Molecular and physiological evidence for the methane oxidation capability of Crenothrix polyspora COHN

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IDENTIFICATION OF BACTERIA CAUSING SOURING AND BIOCORROSION IN THE HALFDAN FIELD BY APPLICATION OF NEW MOLECULAR TECHNIQUES

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ABSTRACT

Nitrate has been added to the injection water in the Halfdan field since January 2001 in an attempt to prevent reservoir souring. As with any new technique, it is important that the treatment is monitored and optimized. In this paper it is demonstrated that cultivation-independent methods are superior for identification of bacteria compared to the traditional cultivation methods. Hence cultivation-independent methods help improve the quality of monitoring.

In situ techniques were implemented on field samples from the Halfdan water injection system. For bacterial quantification and determination of identity fluorescence in situ hybridization (FISH) was applied. The bacterial functional groups were quantified using microautoradiography (MAR). The results demonstrated that:

- The most probable number (MPN) technique measures at least 100 times lower counts than obtained with the MAR and FISH techniques, which impacts on the interpretation.
- Sulfate reducing bacteria (SRB) made up to 10% of all bacteria present when measured by cultivation-independent techniques. By comparison, the MPN method showed no SRB to be present.

- Cultivation-independent techniques can be applied much faster (few hours to few days) compared to traditional MPN techniques (30 days incubation time), resulting in a potentially faster response time to e.g. maltreatments.

Keywords: Halfdan field, souring prevention, sulfate reducing bacteria, nitrate injection, fluorescence in situ hybridization (FISH), microautoradiography (MAR), most probable number (MPN) technique.

INTRODUCTION

Souring of oilfield reservoirs and microbiologically induced corrosion (MIC) of pipelines and process equipment as a result of SRB activity are well established in the oil and gas industry. Typical microbiological monitoring methods using cultivation techniques have an inherent bias regarding strain isolation and growth resulting in only viable and culturable bacteria being detected. While great advances have been made in media and cultivation/enumeration techniques, it is now accepted that depending on the actual ecosystem only 0.001-15% of the viable bacteria are indeed culturable by these classical microbiological methods[1]. Therefore, methods based on microbial enrichment and growth will inherently grossly underestimate the bacterial population size and may not reflect the role of the uncultivated bacteria in the oilfield operation.

The objective of the present study was to evaluate the performance of new molecular detection methods in order to establish improved methods for monitoring, prevention and mitigation of bacterial related phenomena in the oil industry.

The molecular techniques applied in this work on pigging debris samples from the Halfdan water injection system are not based on cultivation, but on the identification of bacterial ribosomes (16S or 23S subunits within the ribosome). Identification and quantification were conducted by fluorescence in situ hybridization (FISH) where specific bacteria or groups of bacteria within a mixed microbial population can be visualized by epifluorescence microscopy[1]. Furthermore, functional bacterial groups were quantified using the uptake of radioactively labeled substrate as a measure of active bacteria. This technique, microautoradiography (MAR)[2], was applied to characterize the bacteria with respect to utilization of an electron donor (organic substrate or hydrogen) and the terminal electron acceptor (oxygen, nitrate or sulfate). The understanding of the complexities of nitrate treatments can be improved with this enhanced monitoring of SRB and nitrate utilizing bacteria (NUB).

THE HALFDAN FIELD

The Halfdan oil field[3] is located in the Danish sector of the North Sea and is operated by Maersk Oil on behalf of the Danish Underground Consortium (DUC). The Halfdan oil and gas accumulation was discovered in December 1998 by a 30,000 ft (9,144 m) long horizontal well drilled from the Dan field. Production of oil and gas commenced in February 1999. Two wellhead platforms are currently installed in the

Halfdan field. Three phase separation is carried out on Halfdan and the stabilized oil is exported to the Gorm-C platform. The gas is exported to the Dan-FF platform.

The Halfdan field is laterally extensive and comprises a high porosity (25-30%) low permeability (0.5-2 mD) chalk reservoir located outside structural closure. The producing horizon is the Maastrichtian Age Chalk with a reservoir temperature of typically 80°C and 4,200 psi (29 MPa). The field is developed with long horizontal wells of 10,000-15,000 ft (3,048-4,572 m) reservoir sections drilled in a dense parallel pattern of alternate producers and injectors with well spacings of 600 ft (183 m). At present, 29 producers and 14 water injectors have been drilled.

Water injection commenced in January 2001 from one injector, HDA-04. Injection water is supplied from the Dan-FF platform and treated in a Minox unit and subsequently transported via a 9 km long sub-sea pipeline made of carbon steel. The Halfdan water injection rate has currently reached a level of 110,000 bbls/day (17,490 m^3/day).

Nitrate has been injected into the Halfdan water injection pipeline from the start of water injection. Presently, the injection rate is 175 ppm sodium nitrate product (40% w/w) which corresponds to 67 mg nitrate/l. Approx. 30% of the injected nitrate is consumed in the sub-sea pipeline, leading to a corresponding reduction of the nitrate level injected into the Halfdan reservoir. The Dan-FF water injection facilities upstream the water injection pipeline are typically treated with a weekly batch treatment of the tetrakishydroxymethylphosphonium sulfate (THPS) bactericide. Pigging of the Halfdan water injection pipeline is carried out twice a month.

Nitrate impacts on SRB activity and biofilm redox potential, as a result of which corrosion due to SRB activity will be reduced[4,5], souring inhibited and previously formed sulfide removed[6,7]. On Halfdan the mechanism applicable is a combination of out-competition of SRB by NUB and inhibition/suppression of SRB activity by NUB due to the formation of nitrite[8]. In the few producing Halfdan wells with partial seawater breakthrough the H_2S production was reduced to a level typically less than half the level measured prior to seawater breakthrough[8].

MATERIALS AND METHODS

The samples analyzed in this study originated from a number of pigging runs performed at the Halfdan-Dan water injection pipeline from April 2003 to March 2004. Pigging debris was sampled in sterile 1 liter glass bottles every 5-10 minutes around the time of pig arrival on Halfdan. During transportation to the laboratory the samples were kept at 5°C without any preservation. The samples with the highest content of debris (measured as solids content) were used for analysis.

Determination of the total number of bacteria in the pigging debris samples was based on the general bacterial stain DAPI (4-,6-diamidino-2-phenylindole) and use of epifluorescence microscopy[9]. DAPI targets the DNA in all bacterial cells: living, inactive and dead cells. The samples were fixed in formaldehyde at a concentration of 2%. An Axioskop 2 plus (Zeiss) epifluorescence microscope with the filter set 01 (Zeiss) and a 100 times magnification objective for oil was used. The counting was performed on black polycarbonate filters (Osmonics Inc.), where 10 randomly chosen fields (100 µm x 100 µm) were selected. Within each of those fields 20-200 cells were counted, with a minimum of 400 cells counted in total per filter.

Identification of bacteria was performed using the FISH technique, which is based on gene probes targeting ribosomal RNA (16S or 23S rRNA) in bacterial cells. Only living and active cells contain sufficient ribosomes that can be detected by FISH. Gene probes consists of two parts: 1) An artificial DNA strand complementary to the ribosomal RNA in the target cell; 2) A fluorescing molecule covalently attached to the probe enables observation of the target bacterium.

FISH was performed with fluorescently labeled rRNA-targeted nucleic acid probes (Thermo Electron Corporation, Germany) on homogenized biofilm (pigging) samples[1]. Fresh samples were filtered onto white 0.2 µm pore size polycarbonate filters (Osmonics Inc.) and fixed in fresh 4% (W/V) paraformaldehyde or 50% ethanol (V/V) directly on the filters for 2 hours. The filters were stained with DAPI (1 mg/ml), washed 3 times in filtered distilled water, and hybridized with gene probes. The filter sets 09 and 10 (Zeiss) were used for the microscopic examination of the FISH labeled cells. A total number of bacteria in the range 20-200 were counted with DAPI at each spot and at the same spots the number of FISH-positive cells was determined. A minimum of 400 DAPI stained cells was counted in triplicate for each hybridization. The following seven probes were used:

1. EUB338(I+II+III) (*Bacteria)*
2. Non-EUB (Nonsense probe)
3. ALF968 (*Alphaproteobacteria*)
4. BET42a (*Betaproteobacteria*)
5. GAM42a (*Gammaproteobacteria*)
6. SRB385 (*Deltaproteobacteria)*
7. SRB385Db (*Desulfobacteriaceae* within the *Deltaproteobacteria*)

Further details about the probes and the hybridization conditions are listed in the database probeBase[10].

Determination of the number of active bacterial groups able to utilize a mixture of carbon sources under different conditions was carried out using the MAR technique[11]. MAR was performed using a broad mixture of the following ^{3}H- and ^{14}C-labeled carbon sources: acetate, glucose, leucine and bicarbonate/H_2 (Amersham Bioscience). The carbon sources were selected since many bacteria are known to assimilate one or more of these substrates. The following electron acceptor conditions were tested in this study: Aerobic; Anoxic with nitrate; Anaerobic (no nitrate nor nitrite). Tracers were added to

sample volumes of 2 ml ensuring an activity of 10 µCi for each substrate. The final concentration of all substrates (labeled and non-labeled) was 0.1 mM, the concentration of sulfate was 28 mM and the concentration of nitrate was 2 mM. For incubations with labeled bicarbonate 10% of the headspace volume was exchanged with H_2. The incubation temperature was 30°C and the incubation time for aerobic/anoxic and anaerobic incubations was 6 and 12 hours, respectively. The specific inhibitors molybdate (30 mM) for SRB and bromo-ethane-sulfonic acid (1 mM) for methanogenic bacteria were used to determine bacterial functional groups[2,12]. The incubations were terminated by addition of freshly prepared PFA/PBS solution to a final concentration of 4%, which was removed after 2 hours of fixation at 4°C by washing 3 times in filtered tap water. Furthermore, incubations with bicarbonate were washed with a glycine buffer (pH 3) for 1 hour to remove precipitated carbonate. The samples were stained with DAPI and 50-100 µl was spread on cover slides. The slides were covered with a radiosensitive film emulsion (LM-1, Amersham, Biotech) and left for exposure for 4 days at 5°C. After development, the total number of DAPI-positive cells and MAR-positive cells were enumerated by fluorescence and brightfield microscopy in the same spots. DAPI-positive cells covered with more than 5 silver grains were evaluated to be positive. Further details about the method (e.g. the use of controls) can be found elsewhere[11,13].

In order to evaluate SRB and NUB presence with traditional cultivation methods, the MPN method was used according to Harrigan and McCance[14]. The SRB growth medium was a modified Postgate medium[15] and the NUB medium consisted of lab lemco (3 g/l), peptone (5 g/l) and potassium nitrate (1 g/l). The samples were inoculated under mesophilic (35°C) conditions with an initial pH of 7.0. SRB were determined to be present when sulfide production was measured. Sulfide was measured colorimetrically by the methylene blue method[16].

In order to relate the total number of bacteria to the contents of solids in the pigging debris, the total solids (TS), suspended solids (SS) and organic content were determined. TS was determined by transferring 50 ml of sample (triplicate) into heat resistant containers and place these at 105°C for minimum 24 hours. SS was determined by filtering 50 ml sample (triplicate) on to 0.45 µm filters (Milipore) and place these at 105°C for minimum 24 hours. All samples were weighed and the remaining material was related to the sample volume. In order to determine the organic content of all the TS and SS samples, these were furthermore placed at 550°C for minimum 24 hours. The mass loss from 105°C to 550°C then gives the organic content of the sample.

RESULTS AND DISCUSSION

Pigging

Microbiological analyses were carried out on samples obtained during three pigging runs in the Halfdan field. During a pigging run deposits are scraped off and carried through the pipeline in suspension (see Figure 1). This study focuses on the microbial community found in such released material (biofilm) with all bacterial numbers related to the amount of total solids in the samples.

Total Number of Bacteria versus Cultivable Bacteria

Traditionally, microbiological monitoring in oilfields is performed by most probable number (MPN) cultivation techniques[14]. MPN counts of different physiological bacterial groups are usually obtained deploying different growth media. However, as only 0.001-15% of the bacteria present in most environmental samples can be cultivated today[1], the MPN technique potentially results in severe misinterpretation of the data. In the present study we have compared the numbers of bacteria achieved by cultivation-based methods to numbers achieved by direct microscopy.

The total number of bacteria in the pigging debris (see Table 1) was obtained by applying the DAPI stain (see Figure 2) and epifluorescence microscopy and the results show the discrepancy between the two methods. In the three samples investigated, total cell counts varied between 10^6-10^9 cells/ml compared to the significantly lower cell counts obtained by the cultivation based MPN technique, which corresponds to less than 0.4%.

Moreover, when MPN was used to estimate the number of nitrate utilizing bacteria (NUB-MPN) and the total number of cultivable bacteria (often assumed to be GAB-MPN + GAnB-MPN) the problems using cultivation-based monitoring of the microbial community becomes clear (see Table 1). It appears that NUB account for more than 100% of the total bacteria in two samples, which is obviously not possible, and to 1% in one sample (November 2003). Thus, in agreement with studies of other environmental microbial systems[1], quantification of bacteria from these oilfield samples using cultivation dependent techniques provide hardly useful quantitative information.

Enumeration by FISH

The DAPI stain includes both inactive and dead cells, so it may overestimate the total number of living bacteria. The FISH technique can to some extent solve this problem. Since FISH labels only bacteria with a certain content of ribosomal RNA, it is only active cells or cells that have recently been active that are enumerated[17]. Unfortunately, the normal FISH procedure is not sensitive enough to detect bacteria with a low activity level (low content of ribosomes), so it may underestimate the real number of active bacteria, particularly in low-nutrient ecosystems. Furthermore, some bacteria

cannot be penetrated by the probe. Thus, as DAPI-staining is also faster and much easier than FISH, it is a good method for a total count.

By using FISH it is possible to identify and quantify certain species and groups of microorganisms (see Figure 2). Thus, by this technique the problem of cultivability is to some extent solved. There are, however, several other difficulties in the application of this method. Presently, the number of reliable gene probes is limited as most bacteria are still unknown. This means that a FISH probe chosen to detect e.g. SRB might detect other bacteria as well - while other so far unknown SRB might not be detected. The probes used in this study to enumerate SRB cover many, but not all SRB. Around 10% of the active bacteria were positive with this probe, thus strongly indicating presence of SRB. Unfortunately, NUB can not be detected by a single or a few gene probes as this physiology is distributed among many phylogenetic groups. In this study we quantified bacteria belonging to *Alpha*-, *Beta*- and *Gammaproteobacteria*. Among these, the *Betaproteobacteria* constituted a large fraction of the bacteria in the pigging sample (around 50% of all active bacteria detected by the EUB338(I+II+III) probe) while no *Alpha*- and very few *Gammaproteobacteria* were present. It is known that some bacteria belonging to these groups can utilize nitrate, so it is likely that a (unknown) fraction of the *Betaproteobacteria* were NUB. However, other major phylogenetic groups which we did not detect in this study, e.g. *Cytophaga* and the Gram-positive *Firmicutes* and *Actinobacteria* also encompass bacteria with this physiological capability, so presently the exact number of NUB cannot be detected by FISH.

FISH is presently one of the best methods to identify and quantify specific bacteria or groups of bacteria, but it is still difficult to link a specific activity or physiology to most probe-defined microbial groups. Only where the physiology is very similar within a certain phylogenetic group, e.g. nitrifiers and to some extent SRB it is possible. However, FISH analyses have the potential to be a significant step forward in the process of making reliable quantification of specific bacteria in oil field related samples. For a better analysis of the microbial communities more detailed molecular analysis must be conducted, but this is still at the research level and not yet ready for general monitoring.

The total number of active bacteria, as determined by FISH and application of a general probe for all *Bacteria*, accounted in this study for 55-66% of the counts obtained by DAPI staining. This is comparable with other environmental ecosystems, but in fact it may be higher as the presence of several major phylogenetic groups (see above) was not investigated. Comparing FISH and MPN counts for GAB+GAnB populations (see Table 1), MPN only accounted for less than 1% of the populations detected by FISH (0.07-0.77%). Thus, FISH counts for specific bacterial groups give a far more reliable estimate of the community structure than the cultivation-dependent data can provide.

Enumeration by MAR and MAR-FISH

By the MAR methodology, bacteria assimilating specific labeled substrate(s) are detected. Hereby, bacteria are detected according to their substrate utilization and hence

their physiology. Physiological groups such as aerobic bacteria, NUB, SRB and other groups can be detected. The MAR technique does not require growth on a certain medium such as the MPN technique, it only requires uptake of a labeled substrate during few hours incubation, which can be carried out under in situ conditions. Thus, a more natural environment can be kept in the sample as only the labeled substrate and maybe a different electron acceptor is added. By quantifying the total number of microorganisms by DAPI or FISH, the fraction of active cells can be estimated (see Figure 3).

The MAR results from the November 2003 data show a bacterial community reflecting anoxic conditions. This is seen from the dominance of NUB ($4.0 \cdot 10^8$ cells/ml) and other anaerobes ($2.2 \cdot 10^8$ cells/ml) over aerobes ($3.7 \cdot 10^7$ cells/ml). Aerobic bacteria might be facultative anaerobes (bacteria able to grow under both aerobic and anaerobic conditions) and, therefore, their presence does not necessarily indicate availability of oxygen.

The dominant physiological group as indicated by MAR was the NUB. Clearly this reflects the influence of nitrate amendment. The dominating bacterial physiology in this system was, therefore, most likely utilizing organic substrates with nitrate as the terminal electron acceptor (possibly denitrification). The finding of SRB by MAR shows that these bacteria were present in the sample and that sulfate reduction may take place in the system. However, these organisms could also use other electron acceptors, e.g. ferric iron, nitrate or other metabolic strategies to sustain their presence in periods. The lack of methanogens could indicate that addition of nitrate may have prevented the strict anaerobic conditions that is demanded by this group of organisms.

Independent FISH and MAR counts show that sulfate reducing bacteria accounted for 4-10 % of the total bacterial counts (DAPI). These numbers underestimate the fraction of living, active cells as they relate to DAPI counts. If the counts are related to the FISH total count SRB accounts for approx. 7-14%. The MAR data show that approx. 27% of the total number of bacteria (or 41-49% of active bacteria) was NUB. This number indicates that a major fraction of the potential NUB as detected by FISH actually were NUB. These data show that NUB dominated the active microbial community with a substantial amount of SRB present as well. Whether the SRB carry out sulfate reduction or use other physiological strategies (such as nitrate reduction) was not determined in this study. The bacteria that did not respond to the applied substrates may still be NUB or SRB, but they were not able to use any of the labeled substrates added to the systems. More detailed studies applying a range of labeled organic substrate could resolve that.

SRB were also detected by cultivation (determined from a significant sulfide production rate), but could not be enumerated using the MPN technique. Thus, from the cultivation data a community totally dominated by NUB was depicted. It can be speculated that fast growing NUB enriched in the nitrate-supplemented system were more cultivable. This may explain the overestimation of NUB in the community as well as the extremely high MPN numbers found for this group in relation to GAB. These results clearly show that the relative abundance of bacterial functional groups was erroneously estimated if based on MPN counts. Also importantly, the monitoring of SRB

by cultivation was not satisfactory, so the study demonstrates that the MAR-FISH approach gave a much more precise and sensitive monitoring of the microbial community in offshore pipelines.

CONCLUSIONS

Cultivation-independent in situ techniques were implemented on pigging samples from the Halfdan water injection system in order to identify and quantify the dominant microbial groups. The study demonstrated that cultivation-independent methods were superior for quantification and partly identification of bacteria compared to the traditional cultivation methods. The MPN technique showed at least 100 times lower counts than obtained by FISH and MAR. These culture independent techniques also demonstrated that SRB made up to 10% of all bacteria present despite being undetectable by the MPN method. Furthermore, the culture-independent techniques could be applied much faster (few hours to few days) compared to traditional MPN techniques (30 days incubation time), resulting in a potentially faster response time to e.g. maltreatments. There are, however, still challenges regarding the use of FISH which should be addressed in the future, but presently a combination of MAR and FISH seems to be the best method for monitoring important microbial populations related to oil fields.

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FIGURES AND TABLES

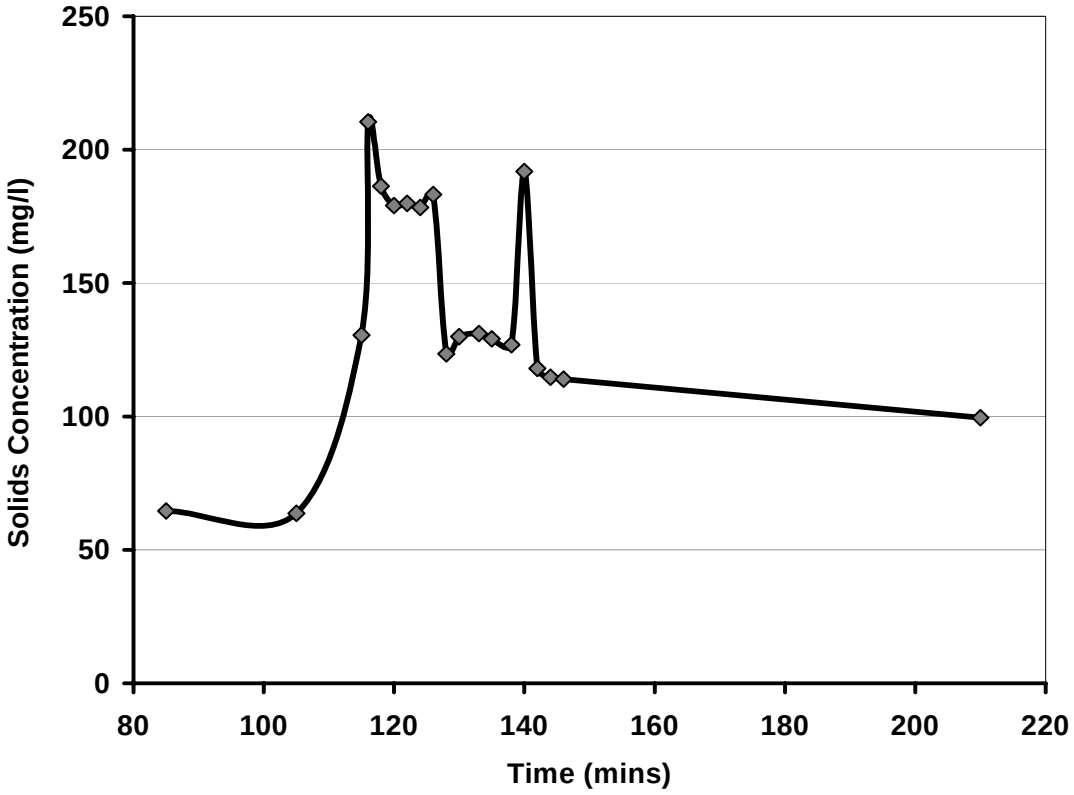

Figure 1 – Solids Removal from Halfdan Water Injection Pipeline during Pigging Operation (pig arrival after 140 mins).

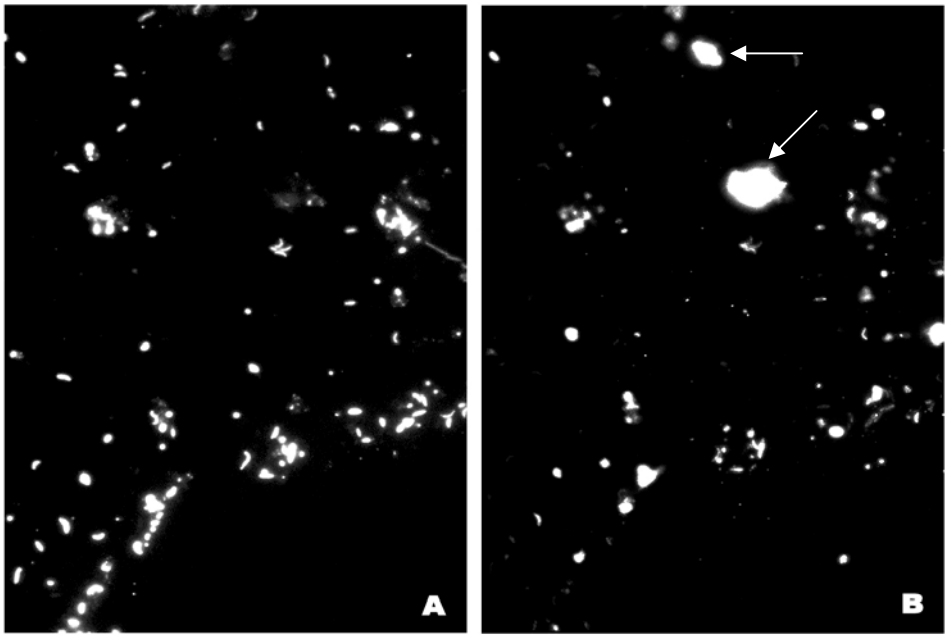

Figure 2 - Epifluorescence Microscopy of Bacteria from a Pigging Sample. A) DAPI staining of all bacteria (blue, here shown as white). B) FISH staining with a gene probe (EUB338(I+II+III)) for all *Bacteria* (green, here shown as white) of the same sample. It is clearly seen that DAPI stains more bacteria than FISH with the broad gene probe. The arrows show examples of non-specific binding of the gene probe to organic debris.

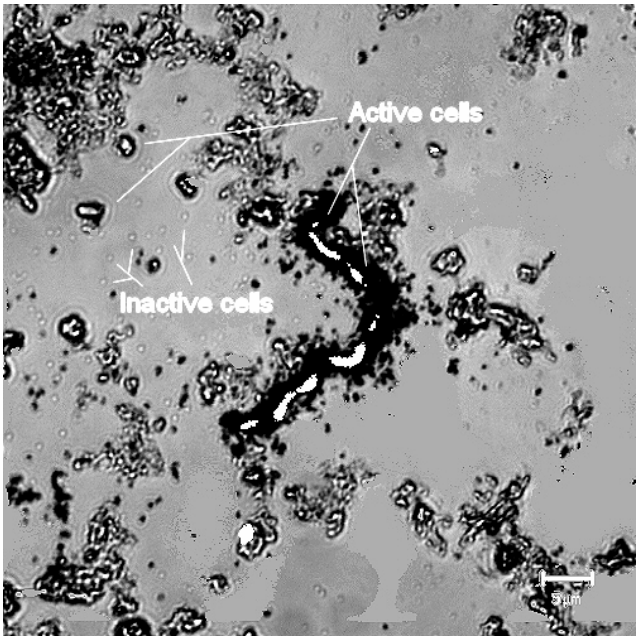

Figure 3 - Illustration of Detection of Bacteria by Microautoradiography (MAR). Black grains illustrate radioactively labeled bacteria indicating that they were metabolic active. White/light gray represent DAPI stained bacteria.

Table 1 - Enumeration of Microbial Groups by Classical and Novel Methods.

Parameter	Explanation	Unit	April 2003	November 2003	March 2004
Total Bacterial Counts					
DAPI	Total number of bacteria	Cells/ml	$3.2 \cdot 10^7 \pm 1.2 \cdot 10^7$	$1.5 \cdot 10^9 \pm 0.02 \cdot 10^9$	$1.4 \cdot 10^6 \pm 0.1 \cdot 10^6$
	Cells per total solids (TS)	Cells/(g TS)	-----	$3.5 \cdot 10^{10} \pm 0.01 \cdot 10^{10}$	-----
	Cells per suspended solids (SS)	Cells/(g SS)	-----	-----	$2.7 \cdot 10^9 \pm 0.1 \cdot 10^9$
	Cells per volatile suspended solids (VSS)	Cells/(g VSS)	-----	-----	$1.0 \cdot 10^{10} \pm 0.2 \cdot 10^{10}$
MPN Counts (Most probable number)					
GAB	General aerobic bacteria	Cells/ml	$4.5 \cdot 10^3$	$1.4 \cdot 10^5$	$1.4 \cdot 10^3$
GAnB	General anaerobic bacteria	Cells/ml	$9.5 \cdot 10^3$	$1.4 \cdot 10^5$	$4.5 \cdot 10^3$
NUB	Nitrate utilizing bacteria	Cells/ml	$4.5 \cdot 10^4$	$2.5 \cdot 10^3$	$4.5 \cdot 10^4$
SRB	Sulfate reducing bacteria	Cells/ml	0	0	0
Presence of SRB (Cultivation)					
SRB	Sulfate reducing bacteria	Yes / No	Yes	Yes	Yes
FISH (Fluorescence in situ hybridization)					
"GAB+GAnB"	Total active bacteria (EUB338(I+II+III))	Cells/ml % of DAPI	$2.1 \cdot 10^7$ 66 ± 12	Non-specific binding[1]	$7.7 \cdot 10^5$ 55 ± 4
Some "NUB"	Some groups containing NUB and many other bacteria (BET42a+GAM42a+ALF968)	Cells/ml % of DAPI	----- -----	Non-specific binding[1]	$3.6 \cdot 10^5$ 26 ± 3
Many SRB	Sulfate reducing bacteria (SRB385 + SRB385Db)	Cells/ml % of DAPI	$\sim 3 \cdot 10^6$ ~10	Non-specific binding[1]	$5.6 \cdot 10^4$ $4 \pm <1$
MAR (Microautoradiography)					
GAB	General aerobic bacteria	Cells/ml % of DAPI	-----	$3.7 \cdot 10^7$ 3 ± 0.1	-----
GAnB	General anaerobic bacteria	Cells/ml % of DAPI	-----	$2.2 \cdot 10^8$ 15 ± 1	-----
NUB	Nitrate utilizing bacteria	Cells/ml % of DAPI	-----	$4.0 \cdot 10^8$ 27 ± 1	-----
SRB	Sulfate reducing bacteria	Cells/ml % of DAPI	-----	$5.6 \cdot 10^7$ 4 ± 0.5	-----
MP	Methane producing bacteria	Cells/ml % of DAPI	-----	0 0	-----

(1) It was not possible to conduct FISH analysis due to non-specific binding of the gene probes to most particles