Identification and characterization of microbial biofilm communities associated with corroded oil pipeline surfaces

Tiffany R. Lenhart, Kathleen E. Duncan, Iwona B. Beech, Jan A. Sunner, Whitney Smith, Vincent Bonifay, Bernadette Biri and Joseph M. Suffita*

Department of Microbiology and Plant Biology, OU Biocorrosion Center, University of Oklahoma, Norman, OK, USA

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Microbiologically influenced corrosion (MIC) has long been implicated in the deterioration of carbon steel in oil and gas pipeline systems. The authors sought to identify and characterize sessile biofilm communities within a high-temperature oil production pipeline, and to compare the profiles of the biofilm community with those of the previously analyzed planktonic communities. Eubacterial and archaeal 16S rRNA sequences of DNA recovered from extracted pipeline pieces, termed ‘cookies,’ revealed the presence of thermophilic sulfidogenic anaerobes, as well as mesophilic aerobes. Electron microscopy and elemental analysis of cookies confirmed the presence of sessile cells and chemical constituents consistent with corrosive biofilms. Mass spectrometry of cookie acid washes identified putative hydrocarbon metabolites, while surface profiling revealed pitting and general corrosion damage. The results suggest that in an established closed system, the biofilm taxa are representative of the planktonic eubacterial and archaeal community, and that sampling and monitoring of the planktonic bacterial population can offer insight into biocorrosion activity. Additionally, hydrocarbon biodegradation is likely to sustain these communities. The importance of appropriate sample handling and storage procedures to oilfield MIC diagnostics is highlighted.

Keywords: microbially influenced corrosion; biocorrosion; petroleum microbiology; pipeline biofilm; hydrocarbon degradation

Introduction

Microbiologically influenced corrosion (MIC), also referred to as biocorrosion, is a process whereby the corrosion kinetic rate of a metallic material is accelerated by the metabolic activity of microorganisms (Beech & Sunner 2004; Beech et al. 2005). In their preferred state of growth, microorganisms can form aggregates of cells, or biofilms, adhered to a surface or substratum and often surrounded and protected by a matrix of extracellular polymeric substances (EPS) (Hall-Stoodley et al. 2004; Petrova & Sauer 2012). Biofilm EPS is composed of cellular metabolites, nucleic acids, and lipids, as well as proteins and polysaccharides (Branda et al. 2005; Flemming & Wingender 2010). Some of these components can facilitate electron transfer between the biofilm and the colonized metallic material (Jones & Amy 2002; Little & Ray 2002; Hamilton 2003), thus contributing to electrochemical interfacial reactions that can result in deterioration of metals and their alloys. The adverse effect of biofilm/surface interactions is of particular concern in petroleum pipelines, where the presence of biofilms has been implicated in metal loss and pipeline deterioration, eventually leading to structural failure and oil leakage (Kilbane et al. 2005). The US Department of Transportation Pipeline and Hazardous Materials Safety Administration (PHMSA) has reported a 20-year average (1994–2013) of 281 ‘Significant Pipeline Incidents’, with estimated damage totaling nearly six billion US dollars (Significant Incidents Summary Statistics 2014). While few incidents were diagnosed as resulting directly from corrosion damage, the fact that microorganisms have been implicated in recent pipeline failures (eg Prudhoe Bay, AK, 2006) (Brouwer et al. 2006) suggests that MIC may be a contributing factor. Detecting both the presence and the activity of biofilm microorganisms within pipeline networks is thus of importance when deciding if and when to implement corrosion mitigation strategies.

A variety of methods, including cultivation using enrichments, DNA sequencing, and metabolite analysis, have been employed for interrogating microbial communities within oil production facilities (Nazina et al. 2006; Li et al. 2007; Dahle et al. 2008; Duncan et al. 2009; Stevenson et al. 2011). These studies indicate that an abundance of physiologically different microorganisms survive in oil–water–gas environments. It is also apparent that the relative microbiological composition can vary considerably, depending on external factors such as temperature, water salinity, the type of corrosion inhibitors used, and if recirculation of injection waters occurs (Magot et al. 2000; Dahle et al. 2008; Duncan et al.

*Corresponding author. Email: jsuffita@ou.edu

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2009; Stevenson et al. 2011). Recently, the authors’ research group conducted a series of extensive molecular analyses on produced water and solid pipeline inspection gage (PIG) samples from a high-temperature oil production facility. Data from these analyses identified thermophilic microorganisms that are capable of biocorrosive activities owing to the formation of electrochemically aggressive metabolic products such as organic acids and H₂S and/or iron reduction/oxidation (Duncan et al. 2009; Stevenson et al. 2011). Production waters from this facility also contained micromolar quantities of alkane-fumarate addition products, known as alkylsuccinates, which are signature metabolites produced during the initial activation step of anaerobic hydrocarbon biodegradation (Wilkes et al. 2002; Davidova et al. 2005). Fatty acid metabolites resulting from further alkylsuccinate degradation were also observed (Grieg & Sulfiita 2005; Duncan et al. 2009). The detection of such diagnostic metabolites suggests that hydrocarbons in the oil production system are likely to serve as a carbon source and/or electron donors for microbial populations residing in the aqueous phase. To determine whether either hydrocarbons or the products of hydrocarbon metabolism would also support the proliferation of corrosive biofilm dwellers required analysis of the surface biofilms within this pipeline system.

The authors’ earlier studies identified microbial community and metabolite profiles in produced waters and PIG samples from the aforementioned high-temperature oil facility (Duncan et al. 2009; Stevenson et al. 2011). In the present study, the microbial community profiles from pipeline surface deposits were obtained to determine whether the planktonic (production water and PIG) pipeline community can be considered an accurate representation of the sessile surface-attached (biofilm) population. An additional goal of this study was to investigate the associations between pipeline biofilm microorganisms, hydrocarbon degradation, and biocorrosion. To this effect cross-sectional pipeline cuttings (referred to as ‘cookies’) were recovered from a corroded oil pipeline within the same oil production facility as in the authors’ previous investigations (Duncan et al. 2009; Stevenson et al. 2011). DNA profiling based on 16S rRNA phylogeny, electron microscopy and profilometry analyses of the cookies were undertaken. Mass spectrometry characterization of acid washes of the cookies was carried out to identify compounds indicative of microbial metabolism of hydrocarbons. The presence of the biofilm on the surfaces of the cookies was verified through field emission scanning electron microscopy imaging, and both eubacterial and archaeal 16S rRNA sequences were successfully recovered from the biofilms. The presence of metabolites associated with hydrocarbon biodegradation pathways was also confirmed. Light microscopy and surface profilometry of cleaned cookie surfaces demonstrated corrosion attack, including pitting, and the chemistry of surface deposits was that of a corrosive environment. With the exception of aerobic mesophilic eubacterial sequences amplified from ‘aged’ (stored) cookies, the DNA profiles of biofilms were generally representative of the previously analyzed PIG material and production water samples from the same facility (Duncan et al. 2009; Stevenson et al. 2011). It is therefore proposed that in well-established recirculating systems, the biofilm community and its impact on corrosion can be characterized through sampling and analysis of the bulk phase of the system.

Materials and methods

Location and sample description

Thirteen cross-sectional carbon-steel (CS) sample cuttings (cookies) were obtained from corrosion-damaged sections of pipelines in February 2008. The production facility is anaerobic, operates at temperatures >50°C, and includes seawater-reinjected processing waters. Specific environmental and geochemical conditions of the field location have been described previously (Masterson et al. 2001). The cookies were extracted from both pipelines and water injection headers. On location, cookies were placed in sterile Whirl-pak bags (Nasco, Fort Atkinson, WI, USA) and shipped under ambient conditions to the laboratory for analysis. The samples were given alphabetical designations A–M. A piece of mild CS, UNS standard steel grade 10180 was used as an unexposed control piece and assigned ‘N’. A description of the cookie samples and the types of analyses performed on each is provided in Table S1 (Supplementary material is available via a multimedia link on the online article webpage).

DNA isolation

DNA extraction was successfully performed on nine cookies (A, B, C, E, F, G, H, K, M). The eubacterial clone data derived from two cookies (A and K) were considered ‘well-preserved’, while from the other seven cookies (B, C, E, F, G, H, M) they were considered ‘aged.’ The term ‘well-preserved’ refers to DNA that was isolated either immediately upon cookie arrival (as with cookie K) or was isolated at a later date from a cookie that was stored at –80°C (as with cookie A). Both of these conditions are recommended to ensure recovery of endogenous DNA and minimize contamination with foreign DNA. All other cookies were maintained at ambient temperature prior to DNA isolation, and the DNA recovered from these cookies was considered ‘aged’.

The corrosion deposits on the surfaces of the pipeline cookies (the surfaces corresponding to the interior of the
pipeline which was in contact with the fluid flow) were removed by scraping with a sterile metal spatula into a sterile Petri dish in a UV-irradiated chamber. The resulting debris was transferred to sterile 1.5 ml plastic centrifuge tubes, and the dry weight of each sample was recorded. The surfaces of cookies from which deposits were removed were covered with 250 μl of DNAzol (Direct Molecular Research Center, Inc., Cincinnati, OH, USA) for 5 min. Following incubation, the DNAzol was removed from the cookie surface by pipette, transferred into a sterile 1.5 ml tube, and used for DNA extraction.

DNA extraction and cloning
DNA was recovered from the cookie scrapings and from the DNAzol samples separately using a PowerBiofilm DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer’s instructions. The resulting DNA eluates were concentrated by ethanol-salt precipitation. For each cookie, the scraping-derived DNA and the DNAzol-derived DNA were pooled prior to amplification. DNA samples were PCR-amplified using gene primers GM5F and 907R for 16S eubacterial rRNA (Santegoeds et al. 1999), and ARC333F and 958R for archaea (Reysenback & Pace 1995), and the resulting PCR products were inspected using agarose gel electrophoresis. Amplified products were cloned into vector pCR4.0 with the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA, USA) for 16S eubacterial rRNA (Santegoeds et al. 1999), and ARC333F and 958R for archaea (Reysenback & Pace 1995), and the resulting PCR products were inspected using agarose gel electrophoresis. Amplified products were cloned into vector pCR4.0 with the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA, USA), transformed into chemically competent E. coli cells, and grown overnight on agar plates supplemented with ampicillin for selection, and isopropyl β-D-1-thiogalactopyranoside and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside for blue-white color screening. In preparation for clone sequencing, individual transformants (white colonies) were cultivated in 96-well microtiter plates containing enrichment medium (LB broth supplemented with 10% glycerol and ampicillin). After growth for 24 h at 37°C, the cultures were sent to the Microgen laboratory for genomics and bioinformatics (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA) for plasmid isolation and subsequent clone sequencing using universal primers M13F and M13R.

16S rRNA sequence analysis
Vector-trimmed sequences were initially prepared in Sequencher (version 4.7, Gene Codes, Ann Arbor, MI, USA). All following eubacterial and archaeal sequence processing and data analysis were performed using the MOTHUR v.1.21.1 platform (Schloss et al. 2009). Trimmed eubacterial and archaeal rRNA sequences were aligned against the SILVA SEED reference databases (bacterial and archaeal, respectively) and screened for chimeras using UChime (Edgar et al. 2011). Putative chimeric sequences were further examined using Pintail (Ashelford et al. 2005). After removing chimeras, sequences were classified, and a column-formatted distance matrix was generated to produce operational taxonomic units (OTUs) at the 97% or higher level of similarity. The final OTU taxonomic data (2.8% dissimilarity cutoff) was utilized to prepare relative abundance graphs. Sequence similarity searches were performed using the NCBI nucleotide Basic Local Alignment Search Tool (BLASTN) by querying cookie FASTA sequence files against the nucleotide collection (nr/nt) search set. Representative bacterial and archaeal sequences were deposited in Genbank under accession numbers KF726875–KF726982, and KF726868–KF726874, respectively.

Cookie cleaning and metabolite sampling
Residual surface deposits and corrosion products that remained after DNA extraction were removed from the pipeline cookies using the ASTM Standard G1-03 corrosion cleaning procedure (ASTM Standard G1-03 2003). Cookies were sonicated and scrubbed with a beaker brush in a soapy water bath for 5 min. After rinsing with deionized water, the cookies were incubated in an acid bath containing 6.2 M HCl and 0.025 M hexamethylenetetramine (an iron corrosion inhibitor). The cookies were then consecutively rinsed with deionized water, acetone, and methanol, followed by N2 drying. Initially, five cookies (E, G, H, J, M) were incubated in the same acid bath, resulting in a combined acid wash. Subsequently, another five pipeline cookies (A, B, C, F, I) were individually acid-washed in 100-ml volumes, resulting in five individual acid baths that contained metabolites from the five cookies indicated above. Additionally, the control cookie N was washed in its own 100-ml acid bath to serve as a metabolite-free negative control. All acid baths (the combined wash, as well as six individual acid washes) were extracted and analyzed by high performance liquid chromatography and quadrupole time of flight (HPLC/QTOF) mass spectrometry (MS), described below, for the presence of bacterial metabolites indicative of hydrocarbon degradation.

Metabolite detection and analysis (solid phase extraction and HPLC/QTOF MS)
Solid phase extractions of the acid wash solutions were performed as follows. Oasis HLB solid-phase extraction cartridges (Waters Corp., Milford, MA, USA) were sequentially conditioned with 1 ml of hexane, 1 ml of ethyl acetate, 1 ml of methanol, and 1 ml of HPLC-grade water. Following conditioning, five ml of an acid wash sample were loaded onto the cartridge. The cartridge was rinsed with 2 ml of water to remove the iron ions. The
samples were then eluted with the following volumes of solvents: 2 ml of water, 2 ml of methanol, 1 ml of ethyl acetate, and 1 ml of hexane. The ethyl acetate fractions were evaporated to ~ 100 μl under nitrogen.

HPLC/QTOF MS of all wash samples was performed in both positive- and negative-ion mode using the following instruments and conditions. High performance LC was performed using an Agilent 1290 autosampler (7°C, 5 μl injection volume), an Agilent 1290 binary pump (0.4 ml min⁻¹ flow, 33 min run time, gradient elution using 100% acetonitrile as eluent A, and 10% acetonitrile/90% water as eluent B), and an Agilent 1290 thermostated column compartment [40°C, ZORBAX SB-C18 Column (2.1 × 100 mm, 1.8 μm)]. Mass spectrometry was performed using an Agilent G6538A QTOF detector with a dual ESI ion source. Negative-ion mode parameters included: dual ESI ion source, negative polarity, m/z 50–1,100 range, 4 GHz data acquisition rate, 1 spectrum s⁻¹ recording rate, 325°C gas temperature, 3,500 V capillary voltage, 160 V fragmentor voltage, and reference masses of m/z 112.985587, 805.985982, and 1,033.9881. Positive-ion mode parameters were the same as above, except for: positive polarity, m/z 50-1,000 range, and reference masses of m/z 121.050873, and 922.009798.

**Metabolic pathway analysis**

Initial raw mass spectrometry data were processed using IDEOM version 17 (Creek et al. 2012) workflow. IDEOM processing uses the XCMS Centwave (Tautenhahn et al. 2008) for the peak detection and mzMatch, R (Scheltema et al. 2011) for alignment of samples, filtering and storage of the data in peakML files. XCMS as well as mzMatch are scripts in the R environment (Team 2008). Detected features were matched against the IDEOM’s version of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolite database. After the identification of extracted features with database metabolites by IDEOM, the putative metabolites were matched with a metabolic pathway database. Pathos (Leader et al. 2011) is a freely available web-based facility that was used to analyze processed mass spectrometry data and graphically display the identified metabolites in the context of their metabolic pathways.

**Microscopic analyses of pipeline cookie biofilms**

A Zeiss Supra 55VP Field emission scanning electron microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with Energy Dispersive X-ray (EDX) was employed for the analysis of cookie L and the control cookie N at the Imaging and Chemical Analysis Laboratory (ICAL) (Montana State University, Bozeman, MT, USA). High-resolution biofilm images were obtained at a 3–5 mm working distance with the electron beam voltage of 1.0 kV while elemental analysis of specific biofilm regions and surface deposits was performed at 20 kV and 15 mm working distance. Cookies were imaged in as-received state, without any additional preparation.

**Surface scanning and pit evaluation**

Surface imaging of acid washed cookies was carried out using a Hixon Digital Microscope KH-7,700 with dual illumination revolver zoom lens (MXG-2500REZ), Z-axis step motor controller (CT-701), and XY stage (XY-G). Surface profilometry was also carried out using a Nanovea ST400 non-contact white light profilometer (Nanovea, Irvine, CA, USA). Hixon-derived images and pit depth profiles were acquired and displayed using proprietary Hixon software. Nanovea-derived height files and pit count analyses were analyzed using the MountainsMap6 software (Digital Surf, Besancon, France).

**Results and discussion**

**Imaging and EDX analysis**

Field emission scanning electron microscopy (FE-SEM) imaging of a surface of a representative cookie L revealed a dense biofilm, with cells embedded within amorphous deposits (Figure 1A). In contrast to the pipeline cookie, biofilms are not visible on the surface of the unexposed carbon steel control cookie N (Figure 1B). While iron and oxygen were dominant elements on surfaces of cookie N (Figure 1C, right panel), EDX spectra collected from discrete locations (spot analysis) on the pipeline cookie L also demonstrated the presence of carbon, calcium, magnesium, sulfur and phosphorus (Figure 1C, left panel). Thus EDX analysis indicates that the surface composition of the unexposed cookie N, which lacks detectable sulfur, calcium or carbon, is very different from the pipeline cookies. The elemental composition of surface deposits on the pipeline cookie L illustrates the accumulation of corrosive chemical species, in particular sulfur. Although the abiotic chemistry of the bulk fluid is likely to contribute to the presence of Ca, Mn and P in surface deposits, it is conceivable that accumulation of these elements occurs due to biotic mineralization processes governed by metabolic activity of biofilm organisms (Gadd 2010; Krause et al. 2012). Indeed, it is documented that both biofilm EPS and exocellular polyphosphate-rich globules released by bacteria under P-limited conditions have a very high affinity for binding divalent cations, in particular Ca²⁺ and Mg²⁺ (Aloisi et al. 2006).

The existence of biofilms within the oil transporting pipelines which are poor in water content is frequently disputed. The FE-SEM images provided in this
study demonstrate that dense biofilms are readily formed on oil-exposed pipeline surfaces. Clearly, the carbon sources required for microbial proliferation and biofilm formation are available and accessible in the petroleum-rich environment of the production facility.

**Eubacterial and archaeal community profiles**

As indicated in Materials and methods (above), the eubacterial clone data were derived from a total of nine cookies. Two cookies were considered well preserved (cookies A and K), and seven cookies (B, C, E, F, G, H, I, J, K).
and M) were considered aged. Phylogenetic analysis of the complete cookie clone sequence data identified 559 eubacterial 16S rRNA sequences, which were clustered into 108 OTUs (sequence similarity of 97% or higher), spanning 10 different bacterial phyla (Table S2 and Figure 2A).

Many of the sequences represent taxa which have previously been identified in petroleum recovery and transport systems that share similar physico-chemical properties (ie anoxic and thermophilic) (Slobodkin et al. 1999; Duncan et al. 2009; Magot et al. 2000; Stevenson et al. 2011). Some of the taxa include Clostridia, Thermodesulfobacteria, Deltaproteobacteria, Bacteroidetes, Synergistetes, and Thermotogae. Of these, the class Clostridia was the most abundant, encompassing 20% of the total sequences (Figure 2A). Most of the clostridial sequences, ~64%, have 99–100% identity to uncultured Thermacetogenium, Thermacetogenium phaeum and Thermoanaerobacter pseudethanolicus (Figure 2A, bar graph). T. phaeum represents the second-largest cookie OTU, containing 41 sequences. Uncultured Thermacetogenium comprises an additional OTU containing 14 sequences. T. phaeum is a strictly anaerobic thermophilic bacterium that oxidizes acetate syntrophically and can also perform homoacetate fermentation (Hattori et al. 2000; Oehler et al. 2012). T. pseudethanolicus is a thermophilic sulfidogenic archaeon that generates H2S from thiosulfate reduction (Zeikus et al. 1983).

The phylum Thermodesulfobacteria constitutes ~6% of the total cookie sequences and represents taxa that have also been found in similar oil pipeline environments (Li et al. 2007; Duncan et al. 2009; Stevenson et al. 2011). All of the Thermodesulfobacteria sequences have 99–100% identity to the sulfate- and thiosulfate-reducing bacterium Thermodesulfobacterium commune (Zeikus et al. 1983), which makes up the third-largest cookie OTU (35 sequences). The cookie sequences also confirm the presence of DNA from Deltaproteobacteria, the Synergistetes, and the Thermotogae with 98–99% identity to Desulfactinum subterraneum, Thermovirga lienii, Anaerobaculum thermoterrenum, and Kosmotoga olearia. Importantly, all of these species were originally isolated from oil production facilities (Rees et al. 1997; Rozanova et al. 2001; Dahle & Birkeland 2006; Dipippo et al. 2009).

In contrast to known indigenous oil reservoir microorganisms, the majority of the cookie eubacterial clone sequences (~60%) represent taxa that are not considered indigenous to petroleum reservoirs. The largest of this group, the Bacilli, make up 29% of the total cookie eubacterial clone sequences (Figure 2A). The Bacilli also represent the largest OTU (120 sequences), containing sequences that are 94% similar to sequences derived from members of the ubiquitous, aerobic and mesophilic genus Tumebacillus. Other cookie taxa which are generally not considered indigenous to oil reservoirs include the Actinobacteria, as well as the Alphaproteobacteria and Betaproteobacteria. These taxa are known to include aerobic soil dwellers, such as Ralstonia, or are members of the normal human microbiome, such as Propionibacterium (Fitz-Gibbon et al. 2013). Additionally, although some members of the phylum Bacteroidetes are known to be strict anaerobes (Chen & Dong 2005), most of the cookie sequences were found to be from the class Sphingobacteria, which includes aerobic species isolated from the environment and from clinical specimens (Sorensen et al. 2005). Aerobic, mesophilic sequences in hydrocarbon reservoirs and hydrocarbon-enriched environments have been noted previously (An et al. 2013; Berdugo-Clavijo & Gieg 2014), although it is still unclear if aerobic bacteria are indigenous populations within these systems. In the facility under investigation, the presence of cookie sequences reflecting mesophilic aerobes may also suggest some level of soil contamination. Indeed, many of the Bacilli are spore-formers,
can withstand transport and survival in ambient conditions. As such, detection of ubiquitous aerobic 16S rRNA sequences highlights the importance of sample handling and/or storage conditions during field investigations.

While bacterial sequences were obtained from all cookies, successful archaeal sequence amplification, resulting in 40 sequences, was only retrieved from one of the nine cookies (cookie K) (Table S2). All sequences represented the phylum Euryarchaeota, which was distributed among five classes: the methane-producing Methanococci, Methanomicrobia, and Methanobacteria, and the H2S-producing Archaeoglobi and Thermococci (Figure 2B). These classes of Euryarchaeota have been identified previously in high temperature oil reservoirs, including in the production waters and PIG samples from the present facility (Dahle et al. 2008; Duncan et al. 2009; Li et al. 2007).

**Comparison of cookie sequence diversity and cookie state of preservation**

Clone sequence numbers varied widely between cookies, making comparisons challenging (Figure S1). However, a generally positive correlation was observed between clone number and sequence diversity (OTU number) for each cookie (Figure S2). The DNA yield for some cookies was comparatively low, offering a small number of sequences and low phylogenetic diversity. In contrast, other cookies yielded higher clone numbers and higher phylogenetic diversity (Figures S1 and S2). A positive correlation was noted between increased cookie sequence diversity and (good) cookie preservation condition. DNA from well-preserved cookies A and K offered both the highest sequence numbers and the greatest diversity (Figure 3A and Figure S1). Importantly, there was also an obvious difference in the distribution of eubacterial taxa between DNA from the well-preserve and from the aged cookies (Figure 3B). The enrichment of Bacilli sequences from the aged cookie-derived DNA is especially evident, with aged cookie-derived DNA resulting in nearly half of the total clone data, compared with ~12% of the fresh cookie-derived DNA (Figure 3B). Overall, fewer than half of the clones (~45%) from well-preserved cookie DNA matched aerobic, mesophilic sequences belonging to the taxa Bacilli, Actinobacteria, as well as Alphaproteobacteria and Betaproteobacteria (Figure 3C). However, these sequences were dominant in DNA retrieved from aged cookies, accounting for ~73% of the total aged cookie sequences (Figure 3C). It is noteworthy that archaeal DNA was isolated only from a well-preserved cookie (K).

**H2S-producing taxa**

Since all cookie sequences were obtained from corroded oil pipelines, of particular interest was the identification of microorganisms implicated in corrosion, especially H2S generating prokaryotes. Sulfate-reducing microorganisms
have routinely been monitored at oil production facilities. Indeed, cookie 16S rRNA clone data indicate a strong presence of sulfate-reducing bacteria (SRBs), including Thermacetogenium, Thermodesulfbacteria, and Desulfacinum, which are indigenous to oil production facilities. However, many anaerobic microorganisms can generate H2S from reduction of electron acceptors other than sulfate. Cookie 16S eubacterial and archaeal rRNA clone data match sequences of microorganisms which are known to utilize a variety of H2S production methods (Table 1). One of the identified Clostridia, Thermoanaerobacter pseudethanolicus, can reduce thiosulfate, and the Synergistetes (Thermovirga lienii, Anaerobaculum thermoterenrenum, and Anaerobaculum hydrogeniformans OS1) are capable of reducing cysteine and elemental sulfur, in addition to thiosulfate (Rees et al. 1997; Dahle and Birkeland 2006; Maune & Tanner 2012). Cookie archaeal sequences include those with 100% identity to Archaeoglobus fulgidus, an organism that reduces both sulfate and thiosulfate (Stetter et al. 1987), as well as Thermococcus litoralis, which reduces only elemental sulfur (Neuner et al. 1990). In oilfield environments mitigation efforts are targeted mainly at SRB, and it is less common to monitor and recognize other H2S-producing microorganisms that can reduce sulfur compounds other than sulfate. The present study demonstrates that when considering MIC in oilfield facilities, it is imperative to investigate the presence of all sulfidogenic microorganisms, and not to focus solely on SRBs.

Comparison of microbial communities from cookie, PIG, and produced water samples

In addition to pipeline cookies, the authors’ research group previously obtained and analyzed liquid produced water samples (PW), as well as debris retrieved from pipeline cleaning operations (PIG samples) from the same sampling event at this oil production facility (Duncan et al. 2009; Stevenson et al. 2011). Thus one of the major interests during the present investigation was to qualitatively compare DNA sequence profiles of the above samples to determine whether the planktonic pipeline community and the surface-associated microorganisms are comparable. Overall, the pipeline cookie taxa are very similar to those identified in both the PW and PIG samples, with a few exceptions. In total, nine of the 10 ‘core’ taxa found in the PW samples were represented in the cookie eubacterial sequences (Duncan et al. 2009), including the second- and third-largest OTUs, which are represented by T. phaeum and T. commune, respectively. Similarly, the PIG bacterial community was also dominated by the thermophilic Clostridia, Thermoanaerobacter and Thermacetogenium. There are, however, notable differences between the cookie eubacterial DNA sequences and those from the PW and PIG samples. One of these differences is found with the Synergistetes T. lienii, which is a dominant representative of both the PW and PIG samples, encompassing ~30% of the total PIG clone and pyrosequencing libraries (Stevenson et al. 2011), and ~70% of the total ‘core’ PW clone sequences (Duncan et al. 2009). In contrast, the cookie survey identified only one T. lienii rRNA sequence (Table S2). The reasons for this discrepancy in the abundance of Thermovirga sequences between the three different communities is unclear and several factors are likely to contribute. Since several produced water and PIG surveys have identified Thermovirga sequences (Dahle & Birkeland 2006; Dahle et al. 2008; Duncan et al. 2009; Pham et al. 2009; Stevenson et al. 2011), it is possible that Thermovirga proliferates successfully in planktonic pipeline communities, but is not a major player in the surface-localized biofilm communities. This suggestion would require further investigation into the ability of T. lienii to thrive within sessile populations under the prevailing conditions, and likely warrants such a study. Alternatively, the discrepancy could also be due to the

Table 1. 16S rRNA sequences representing H2S-producing taxa identified on pipeline cookie surfaces.

| Taxa                  | Representative sequence                      | H2S production method*                      | Sequences |
|-----------------------|---------------------------------------------|---------------------------------------------|-----------|
| **Eubacteria**        |                                             |                                             |           |
| Clostridia            | Thermoanaerobacter pseudethanolicus         | Thiosulfate reduction                       | 15        |
|                       | Garciella nitratireducens                   | Thiosulfate reduction                       | 1         |
| Deltaproteobacteria   | Desulfacinum subterraneum                   | Sulfate reduction                           | 9         |
| Thermodesulfbacteria  | Thermodesulfobacterium commune              | Sulfate reduction                           | 35        |
| Synergistetes         | Thermovirga lienii                          | Cysteine/S0 reduction                       | 1         |
|                       | Anaerobaculum thermoterenrenum, Anaerobaculum hydrogeniformans | Thiosulfate/cysteine/S0 reduction | 1         |
| Thermatogae           | Kosmotoga olearia                          | ND; possibly cysteine reduction             | 2         |
| **Archaea**           |                                             |                                             |           |
| Archaeoglobi          | Archaeoglobus fulgidus                      | Sulfate, thiosulfate reduction              | 7         |
| Thermococci           | Thermococcus litoralis                      | S0 reduction                                | 2         |

*H2S production methods listed for representative sequences of uncultured taxa are presumed.
inability of T. lienii DNA to withstand cookie extraction and transport from the field, resulting in damaged or degraded DNA upon laboratory arrival. It is also possible that the Thermovirga cells were damaged by the cookie extraction procedure itself, which likely subjected the pipeline surface to extreme heat. A molecular survey of pipeline cookies from other high-temperature oil facilities would be required to determine whether this is a true difference between cookies and the planktonic community, or a phenomenon specific to the present study. Note that in another study, Oldham et al. (2012) found that two additional PIG envelope samples from produced water pipelines from the same oil facility differed markedly in the relative abundance of sequences classified as Thermovirga. Approximately 50% of sequences from PIG Sample B were classified as Thermovirga, while <10% of sequences from PIG Sample A were so classified (Oldham et al. 2012). Therefore, if PIG envelope scrapings are representative of pipeline biofilm communities, Oldham et al. (2012) demonstrate that there can be substantial differences between biofilm communities even within the same facility, and that parallel samples of production fluids and biofilm communities must be obtained from the same location at the same time in order to draw conclusions about their degree of similarity.

The other notable difference between cookie, PIG, and PW bacterial community profiles is the large percentage of mesophilic aerobic sequences, such as the Bacilli, Alphaproteobacteria and Betaproteobacteria, as well as the Actinobacteria, that were identified in the cookie survey. In contrast, these signals were only marginally present on the PIG and PW libraries. As indicated previously, it is likely that these signals are a result of cookie sample handling in the field, as suggested by the presence of DNA sequences ubiquitous to soil and the human microbiome, as well as laboratory sample storage conditions (ie ambient temperature). There was an obvious potential for contamination when samples were handled during pipeline cutting, prior to their placement in sterile sampling bags. Due to the nature of the specimens and their close association with the soil, it may be difficult to exclude such sequences from detection, despite careful sample handling and storage. However, extraction of inspected and damaged pipeline surfaces, a procedure which some oil companies execute as part of regular maintenance, is necessary for assessing MIC. Thus it is worthwhile considering modified and improved field sampling and storage techniques when future cookie samples are collected.

A strong similarity was noted between cookie, PIG, and PW archaeal sequence profiles. Owing to differences in sample size, comparison between cookie, PIG and PW archaeal clone sequences presented a challenge. While representatives from all five archaeal classes of cookie sequences were identified in the PW sequence libraries (Duncan et al. 2009), sequences from the PIG samples all fell into the two most abundant cookie-associated archaeal classes, Methanomicrobia and the Methanobacteria (Stevenson et al. 2011). It is unclear why cookie DNA amplification resulted in just 40 archaeal sequences, although it is possible that either low DNA integrity (due to lack of ideal sample handling/storage conditions) or low overall DNA yield led to minimal archaeal 16S rRNA gene amplification.

**Corrosion assessment with light microscopy and profilometry imaging**

Representative examples of cleaned, scanned cookie surfaces revealed evidence of extensive pitting corrosion (Figure 4A and B). Pits of different depths and morphologies were observed on different cookies. For example, representative pits on cookie E were ~104 µm and 150 µm deep (Figure 4A top and bottom panels, respectively), while the depth of pits imaged on cookie M reached nearly 280 µm (Figure 4B). In general, surface

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Three-dimensional imaging of acid-cleaned pipeline cookie surfaces. (A) Images and depth profiles of representative pits from Cookie E were prepared by 3D light microscopy (top images) and surface scanning profilometry (bottom panels). (B) Images and depth profiles of representative pits from Cookie M were prepared by 3D light microscopy (top images) and surface scanning profilometry (bottom panels).
profilometry of each cookie identified between 20 and 288 total pits of at least 50 μm width and 50 μm depth (Table 2). Profilometry of the unexposed control sample identified only one pit matching these criteria. All cookies were recovered from corroded regions of the production pipeline and the imaging confirmed that localized attack occurred in this part of the system.

**Metabolite profiling and analysis of cookie acid washes**

The initial interest in metabolite profiling of the cookie samples was to look for the presence of anaerobic hydrocarbon degradation metabolites, based on the knowledge that hydrocarbons in fuel can provide a suitable carbon source for microbial degradation (Davidova & Sufita 2005). Previously, a series of n-alkane biodegradation metabolites (methyl- to butyl-succinates) had indeed been detected by GC/MS in samples from the same production facility (Duncan et al. 2009).

Acid wash samples from cookies A, B, C, E, F, G, H, I, J, and M were analyzed by HPLC/QTOF MS. More than 5,000 compounds were detected from the samples and, among those, oxygenated chemical features with two to four oxygen atoms were the most abundant. Compounds containing two oxygen atoms included saturated, mono-unsaturated, and di-unsaturated fatty acids. Such fatty acids are anaerobic n-alkane degradation metabolites (Davidova et al. 2005), but they are also ubiquitous in natural environments as well as frequently reported contaminants in HPLC/MS systems.

A careful search was performed for alkylsuccinates, by comparing HPLC retention times with standard succinates and by performing tandem MS experiments on putative succinates. This is because these compounds are diagnostic metabolites associated with anaerobic hydrocarbon decay. However, no evidence for the presence of alkylsuccinates was obtained.

In addition to the oxygenated chemical features with two to three double bonds, there was a strong presence of a variety of oxygenated aromatic compounds. Among these, compounds with three and four oxygen atoms and a double bond equivalence of six were particularly abundant. These are substituted benzenes with two additional double bonds, consistent with compounds such as dicarboxylic acids or a combination of a carboxylic acid and a keto group. A variety of lower-abundance oxidized naphthalene, anthracene/phenanthrene and fluorene compounds, some of which were partially hydrogenated, was also observed. Indeed, by matching the data with a metabolic pathway database, many of the metabolites are known to be associated with the biodegradation pathways of the parent hydrocarbons (Table 3).

The metabolic profiling of the cookie washes revealed extensive degradation and oxidation processes of both saturated and aromatic hydrocarbons. The fact that signature anaerobic hydrocarbon degradation metabolites, such as succinates or malonates, were not detected does not necessarily imply that anaerobic degradation has not taken place. The overall reaction scheme of oxygenated hydrocarbons in the pipeline environment is likely to be quite complex, involving hundreds of major reactions and chemical constituents and little is presently known about this chemistry. In particular, it may well be that the succinates and malonates were metabolized or had undergone chemical changes during the history and processing of the samples.

The metabolic profiling data in this work demonstrate that the metabolomic approach holds great promise for the study and diagnostics of local chemical and microbial environments in oilfield systems. However, it is also apparent that much remains to be learned before the method can reach its full potential.

In addition to metabolic investigation, cookie acid washes were also analyzed for DNA, and PCR amplification procedures were attempted on these samples. Although nanomolar concentrations of DNA were detected in the acid washes, 16S eubacterial rRNA PCR amplification was unsuccessful (data not shown). These results suggest that the DNA, although present, was either damaged during acidification, or that it contained inhibitory compounds, and was not PCR-amplifiable. Other methods of biofilm recovery and preparation, which may better preserve both biofilm metabolites and DNA, are being explored.

| Cookie | Total pit count* |
|--------|-----------------|
| N      | 1               |
| A      | 20              |
| B      | 17              |
| C      | 130             |
| E      | 137             |
| G      | 220             |
| H      | 44              |
| M      | 288             |

*Pits were imaged over a 167 mm² area and the pit parameters are defined as 50 μm in diameter and depth.

| Metabolic pathway                          | Metabolites detected* |
|--------------------------------------------|-----------------------|
| Naphthalene and anthracene degradation      | 17/60                 |
| Methyl-naphthalene degradation             | 10/64                 |
| Fluorene degradation                       | 9/36                  |
| Toluene and xylene degradation             | 9/37                  |

*Cookie metabolites detected/total number of metabolites in pathway.
**Cookie biofilm community composition and pipeline corrosion**

It was of considerable interest to discern which bacteria and/or archaea would reside within biofilms on the pipeline surfaces, as these are considered to play a key role in MIC. Likewise, metabolite analysis was conducted to determine whether the biofilm microorganisms were utilizing petroleum hydrocarbons as a carbon source. Based on DNA sequences and metabolomic data, it is unclear whether anaerobic \( n \)-alkane degradation is a dominant mechanism of carbon substrate utilization for the biofilm microorganisms within this facility. As indicated previously, alkyllsuccinate fumarate addition products (Gieg & Suffita 2005) and/or a homologous series of fatty acid metabolites were not detected in the cookie acid washes. In contrast, MS profiling of the PW samples identified micromolar concentrations of low molecular weight (C1–C4) alkyllsuccinates, as well as the expected metabolites resulting from carbon skeleton rearrangement and decarboxylation of the parent \( n \)-alkanes (Davidova et al. 2005; Duncan et al. 2009). These data suggest that the capability of \( n \)-alkane biodegradation, although present in this production facility, may not occur within sessile communities and that the degradation of the \( n \)-alkanes is carried out by planktonic organisms. In this case, it is likely that the fatty acids produced from planktonic-phase hydrocarbon biodegradation would be utilized by the biofilm dwellers. Indeed, 16S rRNA sequence and metabolic analyses from this and other studies provide support for such a scenario. The major anaerobic microbial cookie DNA sequences were found to be associated with the thermophilic syntroph, *Thermacetogenium*, and the \( \text{H}_2\text{S} \)-producing *Thermodesulfobacterium commune*. *T. commune* can incompletely oxidize fatty acids to produce acetate (Henry et al. 1994), while the physiologically diverse bacterium *T. phaeum* can oxidize acetate in co-culture with a hydrogenotrophic methanogen (Hattori et al. 2000; Oehler et al. 2012). Sequences of rRNA matching those of the hydrogenotrophic methanogen *Methanothermobacter* were also identified in the archaeal clone library. Theoretically then, biofilm-dwelling *T. commune* may utilize fatty acids produced by the planktonic community, and the acetate resulting from incomplete fatty acid degradation may be oxidized by *T. phaeum* in the presence of the methanogen. As a product of fatty acid degradation, acetate is likely to be readily available in this system, and it has been detected in other petroleum reservoirs (Barth 1991; Mayumi et al. 2011). Previous metabolic analysis from PW samples, as well as analysis of cookie acid washes from the current study, demonstrated the presence of various fatty acids in this pipeline facility (Duncan et al. 2009). Thus the biofilm community may have access to a constant supply of carbon sources related to hydrocarbon degradation by the population residing in the planktonic phase. The development of these anaerobic biofilms may then result in increased pitting and pipeline corrosion, due to production of \( \text{H}_2\text{S} \), as well as by organic acid production and its concentration within the biofilm matrix. It is possible, however, that alternative explanations exist for the lack of anaerobic \( n \)-alkane degradation metabolites observed in the surface biofilm. Conceivably, these metabolites were originally present within the biofilms, but were not retained there. The alkane degradation metabolites may have instead preferentially partitioned to the water phase, escaping detection on the cookie surface. The inability to detect those metabolic products in surface deposits does not preclude the likelihood that anaerobic \( n \)-alkane degradation contributes to the metabolic and potential biocorrosive activity of the biofilm population.

Irrespective of the mechanism involved, FE-SEM imaging and surface profilometry confirm, respectively, that prokaryotic biofilms are present on the pipeline surfaces and that pitting of the surface occurs under such biofilms. Furthermore, DNA analysis reveals the presence of sequences representative of organisms implicated in pitting corrosion of carbon steel, suggesting that MIC contributes to localized corrosion of piping material in this installation.

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