Title: Anaerobic Hydrocarbon Biodegradation and Biocorrosion of Carbon Steel in Marine Environments: The Impact of Different Ultra Low Sulfur Diesels and Bioaugmentation

Authors: Deniz F. Aktas^{a,b}, Kathryn R. Sorrell^{a,b}, Kathleen E. Duncan^{a,b}, Boris Wawrik^a, Amy V. Callaghan^a and Joseph M. Suflita^{a,b,*}

Affiliations: ^aDepartment of Microbiology and Plant Biology, 770 Van Vleet Oval, Norman, Oklahoma, 73019

^bInstitute for Energy and the Environment, University of Oklahoma, 100 E. Boyd Street., Norman, Oklahoma, 73019

Corresponding author: *University of Oklahoma, Department of Microbiology and Plant Biology, 770 Van Vleet Oval, Norman, Oklahoma, 73019, USA; Phone: +1(405) 325-3771; Email: jsuflita@ou.edu.

Coauthor emails: DFA - deniz.f.aktas@gmail.com, KRS - katy.r.brown@gmail.com, KED - Kathleen.E.Duncan-1@ou.edu, BW - bwawrik@ou.edu, AVC - acallaghan@ou.edu, JMS – jsuflita@ou.edu

Abstract

The anaerobic biodegradation of ultra low sulfur diesels (ULSDs) by marine microbial communities was examined, along with the relationship of this metabolism to carbon steel biocorrosion. Fuel analysis revealed that the ULSDs differed based on the ratio of low to high molecular weight (LMW/HMW) *n*-alkanes. *Desulfoglaeba alkanexedens*, a sulfate-reducing bacterium capable of degrading LMW *n*-alkanes, was used as a positive control and to explore the impact of bioaugmentation. Metagenomic analysis was conducted to determine the genetic potential for anaerobic biodegradation of a range of hydrocarbons. Initial sulfate reduction rates were faster in incubations amended with ULSDs containing a higher ratio of LMW/HMW *n*-alkanes, but total sulfate loss was similar for all fuels. Sulfate removal in bioaugmented incubations exceeded stoichiometric expectations calculated for the mineralization of all paraffins. In combination with metagenomic analysis, these data confirmed that other microorganisms utilized hydrocarbons beyond the range exhibited by *D. alkanexedens*. A positive correlation between coupon weight loss and sulfate loss was observed, and pitting corrosion was more extensive in non-sterile incubations compared to sterile controls. This study demonstrates that while ULSDs vary in their susceptibility to early stage biodegradation, compositional differences were less important for overall fuel

deterioration and steel biocorrosion patterns. Further, bioaugmentation stimulated other hydrocarbonclastic marine microorganisms.

Keywords: biocorrosion, ultra low sulfur diesel, fuel biodegradation, bioaugmentation, metagenomics, qPCR

1. Introduction

Diesel fuel is a global energy resource used primarily for heating and transportation. The U.S. alone is projected to use 600 million liters of diesel per day in 2016 and forecasts indicate that consumption will increase by over 111 million liters/d through 2040 (Anonymous, 2016). Almost all commercial trucks, most farm and construction equipment, and all freight locomotives are powered by diesel engines. Diesel is also important as a marine fuel. Commercial shipping with diesel vessels accounts for approximately 90% of all world trade (Kaluza et al. 2010). Military vessels are powered with marine diesel, and many use seawater-compensated fuel ballast tanks wherein seawater is used to replace the volume and mass loss of fuel as they are consumed in the ship engines (Lyles et al. 2013; Suflita et al., 2014).

Environmentally, diesel tends to be preferred over other fuel options because it is more energy dense and delivers better fuel economy. However, diesel combustion releases particulates and sulfur emissions to the atmosphere, and unintentional spills occur. In 1996, the U.S. EPA mandated that the sulfur content of diesel be lowered to <15 ppm to produce ultra low sulfur diesel (ULSD) (Kilbane 2006; Song 2003), and full transition to this fuel for highway and off-road vehicles was completed in 2014. Along the North American coastline, sulfur emission control areas are in place, and more than 90% of maritime ships comply with increasingly stringent sulfur fuel standards (Kattner et al. 2015). While decreased sulfur emission is linked with such regulatory mandates, the level of societal reliance on diesel inevitably results in accidental releases. For instance, fuel spills in U.S. waterways related to shipping totaled over 32 million liters between 1990 and 2011 (Anonymous, 2013; Ramseur 2012). Releases to soil and groundwater are well established sources of environmental contamination (Prince and Douglas, 2010). Thus, the environmental consequences associated with widespread diesel reliance are more complex than those associated with atmospheric emissions alone.

In addition, the environmental release of fuels is sometimes associated with the biocorrosion of the carbon steel infrastructure. Fuels that biodegrade too easily serve as carbon and energy sources for the proliferation of microbial communities that ultimately exacerbate biocorrosion through-wall pitting corrosion of pipelines, tanks and other equipment (Aktas et al. 2010, Lenhart et al. 2014, Liang et al. 2016a). Concomitant with the introduction of ULSD were increased reports of infrastructure corrosion (Anonymous, 2012). The essential question is why should diesel reformulated for reduced organosulfur content be inherently more corrosive? The removal of organosulfur compounds from the fuel could conceivably impact its antimicrobial character rendering the resulting formulation more susceptible to microbial attack. It is clear that many organosulfur compounds have important antimicrobial properties (Heldreth and Turos 2005). However, Lyles et al. (2013) examined the effect of the varying sulfur content on fuel

91 stability in marine systems and found that the sulfur content of diesel sampled during
92 progressive stages of oil refining did not significantly influence the rate of anaerobic
93 hydrocarbon biodegradation or sulfate metabolism.

94 Gaylarde et al. (1999) report that one of the major issues in the oil industry is the
95 microbial contamination of stored fuels leading to deterioration in fuel quality and
96 compromise of the fueling infrastructure. A study of the microorganisms in several
97 ULSDs (including one fuel used in this study) revealed that they were classified as
98 obligate or facultative aerobes and not taxa that are generally associated with metal
99 biocorrosion (Suflita et al. 2012). In that study, it was concluded that the bacterial load in
100 those fuels was unlikely to substantively contribute to subsequent biocorrosion problems.

101 Fuel formulations were also changed when environmental regulations in several
102 countries mandated the blending of fatty acid methyl esters (FAME) with petrodiesel in
103 an effort to extend fuel supplies and reduce the net carbon footprint. However, several
104 studies found that these fuel components were labile under both aerobic (Prince et al.
105 2008) and anaerobic (Aktas et al. 2010) conditions. The latter found that FAME
106 biodegradation exacerbated sulfate loss from marine incubations as well as the deposition
107 of reduced sulfides, fatty acid production and metal biocorrosion. Similarly, Fazal et al.
108 (2010) reported that biodiesel was more corrosive on engine parts made of aluminum,
109 copper, and stainless steel relative to petrodiesel. A subsequent study showed that
110 increasing concentrations of rapeseed methyl ester in an ULSD blend exacerbated the
111 corrosion of aluminum and copper (Norouzi et al., 2012). Still, other investigators
112 suggested that the aerobic bioconversion of contaminating ethanol to acetate was
113 responsible for the increase in corrosion in fueling systems storing and dispensing ULSD
114 (Anonymous, 2012).

115 It seems clear that the accidental or intentional introduction of labile components
116 into fuels can impact the integrity of the carbon steel infrastructure. However, this study
117 specifically focuses on the ULSDs themselves, rather than the impact of components
118 either added or removed from the fuels. We hypothesized that the susceptibility of
119 ULSDs to biodegradation was likely a function of hydrocarbon compositional differences
120 and that the more labile fuels would differentially impact biocorrosion. To test this
121 prospect, four different ULSDs were added to incubations containing Gulf of Mexico
122 seawater and sediment. When appropriate, a 1018 carbon steel coupon was incorporated
123 in the assay as well as a known hydrocarbon-degrading, sulfate-reducing, positive control
124 bacterium. Our results indicated that ULSDs possessing a greater proportion of low
125 molecular weight *n*-alkanes proved to be more labile in the presence of a positive control
126 bacterium able to metabolize this group of constituents. However, such differences were
127 less significant in longer-term incubations. Remarkably, the bioaugmented bacterium had
128 a stimulatory impact on other hydrocarbonoclastic marine anaerobes that effectively
129 resulted in an increased extent of sulfate utilization and accelerated biocorrosion.

2. Materials and Methods

2.1 Marine Incubations

Four ULSDs were tested, including one from a refinery (ULSD 1), one from the U.S. Navy (ULSD 2) and two from separate local fueling stations (ULSDs 3 and 4). Coastal seawater and sediment were obtained in January 2013 from the north side of West Ship Island, a barrier island off the Mississippi coast (latitude 30°13'275"; longitude 88°57'243"), and used as both inoculum and growth medium. Sediment was collected by wading into ~0.5 m water depth to scoop sediment into wide mouth high-density polyethylene containers (4 liter) until about 75% full. Seawater was used to fill the remainder of the containers to capacity. Environmental samples were cooled, shipped to the laboratory, and stored refrigerated until use. Anaerobic incubations were prepared in 160-ml serum bottles according to the protocol of Liang and Suflita (2015). The incubations contained 30 ml of seawater, 10 g of sediment, 0.1 ml of a selected ULSD, and a ~1 cm diameter 1018 carbon steel coupon. In addition, a replicate set of incubations contained an inoculum of *Desulfoglaeba alkanexedens* strain ALDC, a model sulfate-reducing bacterium that only mineralizes C₆-C₁₂ *n*-alkanes (Davidova et al. 2006). Several attempts to determine whether other hydrocarbons might be metabolized by this organism proved negative (data not shown). The restricted substrate range exhibited by the inoculum has so far been corroborated by a preliminary annotation of the organism's genome (data not shown). The coupons were suspended in the seawater via teflon threads affixed to the serum bottle closures (Liang and Suflita, 2015). The headspace was adjusted to contain 80:20 N₂:CO₂. Negative controls included sterile incubations (autoclaved 20 min; 121 °C, 20 psi) and ULSD-free controls. The initial experiment was designed to monitor terminal electron acceptor depletion as a function of ULSD amendment with and without the *D. alkanexedens* positive control inoculum. It became apparent that the inoculum exhibited a greater-than-expected impact on biodegradation. Therefore, primers for monitoring the fate of *D. alkanexedens* were designed (below) and used in a repeat experiment. Experiments were conducted in triplicate (unless otherwise noted), and all incubations were maintained without shaking in the dark at room temperature. Benzoate (10 mM) and decane (0.02 ml) were used as positive control substrates. Microbial metabolism was routinely monitored by ion chromatography (Liang and Suflita, 2015) as the loss of sulfate relative to negative controls.

2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

The neat ULSDs were dissolved in ethyl acetate and analyzed by GC-MS as a means of comparing their relative compositions. The ULSD content in the incubations was analyzed at the end of the experiment. The aqueous phase of the incubations was acidified to pH ≤ 2 with HCl, extracted with an equal volume of ethyl acetate, dried over sodium sulfate, concentrated by rotary evaporation, derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and analyzed by GC-MS as previously described (Aktas et al., 2010). Identifications were made by comparison of the GC retention times and MS fragmentation profiles with commercial standards (Sigma-Aldrich, St Louis, Mo) that were similarly analyzed, or by comparison with the NIST Mass Spectral Library (Version 2.0a). Rotary evaporation resulted in the loss of *n*-alkanes

up to C₈. Therefore, the GC-MS analyses showed only *n*-alkanes starting from C₈ in the chromatographic profiles. This was not an issue during the analysis of the neat fuels as no extraction step was employed.

2.3 Corrosion Analysis

The 1018 carbon steel coupons were weighed before and after the incubation to determine the degree of general (e.g. “uniform”) corrosion as evidenced by weight loss. At the end of the experiment, the coupons were cleaned to remove accumulated corrosion products using a modified ASTM International standard method (Anonymous, 2003). Specifically, the coupons were submerged in an acid bath containing 6.2M HCl and 0.025M hexamethylene tetramine for 10 minutes. The coupons were then rinsed with deionized water, acetone, and methanol, dried under N₂, and weighed. As a measure of the degree of surface damage and pitting, the cleaned coupons were scanned using a Nanovea ST400, non-contact, white light profilometer (Nanovea, Irvine, CA) and analyzed using the MountainsMap6 software (Digital Surf, Besancon, France).

2.4 Fate and Impact of *Desulfoglaeba alkanexedens* in the Incubations

2.4.1 Quantitative PCR Analysis

Given the relatively long incubation times and questions regarding the fate of the inoculum, a repeat experiment using ULSD4 was initiated wherein *D. alkanexedens*-specific qPCR primers were developed (see supplementary material). *D. alkanexedens* was monitored in the aqueous phase of the incubations by qPCR amplification of the *dsrA* (dissimilatory sulfite reductase), *aprA* (adenylyl-sulfate reductase), and 16S rRNA genes. Seawater and sediment microbial communities were also quantified by amplification of bacterial *dsrA* and 16S rRNA genes (Ben-Dov et al. 2007, DSR1F and RH3-dsrR; Stevenson et al., 2011, 27F and 338R, respectively). Periodically, the incubation mixtures were sampled, stored at -80°C, and subsequently assayed for the genes of interest. Genomic DNA was prepared by extracting 1 mL of the aqueous phase of the incubations using an automated Maxwell[®]16 instrument (Promega, Madison, WI) according to a protocol described previously (Oldham et al., 2012). Extracted DNA was eluted into 100 µL of nuclease free water and quantified using a Qubit 2.0 fluorometer (Invitrogen/Life Technologies, Carlsbad, CA) with dsDNA High Sensitivity reagents according to the manufacturer’s instructions. Estimates of gene copy number were generated by conducting qPCR on 1:10, 1:100, and 1:1000 dilutions of DNA. qPCR reactions (15 µL) contained 7.5 µL of 2xSYBR[®]Green PCR Master Mix (Life Technologies, Carlsbad, CA), 3 µL of RT-PCR H₂O, and 0.75 µL of forward and reverse primers, and 3 µL of either diluted DNA or RT-PCR H₂O (no template control). Each reaction was performed in triplicate. Standard curves were generated from triplicate dilutions of genomic DNA samples. Standard curves included 10-fold dilutions of DNA ranging from 10³-10⁸ copies µL⁻¹. A StepOnePlus[™] Real-Time PCR System was used for thermal cycling, and StepOne Software v2.1 (Life Technologies, Carlsbad, CA) was used for data analysis.

2.4.2 Functional Gene Abundance

The repeat experiment (amended with ULSD4) was also analyzed via metagenome sequencing. The sediment, aqueous phase, and coupons were separated in

each incubation and the DNA was extracted as indicated above (Oldham et al. 2012), but the PowerMax Soil kit (MOBIO) was used to extract DNA from the sediment. A combination of the PowerSoil and Maxwell protocols was used for DNA extraction from coupons (Dr. Sylvie Le Borgne, Universidad Autónoma Metropolitana-Cuajimalpa, México) and reported in more detail in Liang et al. (2016b). In brief, the PowerBead fluid and beads and lysis solution were transferred into 50 mL centrifuge tubes containing the coupons, subjected to three freeze-thaw cycles, incubated with proteinase K, beadbeat, centrifuged briefly and the fluids further processed in the Promega Maxwell16 instrument as described (Oldham et al., 2012). Given the low DNA yields from coupon samples, barcoded libraries were prepared via a Nextera DNA Library Preparation Kit (Illumina), and libraries were sequenced via Illumina MySeq PE300 technology. All resulting reads were trimmed to remove Illumina and Nextera adapter sequences and overrepresented sequences as determined by analysis via FastQC. Sequences were trimmed using Cutadapt (Martin, 2011). Reads were further trimmed to a quality score of 30 and by removing poly-AAA tail artifacts via HomerTools (Heinz *et al.*, 2010). Only reads longer than 100 bp were retained, and all reads that were unpaired after QC analysis were removed via a custom perl script.

Functional gene abundances in the metagenomic data were determined by blastx analysis via USEARCH (Edgar, 2010). Each read was searched against custom protein databases. Databases for proteins with well-known descriptions in the literature and well automated annotations were generated by extracting the requisite sequences from all complete genomes available through JGI's IMG database (<http://img.jgi.doe.gov>) using the requisite enzyme's KEGG identifier as a search term. Unfortunately, there are no reference databases for genes/proteins associated with anaerobic hydrocarbon degradation to date, and an in-house database was generated for the catalytic subunits of known anaerobic degradation enzymes involved in hydrocarbon activation (Callaghan and Wawrik, 2016). For all blast searches, a minimum column setting of 50 and a 70% identity as a cutoff were used. For taxonomic analysis of functional genes, all reads that generated hits by this method were blastx searched separately against the NCBI NR database using default parameters. The resulting blast output was parsed for taxonomic identifiers via MEGAN (Huson *et al.*, 2007) using its built-in taxonomy browser to generate genus-level phylogenetic assignments. The frequencies of specific gene targets in the metagenomic data were assessed via a ratio to the frequency of bacterial *rpoB*-like sequences. The *rpoB* gene encodes the β subunit of the bacterial RNA polymerase and is thought to be encoded by a single copy in most bacterial genomes. Therefore, a ratio of the frequency of a functional gene to the frequency of *rpoB* roughly estimates the relative abundance of organisms carrying a specific target. However, for varying reasons, such as multiple copies of target genes in genomes and DNA replication, these numbers likely represent an overestimate. The phylogenetic breakdown of 16S-containing reads was determined by first blastn searching all reads against the SILVA 111 16S rRNA gene database (Quast *et al.*, 2013). All reads that contained partial 16S rRNA genes were then analyzed and visualized using QIIME (Caporaso *et al.*, 2010) by conducting closed reference OTU picking against the SILVA reference dataset. QIIME was also used to calculate alpha- and beta-diversity metrics and visualization (see supplementary material).

2.4.3 Accession Numbers

Metagenome sequence data can be found in GenBank under the following identifiers. SRA entry: SRP074411; BioProject: PRJNA319949; BioSample: SRS1422566; Accession Numbers: SRR3476969 (Coupon Biofilm Reads), SRR3476971 (Aqueous Community Reads), and SRR3476972 (Sediment Community Reads). Gene sequences cloned from *D. alkanexedens* to develop specific primers (see Supplementary Material) can be found in Genbank under the following accession numbers: KX810196 (16S rRNA), KX823336 (*dsrAB*), and KX823337 (*aprBA*).

2.4.4 The impact of *D. alkanexedens* on other sediment microorganisms

During the course of the investigation, it was noted that the positive control inoculum had a stimulatory effect on both hydrocarbon and sulfate metabolism. To explore this effect in more detail, seawater/sediment incubations received ULSD 4 and either an amendment of *D. alkanexedens* inoculum, sulfide (1 or 5 mM), and a nutrient solution (20% or 40% v/v) in the form of a mineral marine medium (Widdel and Bak, 1992), or an amendment of spent culture fluids (20% and 40% v/v) obtained by filter-sterilizing an existing 3 month old *D. alkanexedens* culture grown on decane. The headspace was adjusted to contain 80:20 N₂:CO₂. Negative controls included autoclaved incubations and ULSD-free incubations. Benzoate (10 mM) served as positive control substrate to verify that the resident microflora were capable of eliciting a response. Experiments were conducted in triplicate and in the dark at room temperature, and sulfate loss was monitored by ion chromatography as indicated above.

Results

3.1 Short-Term Incubations

Petroleum has been arguably called the world's most compositionally complex organic mixture (Marshall and Rodgers 2004). It is clear that only a relatively small fraction of compounds known to be in structurally diverse hydrocarbon mixtures can be resolved with any particular GC-MS method. Nevertheless, GC-MS analysis was able to demonstrate that the four amended ULSDs shared a similar suite of well-resolved hydrocarbons (Figure 1) and that the most obvious difference among the fuels was the variation in the ratio of low molecular weight (LMW - C₈-C₁₄) to high molecular weight (HMW - C₁₅-C₂₄) *n*-alkanes. That is, the total integrated area under the peaks revealed that the ratios were 0.2, 0.5, 0.8 and 1.1 for ULSDs 1-4, respectively. Other branched, cyclic, and aromatic compounds were also evident in this analysis, but they were resolved as minor peaks relative to the *n*-alkanes.

The GC-MS analysis of the neat ULSDs (Fig. 1) was used to calculate the fraction that individual *n*-alkanes (assuming a uniform analytical response factor) made up of the total paraffin content of each fuel. Since the amount of fuel added to the incubations was the same, the stoichiometric amount of sulfate needed to mineralize each *n*-alkane component in the ULSDs was calculated assuming complete substrate mineralization, according to the example illustrated for decane in equation 1:

The amount of sulfate needed to consume the LMW *n*-alkanes was calculated to be 0.3, 1.2, 1.9 and 2.2 mM for ULSDs 1 - 4, respectively. This is the range of hydrocarbons known to represent suitable substrates for the positive control inoculum, *D. alkanexedens*. After 75 days of incubation, ion chromatography revealed that the amount of sulfate utilized in the various incubations was close to the stoichiometrically expected value. That is, at the relatively early stage of ULSD biodegradation, sulfate loss was consistent with the amount of low molecular weight (C₈-C₁₂) *n*-alkane mineralization by *D. alkanexedens* (Figure 2). In comparable fuel-amended incubations without the bioaugmented positive control organism (data not shown), sulfate loss was less than 1 mM for all fuel amendments and only slightly more than the fuel-unamended control (Fig. 2). These observations suggest that the inoculum, as well as the particular ULSD composition, influences the onset of hydrocarbon biodegradation.

3.2 Long-term Incubations

While initial rates of sulfate reduction varied as a function of the LMW/HMW ratio, this was less apparent with 3 out of the 4 fuels upon extended incubation (Figure 3). The clearest impact on sulfate loss was the presence of the positive control inoculum. Incubations with an amendment of *D. alkanexedens* removed 6.1, 9, 9.3, and 8.8 mM sulfate for ULSDs 1 - 4, respectively, but only 1.9, 3.9, 2.5 and 3.4 mM for the same fuels in incubations without the inoculum (averaged in Fig. 3). The amount of sulfate loss theoretically needed to account for the complete mineralization of all *n*-alkanes in the various incubations is 8.2, 7.7, 8.5, and 7.8 mM for ULSDs 1-4, respectively. Therefore, a comparison of the actual amount of sulfate loss is roughly 75, 116, 109, and 112 % of that theoretically necessary for complete *n*-alkane metabolism in the ULSD 1-4 fuels, respectively. It is therefore likely that other classes of hydrocarbons, besides *n*-alkanes, were also biodegraded in these experiments.

The residual hydrocarbon profiles in the incubations were assessed at the end of the ~6 month incubation (Figure 4). A single, representative, heat-inactivated, sterile control is included to illustrate that the *n*-alkanes would indeed be detectable if they were present in substantial quantities at the conclusion of the experiment. Total ion chromatograms of the ethyl acetate extracts for each ULSD-amended incubation containing *D. alkanexedens* revealed that *n*-alkanes were clearly removed in non-sterile incubations (Figure 4). The degree of hydrocarbon loss in the incubations was consistent with the sulfate loss data (Figure 3) suggesting the mineralization of the *n*-alkane fraction. While the initial substrate utilization may have favored LMW *n*-alkane utilization, GC-MS evidence reveals that the *n*-alkane components were below detection by the end of the incubation period.

A closer examination of the chromatograms revealed that several branched alkanes (C₁₇ and C₂₃) were more prominent at the end of the experiment, and thus, were relatively more recalcitrant than their straight-chain counterparts. This analysis would typically detect signature metabolites of anaerobic *n*-alkane biodegradation (e.g. methylalkylsuccinates), but these compounds were not evident presumably reflecting their low concentrations and transient nature. However, several metabolites of anaerobic naphthalene biodegradation were identified including naphthalene carboxylic acid and

tetrahydronaphthoic acid (data not shown). This observation confirms that other types of hydrocarbons (i.e. more than just the *n*-alkanes) were also amenable to anaerobic biodegradation.

The impact of the positive control inoculum is also apparent when the total ion chromatograms of incubations containing the same fuels but no *D. alkanexedens* were similarly analyzed (Fig. 4). Quite clearly, there are residual *n*-alkane peaks still detectable at the end of the experiment in all fuel-amended incubations. However, the total percentage of *n*-alkane removal is roughly consistent with the amount of sulfate loss based on the aforementioned stoichiometry. Without a suitable inoculum, evidence for the preferential utilization of LMW *n*-alkanes relative to the HMW counterparts is less clear.

3.3 The Fate of the Bioaugmented Inoculum

3.3.1 Quantitative PCR Analysis

Quantitative PCR primers were designed (supplementary material) for the *D. alkanexedens* *dsrA*, *aprA*, and 16S rRNA genes in order to monitor the fate of the positive control inoculum in fuel-amended and unamended incubations (see Section 2.4.4). Changes in the community bacterial *dsrA* and 16S rRNA genes were also monitored in the fuel-amended incubations that had received an inoculum of *D. alkanexedens*. Although there was a quantitative reduction in *D. alkanexedens* genes over time, they persisted over the course of the 187-day incubation in the presence of ULSD 4 (Figure 5). The qPCR assays of both the community *dsrA* and 16S rRNA genes, as well as the *aprA* gene (data not shown), provided corroborating evidence relative to the fate of the inoculum. However, when the same determinations were performed on the fuel-unamended incubations, there was a significant reduction in *D. alkanexedens* genes within the first 30 days of incubation, and they were not detectable after 155 days (Figure 5). Contrary to the fate of the inoculum, there was a significant increase in the community bacterial *dsrA* and 16S rRNA genes after 155 days of incubation (Figure. 5, note the error bars indicate one standard deviation). Upon further incubation, the community bacterial 16S rRNA qPCR signal remained nearly constant, while the community bacterial *dsrA* gene decreased to near the level measured at the beginning of the experiment. Thus, conditions at the end of the incubation were less favorable for the bioaugmented positive control bacterium relative to other sulfate-reducing prokaryotes containing a *dsrA* gene, but the bioaugmented bacterium persisted throughout the experiment.

3.3.2 Functional Gene Abundance

Functional gene analysis was conducted via metagenomic sequencing of DNA extracted from coupons, the aqueous phase, and from sediment originating from incubations amended with ULSD4 and also the *D. alkanexedens* inoculum. With respect to hydrocarbon degradation, the presence of genes associated with both aliphatic and aromatic anaerobic hydrocarbon degradation pathways was observed (for review see Rabus et al., 2016) (Supplementary material, Figure S.1). Most notably, a diversity of sequences encoding the alpha subunit of alkylsuccinate synthase (ASS; alpha subunit encoded by *assA*) was detected (Supplementary material, Table S.8). While the bulk of

these sequences (43%) were identical to *assA* genes in *D. alkanexeden*'s genome, several additional genotypes ranging in 70-90 % identity at the amino acid level to AssA in other alkane-degrading isolates (e.g. *Desulfatibacillum alkenivorans* AK-01, the delta proteobacterium PL12, and *Desulfatibacillum aliphaticivorans*) were also detected. This is consistent with the enrichment for a range of alkane-degrading bacteria in the incubation. Hits for *hbsA*, *ibsA*, and *nmsA*, which encode the alpha subunits of (hydroxybenzyl)succinate synthase, (4-isopropylbenzyl)succinate synthase, and (naphthylmethyl)succinate synthase, respectively, indicate the potential for the degradation of compounds structurally related to cresols (e.g. partially oxidized hydrocarbons), ethylbenzene, and 2-methylnaphthalene. Since these requisite genes are not present in the *D. alkanexeden* genome (unpublished observation), these findings similarly point to the stimulation of additional hydrocarbon (or hydrocarbon-related)-degrading bacteria in the incubations.

With respect to sulfate reduction, *dsrA* genes originating from a wide range of bacterial taxa were detected (Supplementary material, Figure S.2). The dominant group among these was the genus *Desulfovibrio*, as well as several other deltaproteobacterial genera, including *Desulfatibacillum*, *Desulfatirhabdium*, *Desulfosarcina*, *Desulfotignum*, and *Desulfocapsa*. The *dsrA* gene from *D. alkanexeden* was detected in the metagenomic data, but accounted for <1% of *dsrA*-like reads, indicating that it only was a small proportion of the bacteria with the potential for sulfate-reducing activity. This interpretation is supported by analysis of 16S rRNA genes recovered from the metagenomic datasets, that indicate that dominant bacterial groups in the incubation belonged to the Desulfobacterales and Desulfovibrionales, while the Syntrophobacterales, which include positive control organism, only account for a small fraction of the community (Supplementary material, Figure S.3). The principal component and rarefaction analysis (Supplementary material, Figure S.4) indicated that coupon-associated communities were perhaps different from sediment and aqueous populations, though statistical inferences could not be made due to a lack of sufficient replicates for coupon metagenomes. Sediment communities, however, appeared to be significantly more diverse based on rarefaction analysis, which is consistent with the persistence of a range of sediment-associated bacteria that are not necessarily involved in ULSD degradation.

3.4 Corrosion analysis

A topographical analysis of representative coupons recovered from the incubations after the experiment is shown in Figure 6. The coupon from sterile control incubations is relatively smooth, mostly unaltered with radial milling marks still evident at the end of the experiment (Figure 6A). The obvious hole in the coupon was drilled in order to suspend the metal in the incubation mixture. For comparison, Figure 6B shows the surface profile of a comparable coupon recovered from a representative non-sterile incubation after the same amount of time. The scan reveals a highly damaged coupon with milling marks almost completely removed.

Quantification of the degree of localized damage to the coupon surfaces can also be seen in the corresponding histograms that are representative of the depth of pitting corrosion (Figure 6). A pit is defined as surface damage that is at least 10 μm wide and 5 μm deep. The histograms characterize the percentage of pits on the coupon surface in a

particular depth range (Figure 6). The distribution of pit depth in coupons recovered from sterile incubations ranged from 5 – 75 μm with a mean of 35 μm . In contrast, the pit range was 5 – 145 μm with a mean of 72 μm in coupons recovered from non-sterile incubations. Thus, the mean pit depth detected in non-sterile incubations is roughly equal to the maximum pit depth detected in sterile controls. The profilometry findings demonstrate that coupons were heavily damaged in anaerobic marine incubations containing microorganisms that biodegraded various ULSDs under sulfate-reducing conditions but not in the corresponding negative control incubations.

General corrosion was also assessed by coupon weight loss at the end of the incubation. The degree of weight loss (and by calculation the corrosion rate in mmpy) varied as a function of the amount of sulfate loss in the incubations (Figure 7). The average sulfate loss in sterile incubations was 2 mM. Therefore the data in Figure 7 were corrected by this amount. General corrosion as evidenced by weight loss was correlated with sulfate loss ($r^2 = 0.73$), but did not vary as a function of the particular ULSD following extended incubation. However, incubations with and without the positive control inoculum clustered differentially (Figure 7). Incubation mixtures containing *D. alkanexedens* exhibited an average total sulfate loss of about 9 mM for the tested ULSDs. Correspondingly, the total weight loss and general corrosion rates were greater than 0.08 grams and 0.1 mmpy, respectively. Incubations without the bioaugmented organism generally lost less weight (0.04 grams) and exhibited a far more modest corrosion rate of 0.05 mmpy (Figure 7). It is clear that general corrosion of carbon steel coupons was positively correlated with the amount of sulfate loss in the incubations and that the electron acceptor utilization was differentially associated with the anaerobic biodegradation of labile fuel components. While the exact mechanism(s) of biocorrosion cannot be deduced based on this evidence, the results suggest that sulfide induced corrosion largely accounts for the increased corrosion rates in marine incubations amended with fuel.

3.5 The effect of *D. alkanexedens* on other marine microorganisms

The presence of the positive control inoculum in the ULSD-amended incubations resulted in an increase in both the rate and extent of sulfate loss relative to the inoculum-free controls (Figs. 3, 4). It was also clear that the range of *n*-alkane utilization and sulfate loss far exceeded the known capabilities of the inoculum. We conclude that the inoculum had a positive impact on the hydrocarbon-degrading abilities of the indigenous marine microflora. An initial experiment was conducted to explore this impact on hydrocarbon metabolism in more detail using ULSD 4 (Supplementary Figure S.5).

As expected, the presence of the ULSD stimulated sulfate loss relative to the diesel-free control, and incubations amended with *D. alkanexedens* accelerated sulfate loss still further (Fig. S.5). When amended with an easily degraded positive control substrate (benzoate), the microbial community responded with the expected significant increase in sulfate-utilization. The obvious microbial end product associated with such bioconversions is the production of sulfide. However, the exogenous addition of 1-5 mM sodium sulfide to the incubations effectively inhibited sulfate loss (Fig. S.5).

To evaluate whether soluble constituents in culture fluids could stimulate hydrocarbon metabolism, two filter-sterilized preparations (20% and 40% by volume) of fluid from a 3 month-old culture of *D. alkanexedens* were amended to the incubations,

but the degree of stimulation was not significantly different than the incubations amended with *D. alkanexedens* (Fig. S.5). However, when a nutrient-replete fresh marine medium was used as an experimental variable, a significant increase in the degree of sulfate reduction was evident.

4. Discussion

In recent decades, one of the most important transportation fuels in the world, diesel, has undergone major changes in formulation including the reduction of sulfur content and the intentional or unintentional mixing with various biofuel additives. Concomitant with such changes were increased incidents of corrosion in the energy infrastructure (Anonymous, 2012). A direct assessment of how biodiesel and varying sulfur levels impact fuel biodegradation and metal biocorrosion suggests that fatty acid methyl ester additives are labile and can accelerate pitting damage in carbon steel (Aktas et al. 2010), while the removal of sulfur is of far less concern (Lyles et al. 2013). In these cases, the susceptibility of the diesel fuel to anaerobic biodegradation processes under marine conditions was a major determinant in how much sulfate was utilized and the degree of carbon steel biocorrosion. This study goes beyond the intentional alterations in diesel formulation and examines the impact of compositional differences on fuel biodegradation and metal biocorrosion.

While basically similar in overall composition (Figure 1), GC-MS analysis illustrates that several ULSDs differed in their ratio of LMW to HMW *n*-alkanes. In incubations of natural seawater communities amended with *D. alkanexedens*, a bacterium capable of metabolizing C₆-C₁₂ *n*-alkanes, early stage sulfate utilization was nearly equal to the stoichiometrically predicted amount of this electron acceptor needed to mineralize the quantity of LMW *n*-alkanes in each ULSD. It is therefore reasonable to ascribe that metabolism to the bioaugmented bacterium, particularly since the same pattern of sulfate utilization was not evident in incubations without the positive control inoculum. However, we cannot rule out the participation of other hydrocarbon-degrading microorganisms at this stage of metabolism. Minimally, the results suggest that the chemical composition of the ULSDs and the particular inoculum impact the onset of biodegradation, sulfate reduction and biocorrosion.

Although early sulfate reduction rates reflected the LMW/HMW *n*-alkane ratios in the ULSDs, such subtle compositional differences were less important with extended incubation. Total sulfate loss was roughly 9 mM for ULSDs 2-4, and about 6 mM for ULSD 1. The amount of sulfate required for the anaerobic mineralization of all *n*-alkanes is 8.2, 7.7, 8.5, and 7.8 mM for ULSDs 1-4, respectively. Thus, it is reasonable to conclude that most or all of the *n*-alkanes were metabolized. This contention was supported by GC-MS evidence at the end of the experiment wherein the *n*-alkane peaks were removed from non-sterile incubations receiving the positive control organism, but not from the corresponding sterile controls (Figure 4). Further, significantly less sulfate was utilized in non-sterile incubations without the *D. alkanexedens* amendment, and only partial biodegradation of the suite of *n*-alkanes was evident (Figure 4). In addition, incubations that did not receive the positive control amendment did not demonstrate evidence for the preferential biodegradation of LMW *n*-alkanes.

The amount of sulfate in excess of that needed to mineralize the *n*-alkane component in the ULSD suggests that other classes of hydrocarbons in the fuels were

also being metabolized. Partial support for this contention was obtained by the detection of several metabolites associated with the anaerobic metabolism of naphthalene (e.g. naphthalene carboxylic acid, tetrahydronaphthoic acid) (Gieg et al. 2009; Meckenstock et al. 2000; Zhang et al. 2000). However, efforts to detect signature anaerobic metabolites of *n*-alkane biodegradation (Callaghan et al. 2006; Kropp et al. 2000) were unsuccessful, presumably because these intermediates were below GC-MS detection levels.

The factor that had the most pronounced impact on long-term incubations was the presence of the positive control inoculum. Three times as much sulfate loss occurred in non-sterile *D. alkanexedens*-augmented incubations compared to those without bioaugmentation. The qPCR evidence using three specific primers for *D. alkanexedens* clearly illustrates that the organism decreased upon prolonged incubation, yet it persisted over the entire course of the experiment in the presence of fuel (Figure 5). In contrast, the inoculum was removed relatively rapidly in the fuel-free incubations. Presumably, the organism persisted as long as it was able to occupy a LMW hydrocarbon biodegradation niche. In contrast, other bacteria containing *dsrA* transiently increased and eventually declined with prolonged incubation, but returned to a gene copy number after 245 days that was comparable to that observed at the beginning of the experiment (Figure 5). Thus, it can reasonably be argued that the presence of the inoculum had a positive impact on the other sulfate-reducing prokaryotes in the incubation mixtures. This observation is supported not only by the increased level of sulfate reduction in *D. alkanexedens*-augmented incubations, but also by the removal of hydrocarbon substrates well beyond the known nutritional capability of this inoculum.

The nature of the positive impact of the hydrocarbon-degrading sulfate-reducing bacterium on the sulfate-reducing bacterial community remains enigmatic, but such an observation is likely to be particularly important for both fuel stability and metal biocorrosion in marine environments that are hydrocarbon replete (e.g. seawater-compensated fuel ballast tanks). An initial exploration of the reasons for the stimulation revealed that the effect was not due to increased levels of sulfide or water-soluble components present in spent culture fluids (filtered from a *D. alkanexedens* culture). In fact, enhanced sulfate loss beyond that provided by the inoculum itself was only observed in incubations amended with varying amounts of a medium normally used for the cultivation of marine anaerobes. The stimulatory impact of increased inorganic nutrients on hydrocarbon biodegradation is hardly surprising in carbon-rich but nutrient poor marine incubations. In this regard, we cannot dismiss the prospect that the inoculum itself represented a suitable nutritional augmentation. However, as noted above, qPCR determinations assayed with three different *D. alkanexedens* genes indicated that the inoculum tended to persist in fuel-amended incubations, so this argument seems too simplistic. It may be that the culture employed a quorum sensing signaling compound(s) that effectively stimulated the resident anaerobic microflora. While the mechanism of the stimulation is unknown, it is clear that the microflora responded to the inoculum and anaerobic hydrocarbon biodegradation was indeed accelerated. Metagenomic data supports the notion that a range of hydrocarbon degraders beyond *D. alkanexedens* were present at the end of the incubation, though it appears that positive control inoculum remained the dominant phylotype with respect to *n*-alkane degradation. The presence of genes related to anaerobic aromatic hydrocarbon degradation is consistent with the detection of naphthalene carboxylic acid and tetrahydronaphthoic acid metabolites.

Whether the persistence of the requisite bacteria was stimulated by bioaugmentation cannot be assessed from these data, given that metagenomes were not generated for incubations in the absence of *D. alkanexedens*.

Hydrocarbon oxidation linked to sulfate reduction and sulfide production by microorganisms in the presence of a metal coupon inevitably led to biocorrosion that was not evident in sterile control incubations (Figures 6, 7). Mechanistically, the fact that sulfate-reducing bacteria catalyze biocorrosion by multiple mechanisms related to sulfate utilization is not controversial (Coetser and Cloete 2005). Since all coupons from non-sterile incubations displayed a similar degree of coupon damage in the presence of fuel, the differences in biocorrosion as a function of the compositional differences in a particular ULSD are not readily discernable. It may well be that the degree of coupon damage due to biocorrosion became increasingly indistinct over the course of the extended incubation as more hydrocarbon oxidation was coupled with increasing levels of sulfate utilization. That is, we cannot dismiss the fact that the subtle compositional differences in fuel may have been more apparent if the coupons were examined at earlier time points; in particular, when the LMW *n*-alkanes were differentially metabolized. Regardless of the precise mechanism involved, the positive correlation between sulfate utilization and weight loss (and the resulting corrosion rate determinations) argue that the majority of corrosion damage in hydrocarbon-laden anaerobic marine environments is sulfide-related. A similar positive correlation was previously reported in a study designed to assess the corrosivity of various alternative fuels in marine anaerobic incubations (Liang et al. 2016a). While the corrosion rates detailed in this study were comparable or slightly higher than those observed previously (Liang et al. 2016a), the rates would be still be regarded as relatively modest according to NACE international standard assessments (NACE 2005). However, as pointed out above, it may be that the corrosion damage occurred at an early time point and escaped detection. Therefore, the rates are likely to be considered conservative estimates.

5. Conclusion

Many societies have altered the composition of diesel fuels in ways deemed to be beneficial for the environment (i.e. removal of sulfur, addition of fatty acid methyl esters, etc). However, multiple simultaneous changes in fuel formulations tend to obscure the impact of any individual alteration on the mostly carbon steel infrastructure. Subtle compositional differences in ULSD fuels impact their susceptibility to biodegradation by hydrocarbonoclastic sulfide-generating anaerobes. However, such differences are notably less important in longer-term assessments of carbon steel biocorrosion. A known, hydrocarbon-degrading, marine, sulfate-reducing bacterium used as an inoculum persisted in experiments amended with ULSD fuel. The inoculum had an unexpected and significant influence on the overall biodegradation of fuel and biodeterioration of metal. The impact was well beyond what the organism was metabolically capable of achieving alone. The results reinforced the view that biocorrosion is the result of the interplay between the predominant energy sources available in an environment (in this case ULSD), the presence and activity of a suitable inoculum, and the metal undergoing attack. While the exact mechanism(s) of biocorrosion are not discernable in this study, the observation that sulfate utilization was linearly correlated with weight loss suggests that sulfide-induced damage accounts for the major fraction of corrosion in marine incubations amended with fuel. Thus, control efforts should differentially target such processes.

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Conflict of Interest Statement

The authors declare that there are no financial conflicts of interest. JMS is a long-time acquaintance of Dr. J-D Gu (Editor-in-Chief), and they are planning a future scientific collaboration.

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827 **Figure Legends**

828 **Figure 1.** Total ion chromatograms of the four, different, neat ULSDs. The alkanes are labeled
829 with their corresponding carbon chain lengths. The specific peaks are marked with dashed lines
830 to facilitate the comparison among the ULSD profiles.

831 **Figure 2.** Early stage (75 d) sulfate loss in initial marine incubations amended with various
832 ULSDs and a culture of *D. alkanexedens*, relative to a fuel-unamended negative control. Error
833 bars represent the standard errors of triplicate incubations. The dashed lines represent the
834 theoretical amount of sulfate loss expected based on the amount of LMW *n*-alkanes in each fuel.

835 **Figure 3.** Sulfate loss with time in repeat ULSD-amended marine incubations augmented with
836 *D. alkanexedens*. The average loss of sulfate in all negative controls and in incubations without
837 the bioaugmented inoculum are shown for comparison.

838 **Figure 4.** Total ion chromatographic profiles of organic matter extracted from: A) sterile and
839 fuel-amended incubations and (B) sterile and fuel-amended incubations that also received *D.*
840 *alkanexedens*. The *n*-alkanes are labeled with their corresponding carbon chain lengths, and
841 corresponding peaks are marked with dashed lines to facilitate comparisons. The lone sterile
842 control from incubations receiving ULSD 2 (A and B) confirms that *n*-alkanes could be detected
843 using the analytical procedures. Incubations were sampled and analyzed after approximately 6
844 months.

845 **Figure 5.** qPCR analysis of *dsrA* and 16S rRNA gene abundances of community Bacteria (e.g.
846 all bacteria) and *Desulfoglaeba alkanexedens* specifically over the course of the long-term
847 incubation with ULSD 4. qPCR analysis of *dsrA* and 16S rRNA gene abundances of
848 *Desulfoglaeba alkanexedens* is also shown for the fuel-unamended incubations.

849 **Figure 6.** Representative surface images of carbon steel coupons and the associated
850 profilometric depth distribution of pits in sterile (A) and non-sterile (B) incubations of
851 seawater/sediment incubations amended with ULSD 2 and the positive control inoculum.

852 **Figure 7.** The relationship between sulfate loss and general corrosion as evidenced by weight
853 loss in carbon steel coupons retrieved from marine incubations containing various ULSDs. The
854 data points for the incubations without a *Desulfoglaeba alkanexedens* amendment are circled.
855 The general corrosion rate in mmpy (millimeters per year) is calculated based on total incubation
856 time and weight loss evidence.

Relative Response Factor

Total Sulfate Loss (mM)

Sulfate (mM)

A

Relative Response Factor

B

Relative Response Factor

A

B

Graphical Abstract

Anaerobic Hydrocarbon Biodegradation and Biocorrosion of Carbon Steel in Marine Environments: The Impact of Different Ultra Low Sulfur Diesels and Bioaugmentation
Deniz F. Aktas^{a,b}, Kathryn R. Sorrell^{a,b}, Kathleen E. Duncan^{a,b}, Boris Wawrik^a, Amy V. Callaghan^a and Joseph M. Suflita^{a,b,*}