Effect of sodium bisulfite injection on the microbial community composition in failed pipe sections of a brackish-water-transporting pipeline

Hyung Soo Park1*, Indranil Chatterjee1*, Xiaoli Dong2, Sheng-Hung Wang2, Christoph W. Sensen2, Sean M. Caffrey1, Thomas R. Jack1, Joe Boivin3 and Gerrit Voordouw1

1Petroleum Microbiology Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada

2Visual Genomics Centre, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N 4N1, Canada

3Cormetrics Limited, 56 Hawkwood Place NW, Calgary, Alberta, T3G 1X6, Canada

*These authors contributed equally to the study.
Abstract

Pipelines transporting brackish subsurface water, used in the production of bitumen by steam-assisted gravity drainage, are subject to frequent corrosion failures despite addition of the oxygen scavenger sodium bisulfite (SBS). Pyrosequencing of 16S rRNA genes was used to determine the microbial community composition for planktonic samples of transported water and for sessile samples of pipe-associated solids (PAS) scraped from pipeline cutouts representing corrosion failures. These were obtained from upstream (PAS-616P) and downstream of the SBS injection point (PAS-821TP and PAS-821LP, experiencing rapid flow and stagnant conditions, respectively).

Most transported water samples had a large fraction of *Pseudomonas* (1.8-97% of pyrosequencing reads) not found in sessile pipe samples. The sessile population of PAS-616P had methanogens as the main community component (*Methanobacteriaceae*, 56%), whereas *Deltaproteobacteria* of the genera *Desulfomicrobium* and *Desulfocapsa* were not detected. In contrast, PAS-821TP and PAS-821LP had a lower fraction of *Methanobacteriaceae* (41 and 0.6%), but an increased fraction of the sulfate-reducing *Desulfomicrobium* (18 and 48%) and of the bisulfite-disproportionating *Desulfocapsa* (35 and 22%). Hence, SBS injection strongly changed the sessile microbial community. XRD analysis of pipeline scale indicated that iron carbonate was present both upstream and downstream, whereas iron sulfide and sulfur were only found downstream of the SBS injection point, suggesting a contribution of the bisulfite-disproportionating and sulfate-reducing bacteria in the scale to iron corrosion. Incubation of iron coupons with pipeline waters indicated iron corrosion coupled to the formation of methane. Hence both methanogenic and sulfidogenic microbial communities contributed to corrosion of pipelines transporting these brackish waters.

Introduction

Although the microbial communities in oil and gas fields have been analyzed more extensively (3, 5, 12, 17, 21), those inhabiting the walls of pipelines transporting oil, water or
gas are only beginning to be characterized (10, 15, 18, 20, 25, 26, 31). Yet understanding these communities is highly relevant, as they contribute to microbially-influenced corrosion (MIC), which, along with other forms of corrosion, can lead to pipeline failure. As a consequence, considerable effort is made to protect pipelines by injection of oxygen scavengers, corrosion inhibitors and/or biocides.

Corrosion accelerates when the anodic dissolution of metallic iron ($Fe^0 \rightarrow Fe^{2+} + 2e$) is effectively coupled with the cathodic reduction of an available electron acceptor, e.g. $O_2$ under aerobic conditions ($2H^+ + 1/2O_2 + 2e \rightarrow H_2O$), or protons under anaerobic conditions ($2H^+ + 2e \rightarrow 2[\text{H}]$ and $2[\text{H}] \rightarrow H_2$, where $[\text{H}]$ represents atomic hydrogen). Because of the high reduction potential of $O_2$, aerobic conditions are generally more corrosive than anaerobic conditions. Degassing and the addition of the oxygen scavenger sodium bisulfite (SBS) are used in the brackish water gathering system, investigated here, to prevent oxygen-mediated corrosion (Fig. 1).

Under anaerobic conditions, the activity of sulfate-reducing bacteria (SRB) may accelerate corrosion by using cathodic $H_2$ to reduce sulfate to sulfide, which precipitates 1/4 of the ferrous iron as $FeS$. In bicarbonate-rich brackish waters, as encountered here (Table 1), the remainder of the $Fe^{2+}$ formed may precipitate as $FeCO_3$, giving overall equations [1, 2]:

$$4Fe^0 + 8H^+ \rightarrow 4Fe^{2+} + 4H_2 \quad [1]$$

$$4Fe^{2+} + 4H_2 + SO_4^{2-} + 3HCO_3^- \rightarrow FeS + 3FeCO_3 + 4H_2O + 3H^+ \quad [2].$$

In the absence of sulfate, methanogens may contribute to anaerobic corrosion of iron by using cathodic $H_2$ for the reduction of $CO_2$ to methane as indicated by equations [1, 3]:

$$4Fe^{2+} + 4H_2 + 5HCO_3^- \rightarrow CH_4 + 4FeCO_3 + 3H_2O + 3H^+ \quad [3].$$

However, because corrosion is a surface phenomenon, its mechanism is exceedingly complex and some have questioned whether microorganisms contribute in this way (6). The most detrimental forms of corrosion are localized. Given the ability of microorganisms to attach to surfaces and grow in favourable niches, it is understandable that this may lead to alteration of surface properties and ensuing pitting corrosion.

Here we focus on pipelines transporting brackish water from either the Grand Rapids or the McMurray formations near Fort McMurray, Alberta (Fig. 1) for use in the production of bitumen by steam-assisted gravity drainage (SAGD). All of the transported water is
converted into steam, which is injected in the subsurface. Frequent corrosion failures in the cold part of this system require replacement of affected pipe sections. This offers a unique opportunity to determine the microbial community composition at defined pipeline locations. Pipewall community compositions are typically determined through analysis of pigging solids, obtained by scraping an entire line with a spherical metal pig (10, 26) or by placing metal coupons in a line, which are removed after a certain period of time (15, 18, 20). Neither method represents sections of pipe that correspond to actual corrosion failures. Analysis of localized sections of failed pipe, as presented here, can indicate changes in the wall-attached microbial community as a result of treatment (e.g. injection of the oxygen scavenger bisulfite), allowing evaluation whether this has the desired effect of lowering corrosion risk.

**Materials and methods**

**Sample description and handling**

In addition to planktonic samples (E1, E2, and E3), three samples of failed pipe were obtained after being cut out from the leaking line from locations indicated in Fig. 1. These were shipped in pipe-associated water (PAW) in an effort to preserve the microbial community that may have contributed to causing the leak. Samples were obtained both upstream and downstream of the SBS injection point allowing us to focus on the effects of this treatment on the pipeline microbial community. The water and pipeline cutout samples analyzed in this study are listed in Table 2. The pipeline cutouts are shown in Fig. 2A, D and F. Relevant dimensions, inner diameter (ID) and length (L) are indicated in Fig. 2, as well as in Table 2, where the derived internal volume and pipe surface area are also given. Pipe section 616P was collected upstream, while pipe sections 821LP and 821TP were collected downstream of the SBS injection point, 821TP from a line with continuous flow and 821LP from a line that was often stagnant (Fig. 1). The subsurface temperature was 7-10 °C and the temperature of water in the pipeline was on average 13 °C. Samples were shipped at ambient temperature (20 °C) and were received within 1-2 days of collection. The pipe sections were transported in a bucket filled to the brim with PAW and closed with an air-tight lid. Upon arrival, parts of the water samples E1, E2 and E3 were immediately filtered through 0.2 µm
sterile Millipore filters with the remainder being stored in a Coy anaerobic hood (90% N2, 10% CO2). The filters with collected solids, including biomass, were stored frozen at -80 °C. Pipe sections were removed from the bucket with PAW and transferred into the anaerobic hood. PAW was processed, as were E1, E2 and E3. Pipe-associated solids (PAS) were removed by scrubbing the internal pipe surface with a sterile spatula in the anaerobic hood and re-suspending in 0.2 µm filtered PAW. Part of the PAS suspension was again filtered through a 0.2 µm Millipore filter and the filter stored at -80°C, pending DNA extraction. The remainder was stored anaerobically at room temperature for further analyses.

DNA extraction from the water and pipe samples. Genomic DNA was isolated using a procedure modified from Marmur (19). Biomass-containing filtered solids were thawed, resuspended in 280 µl of 0.15 M NaCl and 0.1 M EDTA (pH 8) and treated with lysozyme. Samples were lysed by treatment with 25% SDS and three freeze-thaw cycles (at -70° C and 68° C), except those with amplicon codes 925, 926, 927 and 1351 (Table 2). The latter were lysed by bead-beating in a FastPrep Instrument for 60 sec at a speed setting of 6.0 in 2 ml lysing matrix E tubes (MP Biomedicals). Following further processing, all samples were treated with DNase-free RNase and proteinase K (Roche Diagnostics, GmbH), phenol extraction, precipitation with two volumes of 2.5 M sodium acetate in 95% ethanol, washing with 70% ethanol and drying. The DNA was then dissolved in 10 mM Tris-Cl, pH 8.5 (buffer EB from the Qiagen QIAquick Kit, Qiagen).

Community analysis by pyrosequencing. DNA samples were generally amplified through a two-step PCR amplification. The first PCR was performed with 16S primers 926Fw (AAACTYAAKGAATTGRCGG) and 1392R (ACGGGCGGTGTGTRC). The PCR mixture (50 µL) contained 20 pmol/µL each of forward and reverse primers in 2 µL, 25 µL of 2 x PCR master mix [containing 0.05 units/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2 and 0.4 mM of each dNTP (Fermentas)], 21 µL of nuclease-free water and 2 µL of DNA template (10-100 ng). PCR was for 3 min at 95°C, followed by 25 cycles of 30 sec at 95°C, 45 sec at 55°C and 1.5 min at 72°C, and then 10 min at 72°C. The PCR product was checked on an 0.7% agarose gel, purified with a QIAquick PCR Purification Kit (Qiagen), and its concentration was determined by a Qubit Fluorometer (Invitrogen), using a Quant-
iT™ dsDNA HS Assay Kit (Invitrogen). The second PCR (10 cycles) was performed with 100 ng of PCR product and the FLX titanium amplicon primers 454T_RA_X and 454T_FwB, which have the sequences for 16S primers 926Fw and 1392R as their 3’-ends. Primer 454T_RA_X has a 25 nucleotide A-adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and a 10 nucleotide multiplex identifier barcode sequence (Table 2: MID), whereas primer 454T_FwB has a 25 nucleotide B-adaptor sequence (CTATCGCCTTTGCCAGCCCGCTCAG). The second-round PCR product was similarly checked, purified and its DNA concentration determined. Amplicons for DNAs with sequence codes 937, 939, 940 and 941 (Table 2) were obtained by a single 35-cycle amplification with bar-coded primers. 16S PCR amplicons (typically 25 μl of 5 ng/μl) were sent for pyrosequencing (16S profiling) to the Genome Quebec and McGill University Innovation Centre, Montreal, Quebec. Pyrosequencing was performed with a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation).

Analysis of pyrosequencing data. Analysis was conducted with Phoenix 2, a 16S rRNA data analysis pipeline, developed in house (See Fig. S1, Supplementary material). Raw pyrosequence data were subjected to stringent systematic checks to remove low quality reads and minimize sequencing errors, which could have been introduced during the pyrosequencing process (13). Eliminated sequences included those that: (i) did not perfectly match the adaptor and primer sequences, (ii) had ambiguous bases, (iii) had an average quality score below 27, (iv) contained homopolymer lengths greater than 8, or (v) were shorter than 200 bp after primer removal, respectively. The remaining high quality sequences were compared against the non-redundant SSU_Reference data set SILVA102 (22) using the Tera-Blast algorithm on a 16 board TimeLogic Decypher system (Active Motif, Inc.). Sequences with an alignment of less than 90% of the trimmed read to the best BLAST match with a greater than 90% sequence identity were identified as potential chimeras and were excluded from further analysis. Sequences, which passed quality control and chimeric sequence removal, were clustered into Operational Taxonomic Units (OTUs) at 3% distance by using the average linkage algorithm (24). After grouping sequences into OTUs, alpha diversity indices were calculated for each sample, including the total number of OTUs, the
Simpson's diversity index and the Shannon diversity index. A taxonomic consensus of all representative sequences from each OTU was derived from the recurring species within 5% of the best bitscore from a BLAST search against the SILVA database. The entire set of the raw reads is available from Sequence Read Archive (SRA) at NCBI under accession number: SRP005237.

Amplicon libraries (Table 2) were clustered into a Newick-formatted tree using the UPGMA algorithm with the distance between communities calculated with the thetaYC coefficient (30) as a measurement of similarity between the structures of two communities in the Mothur software package (23). The Newick format of the sample relation tree was visualized using Dendroscope (14). Differences and similarities among amplicon libraries were also explored with Non-Metric Multidimensional Scaling (NMDS) ordination analysis based on the thetaYC distance matrix in Mothur using the majorization algorithm from Borg & Groenen (4). Statically significant differences in community structures were determined using analysis of molecular variance (AMOVA) based on the thetaYC coefficient.

XRD analysis of PAS. PAS samples were sent to DNX Inc., Calgary, Alberta for analysis of crystalline components by X-ray diffraction (XRD). Powder diffraction analysis by reference intensity ratio was performed using a Rigaku Miniflex instrument with iron K-alpha radiation. The diffraction patterns for wall or pit scale were matched to standard patterns for siderite (iron carbonate), iron carbonate hydroxide, goethite (iron oxide hydroxide), elemental sulfur, mackinawite (iron sulfide) and elemental iron. Findings are presented as fractions (%) of these components in the scale.

Iron corrosion under methanogenic conditions. Iron coupons (4x1x0.1 cm) were cut from ASTM A366 carbon steel with 0.015% (w/w) carbon (ASTM International Designation A 1008/A). These were cleaned according to a standard protocol (NACE RP0775-2005). Briefly, the coupons were polished with 400-grit sandpaper and then placed in a dibutyl-thiourea-HCl solution for two minutes. The coupons were then neutralized in a saturated bicarbonate solution for two minutes, rinsed with deionized water and then with acetone, and finally dried in a stream of air. Coupons were weighed three times and the average weight
was recorded as the starting weight. Duplicate coupons were placed in small plastic holders to prevent them from contacting the glass wall or each other during tests.

For the corrosion experiments, 120 ml serum bottles were filled with 70 ml of brackish water (E1-3, E2-3, or E3-3) or with a Milli-Q purified water control (MQ) under anaerobic conditions. Two coupons were added and the bottles were closed with a butyl rubber stopper. The headspace was 90% (v/v) N₂ and 10% CO₂. Each bottle was incubated in duplicate at 32°C while shaking at 100 rpm. After incubation for 7 h coupons were cleaned and dried according to the NACE standard protocol. The corrosion rate (CR) was determined from the metal weight loss $\Delta W$ as: $\text{CR} = 87,600 \times \frac{\Delta W}{A \times T \times D} \text{mm/yr}$, where $A$ is the coupon area (cm²) and $D$ is the density of steel (7.85 g/cm³). A typical coupon weighed 4 g, had $A = 8.9$ cm² and had a weight loss of 0.019 g over 2688 h giving a $\text{CR} = 0.009 \text{ mm/yr}$.

During the course of the corrosion experiments, methane production was detected by injection of 0.2 ml of culture headspace into an HP 5890 gas chromatograph equipped with a stainless steel column (0.049 cm x 5.49 m) Porapak R 80/100. Injector and detector temperature were 150 and 200°C, respectively. For determination of dissolved ferrous iron, a 100 µl aqueous sample was taken without shaking and added to 500 µl 0.5 M HCl and mixed with 3 ml of ferrozine solution (1g/L in 50 mM HEPES). Following incubation for 10 min at room temperature the OD₅₆₂ was determined.

**Results**

**XRD analysis of pipeline scale**

Brackish waters, produced from the Grand Rapids and McMurray formations, had significant concentrations of sodium ion, chloride, and bicarbonate, but lacked sulfate, sulfide, nitrate, nitrite and organic carbon (Table 1). Concentrations in McMurray waters were generally higher than in Grand Rapids waters.

The surface of pipeline cut-outs was covered by scale (Fig. 2B, E, F). Many deep pits were observed once this scale was removed (Fig. 2C). In the case of pipe that was close to the subsurface water intake point (Fig. 1: 616P), erosion corrosion through impingement of suspended sand, cavitation by bubbles of gaseous CO₂ (formed when the water depressurizes...
at the surface) and CO₂ corrosion are possible mechanisms for pit formation. Electrochemical carbonic acid or hydrogen sulfide corrosion (Fe⁰ + H₂CO₃ → FeCO₃ + H₂; Fe⁰ + H₂S → FeS + H₂), potentially stimulated by adhering microorganisms (equations 2 and 3), must also be occurring to explain the presence of ferrous iron-containing minerals (FeCO₃, Fe₂(OH)₂CO₃ and FeS) in the scale covering the corroded pipe (Table 3).

The pipeline scale upstream from the SBS addition point near well 616P (Fig. 1) had a high fraction of iron carbonates (FeCO₃, 85-95%; Fe₂(OH)₂CO₃, 4-8%), some sand (SiO₂, 4-8%) and trace metallic iron. The presence of sand and metallic iron is consistent with an erosion corrosion process, i.e. sand abrasion of the iron surface. Neither sulfur (S₈) nor mackinawite (FeS) were observed. However, these were major components of the scale downstream from the SBS addition point (Table 3: 20-30% and 10-20%, respectively), which had a smaller fraction of FeCO₃ (55-65%). Clearly, the S₈ and FeS components arose from the injected SBS and microbial community analysis indicated that bacteria were likely involved in the formation of these scale components.

**Dendrogram and NMDS ordination of microbial community compositions**

PCR amplification and sequencing of 14 samples gave 22 amplicon libraries (Table 2). The number of sequences in each library following processing by the Phoenix 2 pipeline is indicated in Table 2. Their relatedness was determined through the generation of the amplicon library tree (Fig. 3) visualized with the UPGMA dendrogram program and NMDS ordination analysis (Fig. 4). Multiple amplicon libraries generated from the same sample always clustered together (Fig. 3), even with variations of DNA isolation methods (with or without bead beating) or PCR methods (two-step or single step amplification), as indicated in the Methods section. The tree also indicated that amplicon libraries from sessile, pipe-associated solids samples PAS-616P, PAS821LP and PAS-821TP, as well as from water sample PAW-821LP clustered separately from all other planktonic water samples (Fig. 3). The latter were all from pipe-sections experiencing rapid flow, except PAW-821LP, which was from a stagnant pipe-section. Apparently stagnancy led to a decreased difference between the planktonic and sessile community, causing PAW-821LP to cluster together with PAS-821LP (Fig. 3).
The AMOVA analysis of differences between amplicon libraries for samples from planktonic and sessile (biofilm) samples was restricted to samples of PAS and PAW (pipe-associated solids and pipe-associated water), which arrived in the lab in the same bucket. Planktonic water samples E1, E2 and E3 were excluded from this analysis. Hence we compared amplicon libraries 941, 940, 929, 930, 516, 1351 and 1718 for sessile PAS samples (Table 2) with libraries 939, 928, 1717, 515 and 514 for planktonic PAW samples. Alternatively, differences between amplicon libraries obtained for samples collected either upstream (516, 1351, 515 and 514) or downstream (941, 940, 929, 930, 928, 1718 and 1717) from the point of bisulfite injection were analyzed. The observed separation of amplicon libraries before and after SBS addition (Fig. 4) was statistically significant as determined by the AMOVA analysis (Table S2: p < 0.001). Hower, planktonic and biofilm communities had no significant differences in community structure and membership at the given sample size (Table S2: p = 0.116, 0.212). Planktonic community libraries from water samples E1, E2, or E3, collected at different times (T1: libraries 364 and 365; T2: 938, 927, 926, 937 and 925; T3: 1714, 1716 and 1715; as indicated in Table 1) were also compared (Fig. 4: blue points). Amplicon libraries from samples collected at T1 were more distant from those collected at T2 and T3 and their spatial separation was statistically significant, as tested with AMOVA (Table S2).

Microbial community composition of brackish waters

The grouping of pyrotags into taxons by BLAST searches of the SILVA database is shown for all amplicons in Table S1. Sequences for multiple amplicon libraries from the same sample were combined into a single taxon distribution for each of the 14 samples (Table 4). Amplicons from subgroup e (Fig. 3) contained a very high fraction of taxons of the genus *Pseudomonas* (70-97%), giving this subgroup a low species richness and evenness with a Shannon index (SHIN) of 0.34-1.69 and a Simpson index (SIM) of 0.42 to 0.90 (Table 2). Subgroup f contained 28-57% *Pseudomonas* spp. and 28-37% *Methanobacteriaceae*, increasing the evenness and richness of the community (Table 2: SHIN = 1.61-2.82). The taxon distributions of subgroups g, h and i were distinct (Fig. 3) and among the most even (Table 2: SHIN = 3.57-4.16). All three contained 1.8-3.4% *Pseudomonas*, but only subgroup g had a high fraction of *Methanobacteriaceae* (8.5%). This subgroup also had a high fraction...
of the methanogenic genera *Methanolobus* (11.3%) and *Methanocalculus* (12.9%). Subgroup h contained high fractions of *Methylphilaceae* (30%) and *Flavobacterium* (10.8%), whereas subgroup i had high *Anaerolinaceae* (11.3%), *Rhodocyclaceae* (10.1%) and *Streptococcus* (13%).

Overall the data indicated that the 10 planktonic samples of rapidly flowing water harbored diverse communities in which minimally 47 OTUs (26 taxa) and maximally 522 OTUs (179 taxa) were observed at the given sequence depth (Table 2).

### Microbial community composition of pipe wall samples

*Pseudomonas* spp., a dominant genus in amplicon libraries from planktonic water samples, were only a minor community component (0.02-0.67%) in amplicon libraries from sessile pipeline samples (Table 4: PAS-821LP, PAS-616P and PAS-821TP). We have used the sample codes to refer to these, as these can be directly related to their position in the brackish water production operation (Fig. 1). The pipe wall upstream from the SBS addition point (Table 4: PAS-616P) harbored a very high fraction of *Methanobacteriaceae* (56%), as well as of *Coriobacteriaceae* (13%) and *Desulfuromonas* (9.8%). The physiological function of the latter is unknown, because no sulfur is present in the pipe scale at 616P (Table 3). *Desulfuromonas acetoxidans* couples oxidation of acetate to the reduction of sulfur to sulfide. The pipewall downstream from the SBS addition point (Table 4: PAS-821TP) showed *Methanobacteriaceae* (41%), but also *Desulfocapsa* (35%) and *Desulfomicrobium* (18%) as major community components. *Desulfocapsa* derives energy for growth from the disproportionation of sulfur, thiosulfate or bisulfite to sulfide and sulfate. For bisulfite the reaction is:

\[
4\text{HSO}_3^- \rightarrow \text{HS}^- + 3\text{SO}_4^{2-} + 3\text{H}^+; \Delta G^\circ = -59 \text{kJ/mol of bisulfite} \quad [4]
\]

*Desulfocapsa sulfoexigens* is an autotroph, which requires only CO₂ as the carbon source (11). Some SRB, including *Desulfovibrio sulfodismutans* can also catalyze reaction [4], but in keeping with their status as incomplete oxidizers, these require acetate and CO₂ as the carbon source (1, 2). *Desulfurivibrio*, which was found in PAS-821TP at 0.3% of the reads (Table 4), can reduce sulfur and polysulfides with H₂ as the electron donor. The reactions reported to be catalyzed by these microorganisms do not lead to formation of elemental sulfur.
sulfur ($S^0$). The finding of 20-30% sulfur in the scale near 821TP (Table 2) suggests that bisulfite may also be disproportionated to sulfur and sulfate:

$$3\text{HSO}_3^- \rightarrow S^0 + 2\text{SO}_4^{2-} + \text{H}_2\text{O} + \text{H}^+; \Delta G^0 = -62 \text{ kJ/mol of bisulfite} \quad [5].$$

This reaction is not proven by our study and alternative explanations for the observed production of sulfur must be considered. The SRB *Desulfomicrobium* may use cathodic hydrogen to reduce sulfate to sulfide (reactions 1, 2), forming FeS and FeCO$_3$ of which 10-20% and 55-65% were present in the scale downstream of the SBS injection point (Table 2).

The community in PAS-821LP, which often experienced stagnant conditions, had a low fraction of *Methanobacteriaceae* (0.6%), while harboring high fractions of *Desulfomicrobium* (48%), and *Desulfocapsa* (22%), as well as some *Desulfurivibrio* (3.5%). Hence, this community was dominated by *Deltaproteobacteria* capable of reducing sulfate, sulfur or polysulfide, or of disproportionating sulfur and bisulfite. Overall, the results indicated a large community change as a result of the injection of SBS, with *Deltaproteobacteria* of the genera *Desulfocapsa* and *Desulfomicrobium* becoming major community components. Pipe sections subjected to rapid flow (821TP) appeared to maintain a high fraction of *Methanobacteriaceae* in the sessile population not seen in stagnant pipe sections (821LP).

### Contribution of methanogens to iron corrosion

In view of the high fraction of methanogens in the brackish waters and on the pipeline walls (Table 4), it was of interest to check whether these contribute to corrosion. Incubation of metal coupons with brackish waters E1-3, E2-3 or E3-3 gave methane evolution as shown in Fig. 5A. Production of 3-4 mM methane was observed in the headspace over a 16-week period. No methane production was seen in the MQ control (Fig. 5A), or in incubations of brackish waters without metal coupons (results not shown). The organic carbon content of brackish waters is only 0-10 $\mu$g/L (Table 1), which if converted into methane, would give rise to maximally 1 $\mu$M of methane. Hence, the methane formed in the incubations in Fig. 5A, resulted from using Fe$^0$ as electron donor for CO$_2$ reduction.

The corrosion rates observed by weight loss were low (Fig. 5B: 0.008-0.009 mm/year), although significantly higher than those observed for the MQ-control. The
average weight loss of the coupons used (Fig. 5C: E1, E2, E3) was 0.0188 g, which corresponds to a corroded iron concentration of 3.3 mM. This value is lower than the observed ferrous ion concentration of 4-6 mM (Fig. 4B), which in turn is lower that the maximal ferrous ion concentration of 6-10 mM expected based on the concentration of methane in the headspace (Fig. 4A: 2.4-4 mM). Precipitation of ferrous ions as iron carbonate and its incomplete removal during coupon cleaning, underestimating the actual weight loss, is a possible cause of these discrepancies.

Discussion

The pyrotag sequencing strategy used here to determine community compositions in pipelines transporting brackish waters has indicated that (i) these pipelines harbor a significant fraction of *Methanobacteriaceae*, which colonize the pipeline surface and contribute to corrosion and that (ii) injection of SBS gives rise to a significant change in the wall-attached microbial community to one which has a high fraction of the bisulfite-disproportionating genus *Desulfocapsa* and the SRB *Desulfomicrobium*. Although NMDS ordination analysis indicated that, overall, the biofilm and planktonic communities were not significantly different (Table S2), some genera showed a strongly biased distribution, e.g. *Pseudomonas* which was predominantly planktonic. Stevenson et al. (26) also reported that that sessile and planktonic communities in a flowing pipeline were different. Flow appeared to be important, as similar sessile and planktonic communities were observed under stagnant conditions (Fig. 3: subgroups a and b, as opposed to subgroups c and g or d and f).

We have shown that the *Methanobacteriaceae*, present in a substantial fraction on the pipeline surface in pipe section 616-P and 821-TP, likely contributed to the corrosion. The potential of methanogenic Archaea to use cathodic hydrogen to reduce CO$_2$ to methane was first demonstrated by Daniels et al. (7), who showed that a number of pure cultures of hydrogenotrophic methanogens were able to form methane from Fe$_0$ and CO$_2$. The strains tested included *Methanococcus thermolithotrophicus*, *Methanobacterium thermoautotrophicum* and *Methanobacterium bryantii*, *Methanosarcina barkeri*, and *Methanospirillum hungatei*. All were able to form methane from Fe$_0$ in anaerobic tubes with an N$_2$-CO$_2$ headspace. Because these strains were not preselected, it would seem that the
ability of methanogens to oxidize Fe\(^0\) is widespread. In contrast Dinh et al. (9) used enrichment techniques to isolate strain IM1, which was able to produce up to 4 mM CH\(_4\) from iron granules in 20 days, an activity comparable to that found here (Fig. 5A). Strain IM1 belonged to either the genus *Methanobrevibacter* or *Methanobacterium*. Likewise, Uchiyama et al. (27) isolated the corrosive methanogen *Methanococcus maripaludis* strain KA1 from an Fe\(^0\)-corroding enrichment culture, using a sample obtained from the bottom of a crude oil storage tank. Interestingly, the type strain *Methanococcus maripaludis* strain JJ1 was found not to be corrosive, indicating that this trait can be strain-dependent.

The effect of the oxygen scavenger bisulfite on the microbial community of a high-temperature (50 °C) wash tank in the Berkel oil field in the Netherlands was reported recently (28). The produced water, oil and gas were essentially free of sulfide and sulfate. Ammonium bisulfite (ABS), injected at 15 ppm, was the only input of inorganic sulfur into the system. A downstream wash tank had elevated levels of *Thermodesulfovibrio yellowstonii*, a thermophilic SRB not found in other field samples. Increased corrosion was observed downstream from the ABS injection point, which was ascribed to this SRB and caused the authors to question the utility of ABS as an oxygen-scavenging, corrosion-preventing agent. The wash tank had decreased levels of *Deltaproteobacteria*, indicating that these were unlikely to contribute to sulfite disproportionation and/or sulfite or sulfate reduction.

In the brackish water-transporting lines studied here, sodium bisulfite (SBS) was injected to maintain a residual concentration of 25 ppm downstream from the injection point. This led to a proliferation of *Deltaproteobacteria* disproportionating sulfite to sulfate and sulfide (equation 3), or sulfate and sulfur (equation 4). The latter reaction has not been described for known isolates (2, 11, 16), but is thermodynamically feasible and appears likely in view of the high fraction of sulfur in the scale downstream from the SBS injection point (Table 3). If the injected SBS reacted exclusively with O\(_2\), as intended, only sulfate would be formed (H\(^+\) + HSO\(_3^-\) + 1/2O\(_2\) → SO\(_4^{2-}\) + H\(_2\)O), which can then be used by SRB such as the *Desulfomicrobium* species found as a major community component in 821TP and 821LP. The high sulfur content in pipeline scale indicates the presence of excess SBS beyond what is required for O\(_2\) scavenging.

In conclusion, it appears that culture-independent methods to determine pipeline microbial community composition contribute to the understanding of which groups...
potentially participate in iron corrosion. These methods also indicate microbially-mediated
disproportionation of bisulfite, a process not previously described for this environment. The
collected information is thus both of fundamental and of practical interest

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subjected to nitrate injection decrease through formation of denitrifying biomass and


**Figure legends**

**Fig. 1.** Schematic of the brackish water gathering system. Water was collected through multiple pipelines, transporting $10^3$ m$^3$/day each from the Grand Rapids formation (E1) and the McMurray formation (E2). The co-mingled water (E3; $10^4$ m$^3$/day) was transported to the water treatment facility where sodium bisulfite (SBS) was added to scavenge oxygen. Samples of pipe-associated solids (PAS) were obtained from sections of failed pipe collected on November 12, 2017.
either upstream (616P) or downstream (821TP, 821LP) from the SBS addition point. The main direction of flow is indicated by the arrows; 821LP was often stagnant.

Fig. 2. Pipe sections analyzed in this study. (A) 616P, 6 and 8 cm are the inner diameter (ID); (B) Corrosion and scale on the inside pipe surface of 616P; (C) Close-up of pipe surface from a pipe section near 616P after removal of scale and cleaning; (D) 821LP, 22 cm is the outer diameter (OD); (E) Corrosion and scale on the inside pipe surface of 821LP; (F) Corrosion and scale on the inside pipe surface of 821TP.

Fig. 3. Amplicon library relational tree. The tree was generated using the UPGMA algorithm with the distance between communities calculated with the thetaYC coefficient in the Mothur software package. The tree was visualized with Dendroscope. Dendrogram subgroups (a) to (i) are indicated. Amplicon libraries from sessile PAS samples (a, c, d) treed separately from those for planktonic samples of fast-flowing water (e-i). Amplicon libraries for PAW-821LP (c), the single sample of stagnant water, treed with those for the PAS samples. Bar represents 0.01 substitutions per nucleotide position.

Fig. 4. NMDS ordination of amplicon libraries calculated using the majorization algorithm based on the thetaYC coefficient matrix. Each point in the plot represents a single amplicon library, indicated with its amplicon library code (Table 1). The distance between points represents the differences between these amplicon libraries. The red and green points represent amplicon libraries from sessile (PAS) and planktonic samples (PAW), respectively, which were shipped together. There was no clear separation between sessile and planktonic amplicon libraries. However, there was a significant community structure shift between amplicon libraries from samples collected before and after the point of SBS addition. The blue points represent amplicon libraries from planktonic water samples E1, E2, or E3 (Fig. 1), which were received on different times T1 (10/28/09), T2 (9/24/09) or T3 (1/27/10). Amplicon libraries from samples collected at T1 are more distant from those collected at T2 and T3.
Fig. 5. (A) Headspace methane and (B) ferrous iron formation by brackish waters incubated with iron coupons. (C) Corrosion rate of iron coupons as determined by metal weight loss. Data are averages for two separate incubations with two coupons each. Brackish water samples E1-3, E2-3 and E3-3 (Table 2) were used for the experiment.
Table 1. Concentrations (ppm) of selected analytes in brackish water wells. Data give the range observed for ten Grand Rapids and two McMurray wells and were provided by the pipeline-operator.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Grand Rapids</th>
<th>McMurray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate</td>
<td>390-560</td>
<td>1500-1600</td>
</tr>
<tr>
<td>Chloride</td>
<td>2500-3600</td>
<td>5200-5300</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0-1</td>
<td>2</td>
</tr>
<tr>
<td>Sulfide</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrate + nitrite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium</td>
<td>1700-2300</td>
<td>3500-3600</td>
</tr>
<tr>
<td>Calcium</td>
<td>12-24</td>
<td>30-40</td>
</tr>
<tr>
<td>Total iron</td>
<td>0.16-0.61</td>
<td>2.6-2.8</td>
</tr>
<tr>
<td>DOC(^1)</td>
<td>0-10</td>
<td>0-10</td>
</tr>
<tr>
<td>TOC(^1)</td>
<td>0-10</td>
<td>0-10</td>
</tr>
</tbody>
</table>

\(^1\) Dissolved and total organic carbon
Table 2. Samples processed during the study. Multiple entries for a sample were for amplicons obtained through different DNA isolation and/or PCR procedures as explained in the text. The multiplex identification sequence (MID) and the number of good 16S pyrotags obtained for each amplicon library (N) are indicated. The number of OTUs observed, the Shannon index (SHIN) and the Simpson index (SIM) and the number of derived taxons, describing microbial community diversity are also indicated. The data are ordered according to the dendrogram in Fig. 3.

<table>
<thead>
<tr>
<th>Date received</th>
<th>Description</th>
<th>Sample code</th>
<th>Amplicon code</th>
<th>MID</th>
<th>N</th>
<th>Grp</th>
<th>OTUs</th>
<th>SHIN</th>
<th>SIM</th>
<th>Taxons</th>
</tr>
</thead>
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<tr>
<td>10/28/09</td>
<td>Pipe-associated solids</td>
<td>PAS-821LP³</td>
<td>941</td>
<td>TACGCTGTCT</td>
<td>6251 a</td>
<td>174</td>
<td>2.16</td>
<td>0.29</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAS-821LP³</td>
<td>940</td>
<td>TACAGATCGT</td>
<td>5908 a</td>
<td>252</td>
<td>2.34</td>
<td>0.26</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>PAS-821LP³</td>
<td>929</td>
<td>ATATCGCGAG</td>
<td>4850 a</td>
<td>313</td>
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<td>0.28</td>
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<td></td>
<td></td>
<td>PAS-821LP³</td>
<td>930</td>
<td>CGTGTCTCTA</td>
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<td>0.2</td>
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<tr>
<td>10/28/09</td>
<td>Pipe-associated water</td>
<td>PAW-821LP</td>
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<td>TACACGTGAT</td>
<td>5318 b</td>
<td>212</td>
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<td>PAW-821LP</td>
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<td>ATCAGACACG</td>
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<td>8/13/2009</td>
<td>Pipe-associated solids</td>
<td>PAS-616P⁴</td>
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<td>AGACCGACTC</td>
<td>2329 c</td>
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<td>2.19</td>
<td>0.23</td>
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<td></td>
<td></td>
<td>PAS-616P⁴</td>
<td>1351</td>
<td>TAGTATCAGC</td>
<td>9785 c</td>
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<td>PAS-821TP⁵</td>
<td>1718</td>
<td>ATACGACGTA</td>
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<tr>
<td>9/24/09</td>
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<td>E2-2</td>
<td>938</td>
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<td>3796 e</td>
<td>47</td>
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<td>E2-2</td>
<td>926</td>
<td>ACCTCGACAC</td>
<td>4829 e</td>
<td>94</td>
<td>0.65</td>
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<tr>
<td>9/24/09</td>
<td>Comingled water</td>
<td>E3-2</td>
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<td>Grand Rapids water</td>
<td>E1-3</td>
<td>1714</td>
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<tr>
<td>9/24/09</td>
<td>Grand Rapids water</td>
<td>E1-2</td>
<td>937</td>
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<td>Pipe-associated water</td>
<td>PAW-821TP</td>
<td>1717</td>
<td>CGAGAGATAC</td>
<td>9488 f</td>
<td>354</td>
<td>2.82</td>
<td>0.16</td>
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<tr>
<td>1/27/10</td>
<td>Comingled water</td>
<td>E3-3</td>
<td>1716</td>
<td>CATAGTAGTG</td>
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<td>267</td>
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<td>McMurray water</td>
<td>E2-3</td>
<td>1715</td>
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<td>9396 f</td>
<td>187</td>
<td>1.61</td>
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<tr>
<td>8/13/2009</td>
<td>Pipe-associated water</td>
<td>PAW-616P</td>
<td>515</td>
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<td>2352 g</td>
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<td></td>
<td></td>
<td>PAW-616P</td>
<td>514</td>
<td>ACAGTGCGT</td>
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<td>352</td>
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<tr>
<td>9/7/09</td>
<td>Comingled water</td>
<td>E3-1</td>
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<td>TGATACGTCT</td>
<td>3211 h</td>
<td>250</td>
<td>3.57</td>
<td>0.06</td>
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</tr>
<tr>
<td>9/7/09</td>
<td>McMurray water</td>
<td>E2-1</td>
<td>364</td>
<td>TCTCTATCGC</td>
<td>6055 i</td>
<td>522</td>
<td>4.15</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) Dendrogram subgroup (see Fig. 3).
2) Number of taxons is the number of entries in Table S1 with at least one good read.
3) Pipe section 821LP had inner diameter ID = 20 cm, length L = 14.5 cm (Fig. 2C), internal surface area A = 910 cm² and internal volume V = 4553 cm³.
4) Pipe section 616P had ID = 6-8 cm, L = 21.5 cm (Fig. 2A), A = 473 cm² and V = 827 cm³.
5) Pipe section 821TP had ID = 17.2 cm, L = 38.7 cm (Fig. 2E), A = 810 cm² and V = 2376 cm³.
Table 3. Composition of pipewall scale upstream or downstream from the SBS injection point, as determined by XRD analysis.

<table>
<thead>
<tr>
<th>Mineral/compound</th>
<th>Chemical Formula</th>
<th>Upstream (%)</th>
<th>Downstream (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siderite</td>
<td>FeCO₃</td>
<td>85-95%</td>
<td>55-65%</td>
</tr>
<tr>
<td>Iron carbonate hydroxide</td>
<td>Fe₂(OH)₃CO₃</td>
<td>4-8%</td>
<td>0%</td>
</tr>
<tr>
<td>Quartz (sand)</td>
<td>SiO₂</td>
<td>4-8%</td>
<td>0%</td>
</tr>
<tr>
<td>Sulfur</td>
<td>S₈</td>
<td>0%</td>
<td>20-30%</td>
</tr>
<tr>
<td>Mackinawite</td>
<td>FeS</td>
<td>0%</td>
<td>10-20%</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe⁺</td>
<td>trace</td>
<td>0%</td>
</tr>
</tbody>
</table>
Table 4. Phylogenetic classification and distribution of pyrotags into taxons in combined amplicon libraries. All good reads N for multiple amplicon libraries from a single sample were combined, as indicated by the amplicon code(s). The fraction of reads (%) is indicated for each taxon. The sum of all reads is 137771. The total number of reads (N) and the sum fraction of reads (%), for each taxon are also indicated and were used to rank the table. Entries with an f(%) of 0 are not shown, but can be found in Table S1.

| Order | Family | Genus | Sample code | # of reads | 0.000 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 | 0.030 |
|-------|--------|-------|-------------|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|       |        |       | PAS-811LP   |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | PAS-616P   |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | PAS-321TP  |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E2-2       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E3-2       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E1-3       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E1-2       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E3-1       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E2-3       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | E3-3       |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Order | Family | Genus | Sample code | # of reads | 0.000 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 | 0.034 |
|-------|--------|-------|-------------|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|       |        |       | PAW-616P   |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | PAW-821LP  |            |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       |        |       | PAW-821TP  |            |       |       |       |       |       |       |       |       |       |       |       |       |       |

1Taxonomic classification of all reference sequences provided by Phoenix2 were checked with the SINA alignment service (22), the RDP classifier (29), and the green genes classification tool (8).