field, as is the detection of sequences related to the microaerophile *T. thermophila*. Most *Marinobacter* species are aerobic, although some members can denitrify. As oil fields are deprived of oxygen, the presence of these *Marinobacter* as indigenous species in the field is dubious at best. They are, however, frequently found in many other studies on the microbial diversity of oil field production waters (see Results). They are extremely salt-tolerant hydrocarbon degraders and are often detected in oil-contaminated sites. This clearly displays their affinity with this type of ecosystem (Yakimov *et al.*, 2007). Considering the overall community found in the production water, it seems that the halophilic species are a mixture of microorganisms indigenous to oil fields (anaerobes) and microorganisms that are associated with the surface facilities (e.g. piping system of the well, aerobes). Some species are also indicative of the presence of small amounts of oxidized sulphur compounds. Because the community is indeed specific for oil fields and oil-associated environments, some species can be used as indicators of such a system in future research.

Temperature-induced community changes

When comparing the microbial community in the primary separator tank and the production water, it is observed that there is a substantial overlap between the two communities. This is logical because the tank is only used for separation of the collected water/oil mixture, no chemicals are added and no aeration is applied in the tank. There are, however, notable differences. The main difference is the occurrence of *Marinobacterium* species in the first tank. Most members of the genus *Marinobacterium* are strictly aerobic, with the exception *Mb. litorale* (also detected), which can denitrify. All the detected *Marinobacterium* members have a lower temperature range and a lower optimum growth temperature (30–40 °C) with respect to the species detected in the production water. They are halophillic and are commonly associated with seawater and can be associated with marine oil-related ecosystems (Yakimov *et al.*, 2005). Despite this, there is probably no association between the oil field and the occurrence of the *Marinobacterium* species, considering their strict aerobic nature and maximum growth temperatures, which are below the field temperature. They have, however, characteristics that match the environment of the primary cold oil–water separator tank. The appearance of sequences related to strict aerobes such as *Mb. halophilum* and *Mb. georgiense* and the disappearance of sequences related to some strict anaerobes such as *A. thermoterrenum* points towards an increased oxygen intrusion and a decrease in temperature. Another difference is the disappearance of the *Alphaproteobacteria*; an explanation for this observation cannot be given. It is likely that due to the pumping, the oil–water mixture becomes aerated, inducing microbial activity. It should be noted that even small amounts of oxygen lead to a substantial increase in the bacterial population (the presence of 1 mg of oxygen can support the aerobic growth of one billion cells, given no other limitations). The sensitive PCR methods can therefore already indicate small variations in the system when comparing the first tank and the production water. This implies the possibility that

biologically, there is a large shift while chemically almost no difference can be detected.

The archaeal community from the production water is comparable to the primary oil–water separator tank. A clear difference is the occurrence of *Methanolobus*, specifically sequences related to the species *M. zinderii* (*Methanolobus* sp. SD1), which is a methylotroph isolated from a subsurface coal seam. The proliferation of this archaeon is most likely also an effect of the decreasing temperature. The microbial community detected in the primary oil–water separator tank (35 °C) was similar to the secondary oil–water separator tank (50 °C). It is highly unlikely that all these species can indeed survive at these elevated temperatures. The fact that these species are still detectable is probably due to the fact that despite their inactivation, the dead cells are still detectable by the DNA analysis in the water phase as it enters the secondary hot oil–water separator tank from the cold one. The secondary oil–water separator tank does display sequences from or related to two bacterial species not detected in the previous environments. One is the clostridium *Desulfotomaculum thermosapovorans*, which is a thermophilic sulphate reducer utilizing long-chain fatty acids. Sequences related to the *Flexistipes* genus were also detected.

Negative effects of NH_4HSO_3 addition

Before reinjection of the produced water in a deeper subsurface layer, the oxygen mainly introduced via the tap water that is used to reduce the salt concentration is scavenged from the system by addition of NH_4HSO_3 in the wash tank. In the oil industry, this is a common practice to prevent heavy corrosion of the injection wells by a combination of O_2, NaCl and elevated temperatures down hole. NH_4HSO_3 is used specifically for its quick reactivity with oxygen, which is often required if the water is to be completely oxygen free within a short time frame. From a production chemist point of view, this is more efficient than the use of slower reacting compounds such as sodium bisulphite or other oxygen scavengers. From a microbiological point of view, this exercise seems illogical. The addition of NH_4HSO_3 means the introduction of an activated form of sulphate in combination with the addition of a nitrogen source, which undoubtedly leads to an establishment of an opportunistic sulphate-reducing community. Such a community, indeed, has been detected in the wash tank from the surface facility. The community in the wash tank consisted predominantly of specific thermophilic sulphate-reducing bacteria. Most dominant in the bacterial clone library were sequences related to *T. yellowstonii*; this species can utilize sulphate, thiosulphate and sulphite with various organic acids and alcohols as electron donors. The species belongs to a deep lineage branching near the division between bacteria and Archaea. Other detected deep-lineage bacteria included *Sulfurihydrogenibium azorense* and the members of *Aquificales*. They are thermophiles, which can grow well in the presence of sulphur compounds utilizing them both as electron donors and as acceptors. Their presence indicates elevated temperature plus availability of inorganic sulphur compounds.

Sequences affiliated to two specific Archaea were detected in the water from the wash tank, namely *Methanomethylovorans thermophila* and *Methanothrix thermophila*. The first can only utilize methanol and methylamines; the second can only use acetate. The presence of sulphate-reducing bacteria selects for these types of methanogens. They use substrates that are poorly used by sulphate-reducing bacteria, allowing them to survive in this sulphidogenic environment. Overall, the microorganisms detected in the wash tank are related to sulphur-associated hydrothermal vent systems. It is observed that the NH_4HSO_3, in combination with fermentation end products already present in the water, provides the perfect environment for a specific thermophilic sulphate-reducing community. That H_2S is indeed produced is proven by the fact that it can be found in the off-gas measurements of tank T103. H_2S levels are found up to 250 p.p.m. This NH_4HSO_3 addition seems to be in contradiction with many large-scale investigations towards the prevention of reservoir souring and microbial-induced corrosion. For example it is reported that the pipeline after this wash tank is indeed subjected to heavy corrosion. This addition is therefore not advised and it is encouraged to search for a good alternative oxygen scavenger, which does not contain SO_3^{2-} or ammonia.

Combined use of different techniques in community analysis

The DGGE technique is widely used in the oil industry to assess the microbial community present in oil-associated environments. It was shown that DGGE indeed provides a good first overview of the microbial diversity present in the environments of interest. The observed differences are likely due to primer biases and mismatches causing preferential amplification of some of the present species (Suzuki & Giovannoni, 1996). The clone libraries provided a more detailed overview of the community in respect to for example individual populations ('singletons'). An example of such a singleton is a sequence from the production water related to *Halomonas salina* (*Deleya salina*) (Valderrama *et al.*, 1998). Other techniques such as Tag-sequencing (Huse *et al.*, 2007) and metagenomics (Singh *et al.*, 2009) will provide even more details on these ecosystems.

In this study, we described the microbial diversity of oil field production water and the associated surface facility separation units using a combination of two culture-

independent methods. Summarizing the results from the DGGE and clone library, it was concluded that the communities found, although diverse, match with the characteristics of the specific conditions, such as temperature, available electron acceptor and salinity. The fact that these changes in the community may be linked to changes in their environment has the potential to indicate changing conditions in an oil reservoir upon for example water flooding. However, the results in this paper have been deduced from 16S rRNA gene sequences and do not give full certainty on the metabolic properties of the species, which should be considered in future studies. The communities in the production water and in the two oil–water separator tanks, although all found to be different, displayed an association with other oil-related ecosystems. This is a first step in the use of microorganisms as information carriers of reservoir conditions. The addition of NH_4HSO_3 in the wash tank led to a substantial enrichment of sulphate reducers. From a souring and corrosion point of view, it is advised to look for alternative oxygen scavengers that does not contain SO_3^{2-} and ammonia.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Affiliation of sequences found with relevant known/described species or sequences.

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