Molecular tools to track bacteria responsible for fuel deterioration and microbiologically influenced corrosion

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Investigating the susceptibility of various fuels to anaerobic biodegradation has become complicated with the recognition that the fuels themselves are not sterile. Bacterial DNA could be obtained when various fuels were filtered through a hydrophobic teflon (0.22 μm) membrane filter. Bacterial 16S rRNA genes from these preparations were PCR amplified, cloned, and the resulting libraries sequenced to identify the fuel-borne bacterial communities. The most common sequence, found in algal- and camelina-based biofuels as well as in ultra-low sulfur diesel (ULSD) and F76 diesel, was similar to that of a *Tumebacillus*. The next most common sequence was similar to *Methylobacterium* and was found in the biofuels and ULSD. Higher level phylogenetic groups included representatives of the Firmicutes (*Bacillus*, *Lactobacillus* and *Streptococcus*), several Actinobacteria, Deinococcus-Thermus, Chloroflexi, Cyanobacteria, Bacteroidetes, Alphaproteobacteria (*Methylobacterium* and Sphingomonadales), Betaproteobacteria (*Oxalobacteraceae* and *Burkholderiales*) and Deltaproteobacteria. All of the fuel-associated bacterial sequences, except those obtained from a few facultative microorganisms, were from aerobes and only remotely affiliated with sequences that resulted from anaerobic successional events evident when ULSD was incubated with a coastal seawater and sediment inoculum. Thus, both traditional and alternate fuel formulations harbor a characteristic microflora, but these microorganisms contributed little to the successional patterns that ultimately resulted in fuel decomposition, sulfide formation and metal biocorrosion. The findings illustrate the value of molecular approaches to track the fate of bacteria that might come in contact with fuels and potentially contribute to corrosion problems throughout the energy value chain.

**Keywords:** Fuel; biofuel; biodegradation; biocorrosion; 16S rRNA genes; bacterial identification

**Introduction**

The microbial biodegradation of fuels is associated with decreased product quality, compromised equipment performance, and the biocorrosion of metal surfaces. Such issues may possibly become more acute as alternative fuel formulations are increasingly used in the existing carbon steel infrastructure (Aktas et al. 2010; Sorensen et al. 2011). Addressing such problems requires reliable information on where and how problematic microorganisms come in contact with and proliferate on metallic structures, eventually leading to microbiologically influenced corrosion or biocorrosion. A copious literature exists on the subject of biocorrosion resulting from fuel/seawater exposures (eg see Lee et al. 2006, 2010 and references therein). There is also a growing literature on metal corrosion in seawater systems exposed to alternative biofuels (Aktas et al. 2010; Lee et al. 2010, 2012; Sorensen et al. 2011). Attention has been concentrated on corrosion due to microbial sulphide production that is often the product of sulphate-reducing prokaryotes. Such a scenario can be of particular consequence onboard ships equipped with seawater-compensated fuel ballast tanks. In these vessels, seawater is used to compensate for weight and volume loss as the engine consumes fuel. Therefore, there is regular and direct contact between fuel and seawater and the opportunity for the development of problematic biofilms. It is hypothesized that the fuel-induced corrosion is a function of: (1) the chemical composition of the fuel, (2) its inherent propensity to undergo biodegradation and (3) the contact of the fuel with microorganisms that may ultimately accelerate biocorrosion processes.

All microbes, regardless of the habitat where they primarily reside, are essentially aquatic organisms and require liquid water. This would seem incongruous in a largely hydrophobic fuel infrastructure. However, there are multiple ways by which water can contaminate fuel supplies. The interaction of seawater and fuel is obvious onboard ships. The more subtle ways fuels can come into contact with water include condensation in fuel tanks or by accidental contamination of storage facilities via precipitation events. The mix of more hygroscopic biofuels with traditional fuels increases
the tendency of the resulting product to absorb moisture from the atmosphere. Such water/fuel mixtures represent environments that can be conducive for the proliferation of microorganisms should a suitable inoculum be available.

This recognition leads to a series of interrelated questions which requires an exhaustive investigation to be answered, including: Do microorganisms typically contaminate fuels? How might fuels contract such an ‘infection’? Does the presence of microorganisms represent a substantive threat to fuel use? How should the microbiological status of fuels be monitored? This study represents an initial attempt to address such questions.

Traditionally, microbial ecologists relied on the cultivation of microorganisms or the measurement of microbial activities to assess questions of ‘who is there?’ and ‘what are they doing?’ in various environments. While much has been learned with these approaches, it has been clear for decades that scientists could only cultivate a small fraction of the microorganisms they knew to be present. Since the mid-1980s, molecular approaches have allowed practitioners to directly assess the identities of microbial communities without the need to cultivate them. Most of the advances in molecular biology have focused on the sequencing of nucleic acids extracted from particular environments. The sequencing of the DNA obtained provides valuable phylogenetic information about the specific members of complex microbial communities and how they compare to other known microorganisms. This approach has proven highly valuable for characterizing microbes that inhabit the various environments and for developing testable hypotheses on their prospective niche. However, DNA sequence data can easily be over-interpreted. Because microorganisms belong to a common taxon does not necessarily mean they share the same metabolic capacity. Further, DNA recovered and sequenced from a particular habitat may also originate from dead or dormant cells. Nevertheless, baseline investigations can often be advanced with molecular surveys and sources of contamination can be traced.

The molecular survey of traditional petro-based diesel fuels as well as first-, and next generation biofuels performed in this study revealed that most harbored a characteristic microflora. However, the fuel-associated microorganisms were very unlike the bacteria that dominated anaerobic seawater incubations in either the absence or presence of fuel. Anaerobes are believed to be differentially associated with carbon-steel biocorrosion processes. Thus, it is concluded that microorganisms found in the fuels do not represent a significant biocorrosive threat under seawater incubation conditions. However, their potential proliferation in the fuel infrastructure has the prospect of exacerbating other types of fuel problems.

Materials and methods

Fuels and microorganisms

Military fuels F76 (a marine diesel, 600 ml) and JP5 (jet propellant, 500 ml), as well as algal-F76 (400 ml), camelina-JP5 (450 ml), ultra low sulfur diesel (ULSD from a refinery) (1.7 l), ULSD (from a typical supplier) (500 ml), and soy-based biodiesel (1 l) were filtered through a hydrophobic teflon (0.22 μm) membrane filter (Millipore). DNA was extracted from the filter using the Power Biofilm DNA Isolation Kit (MO BIO Laboratories). Partial bacterial 16S rRNA gene fragments were amplified with the primers 27F (JGI) and 907R (Muyzer and Smalla 1998) using the protocol for PCR amplification specified in the Department of Energy Joint Genome Institute (JGI) protocol for RNA Library Creation (http://my.jgi.doe.gov/general/protocols/SOP_16S18S_rRNA_PCR_Library_Creation.pdf). Clone libraries were created using the TOPO TA Cloning Kit (Invitrogen Corp, Carlsbad, CA). The number of sequences in each library was: ULSD (51), Algal F76 (51), Camelina JP5 (51), F76 (50). Sequencing was performed on an ABI model 3730 capillary sequencer using the M13 flanking regions of the TOPO cloning vector as sequencing primer sites (Microgen: The Laboratory for Genomics and Bioinformatics, Oklahoma City, OK). Sequencher (Gene Codes Corp., Ann Arbor, MI) was used to examine the chromatograms and create consensus sequences from the forward and reverse reads. Duncan et al. (2009), describe the sequence analysis protocol in detail.

Q-PCR procedures

The numbers of bacterial 16S rRNA gene copies were estimated using q-PCR. Briefly, 30 μl of Syber Green-containing PCR reactions contained 4 μl of 1X or 8 μl of a 1/10X dilution of template DNA, 15 μl 2× Syber mix (Applied Biosystems, Carlsbad, CA), 0.5 mM Betaine, 0.15 μl of 100 μm 27F primer and 0.075 μl of 100 μm 338R primer (Hamady et al. 2008; Stevenson et al. 2011). Real-time thermal cycling was carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and data acquisition and analyses were carried out using StepOne Software v2.1 (Applied Biosystems, Carlsbad, CA). Cycling conditions for the 331 bp amplicon were: 95°C for 10 min followed by 40 cycles of 96°C for 30 s, 55°C for 45 s, and 72°C for 45 s. Serial dilutions of a control DNA plasmid containing a single copy of a bacterial 16S rRNA gene sequence (a gift from Dr B. Stevenson, University Oklahoma) was run in triplicate.
alongside triplicate reactions of template DNA isolated from the various fuels. Copy number estimates were based on the following equation, assuming the average molecular mass of a dsDNA bp at $6.6 \times 10^{11}$ ng mol$^{-1}$, and Avogadro’s number of copies per mol, $6.022 \times 10^{23}$:

$$\text{Copies} = (\text{plasmid concentration (ng} \mu\text{l}^{-1}) \times 6.02 \times 10^{23}\text{ copies mol}^{-1})/(\text{plasmid length (bp)} \times 6.6 \times 10^{11}\text{ ng mol}^{-1})$$.

**Seawater incubations**

The microflora found in fuel was compared with the microbial communities associated with seawater samples that might be used to fill ballast tanks. Pristine aerobic coastal seawater from Key West, FL as well as associated marine sediment was collected as described by Lee et al. (2007). One liter of seawater was filtered through Nalgene filter units (PES membrane, 0.2 micron pore size, catalog number 568-0020) as soon as possible after sampling and the filters stored at ultralow temperatures ($-75^\circ$C). DNA was extracted from the filter using the MegaPrep UltraClean Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA) and concentrated by ethanol precipitation per manufacturer’s instructions. The same seawater was then used to construct incubations under strictly anaerobic conditions (Widdel and Bak 1992) in either the presence or absence of ULSD (0.25 g). Thirty ml of pristine Key West seawater and 15 g of sediments were added to the incubations. Sulphate depletion from Key West seawater incubations was monitored by ion chromatography as previously described (Aktas et al. 2010). All incubations were in sterile serum bottles and the experiment was conducted in triplicate. The serum bottles were closed with teflon stoppers that were held in place with aluminum crimp seals. The headspace was adjusted to N$_2$/CO$_2$ (8:2). Incubation of the bottles was in the dark at room temperatures for more than a year.

After incubation, each serum bottle was vortexed at high speed and as much fluid as possible immediately removed into a sterile 50 ml centrifuge tube. The fluid was centrifuged for 30 min at $6000 \times g$, the supernatant removed, and 250 ul PCR-grade water added to the pellet. The tubes were stored at ultralow temperatures until DNA extraction. The PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) was used to extract DNA, with the following modifications made to enhance lysis prior to bead-beating. The resuspended pellet was transferred into a screw-capped tube containing Lysing MatrixE Beads (MP Biomedicals, Irvine, CA), then the contents of the Powerbead Solution tube and 100 ul of solution C1 (both are components of the PowerSoil™ DNA Isolation Kit) were added to each screw-topped tube, the tubes vortexed then bead beaten for 90 s at 25,000 rpm (Mini-BeadBeater, Biospec Products, Bartlesville, OK). Extracted DNA from all seawater incubations was quantified using the Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay kit (Invitrogen Corporation Life Technologies, Carlsbad, CA). Clone libraries were created using the TOPO TA Cloning Kit (Invitrogen Corp, Carlsbad, CA). The number of sequences in each library was: Key West seawater (72), seawater/sediment rep 1 (40), seawater/sediment rep 2 (44), fuel unamended anaerobic incubation (44), and anaerobic incubation of seawater/sediment with ULSD (42).

**Sequence analysis of clone libraries**

Sequences were analyzed using the bioinformatics software package, mothur (Schloss et al. 2009). Unique sequences were trimmed to overlap a minimum of 450 bp and aligned using the NAST-aligner (DeSantis et al. 2006). Sequences were screened for chimeras using UChime (Edgar et al. 2011). A distance matrix was generated and used to cluster sequences into OTUs at the 97% similarity level using the average neighbor algorithm (Huse et al. 2010). A representative sequence from each OTU was assigned a taxonomic classification based on the naïve Bayesian classifier (RDP, Wang et al. 2007), set at 60% threshold level of confidence.

**Results**

**Bacterial communities in fuel**

A molecular survey of the microbial communities in fuel samples was conducted using two petroleum-based military fuels (JP5 and F76) and two ultra-low sulfur diesels (ULSD); one collected from a refinery and another from a typical supplier. In addition, a first-generation biodiesel (BD) composed of a mixture of soy-derived methylesters was similarly analyzed, as were two next generation biofuel mixtures consisting of the aforementioned petro-based fuels and hydropyro-processed bio-lipids from algae (algal-F76) or the camelina plant (camelina-JP5). Thus, the molecular survey was conducted for a total of six fuel samples. The analysis revealed that DNA could be extracted and amplified from all of the fuel samples except the ULSD and the methylester-based biodiesel. These two fuels obtained from the refinery were devoid of extractable DNA. Subsequent analysis focused on the remaining four fuel types and the bacteria were assayed in particular.

The dominant microflora associated with the other four fuels as detected by clone library analysis is...
depicted in Figure 1. The Proteobacteria was one of the most dominant phyla detected and ranged from a relative ratio of 35–61% in F76 and camelina-JP5, respectively. This phylum is divided into six classes (alpha through zeta), but only three of these taxa were represented in this survey. This included the Alpha-proteobacteria that exhibited a relatively narrow range of 22–35% depending on the particular fuel (Figure 1). A broader range (2–25%) was found for the Betaproteobacteria and only F76 harbored members of the Deltaproteobacteria (Figure 1). The absence of Deltaproteobacteria in fuels is notable since this group includes many genera of the sulfate reducing bacteria that have most regularly been associated with the biocorrosion of metal surfaces. However, even the F76 fuel had only a single representative of this class and it was a 97% match to the genus *Cystobacter*, a myxobacterium, that is not known to be associated with biocorrosion. The myxobacteria are noted for tolerating extreme conditions of temperature and desiccation characteristics (Reichenbach 1999) that might aid in their survival in high strength organic solvents.

Another prominent bacterial phylum found in the same four fuels was the Firmicutes. This taxon varied more widely as a proportion of the total fuel microbial communities (Figure 1). The lowest fraction of Firmicutes was found with F76 and constituted only 8% of the community. In contrast, this phylum represented from 29–45% in the other fuels. Unlike the Gram-negative Proteobacteria, most of these microorganisms are Gram-positive and have a low G+C (guanine-cytosine) content. Many Firmicutes produce endospores that tend to resist adverse environmental settings (Onyenwoke et al. 2004). Thus, these microorganisms are often found to at least tolerate extreme conditions of temperature, radiation, desiccation or other abiotic factors. The Firmicutes are typically divided into the anaerobic Clostridia and the aerobic or facultative Bacilli. Clostridial DNA was not recovered during the fuel analysis. All the Firmicutes detected were associated with the class Bacilli and *Tumebacillus* was, by far, the predominant Firmicutes found in the fuels. This genus includes two recognized species of Gram-positive, aerobic, spore-forming, rod-shaped, non-motile bacteria and both were isolated from soil (Steven et al. 2008; Baek et al. 2011).

The Actinobacteria comprised the next prominent bacterial phylum found in fuel. This group of microorganisms was readily detected in the fuels at levels that ranged from 8% of the microbial community in camelina-JP5 to 27% in the F76 fuel. The sequences were quite diverse and associated with 12 different families of Actinobacteria. The Actinobacteria are Gram-positive aerobes with a high (>50%) G+C content in their DNA, a feature that helps distinguishes them from the Firmicutes. As a group, they tend to be widely distributed in nature and representatives are known to survive for extended periods of time under adverse environmental conditions.

Most of the other types of bacteria detected were relatively minor members of fuel microbial communities. These included microorganisms belonging to the phyla Bacteroidetes, Chloroflexi, Deinococcus-Thermus, and the Gemmatimonadetes. Individually, these microbial groups constitute from 0–8% of the total fuel microflora. Most members of these phyla are composed of obligate or facultative aerobic bacteria. The last major group of microorganisms detected occurred strictly in the F76 diesel. This fuel has a 24% relative abundance of microorganisms belonging to the phylum Cyanobacteria (RDP Classifier) and also 99% identical to the sequence of chloroplasts from a variety of land plants (BLASTN searches, also RDP Classifier: Cyanobacteria_order_incertae_sedes, see Table S1) [Supplementary material is available via a multimedia link on the online article webpage]. It was difficult to

![Figure 1](image.png)
further characterize this group of bacteria (eg into class or family) of the Cyanobacteria as the quality of matches to known microorganisms was often <50%. Cyanobacteria is a phylum of bacteria that obtain their energy through photosynthesis, a process that is very unlikely in the fuel infrastructure. However, members of this group are known to produce resting structures such as akinetes (thick-walled dormant cells derived from the enlargement of a vegetative cell) that help them survive adverse environmental conditions.

**Q-PCR analysis**

Q-PCR was used to estimate the bacterial abundance in the various fuels. In this molecular determination, the number of bacterial 16S rRNA gene copies was estimated per volume of sample. The number of 16S rRNA gene copies ranged from 138 ± 13 to 12,800 ± 400 cell equivalents per ml of fuel (Table 1). The F76 fuel was associated with the greatest number of 16S rRNA gene copies per ml, while the other fuels contained ~100-fold fewer gene copies per ml.

**Bacterial communities in seawater and seawater incubations**

The fuel itself can clearly be associated with a bacterial microflora. Therefore, how the microorganisms in fuel compared to those in seawater (that would come in contact with the fuel under normal shipboard operations) was questioned. The dominant microbial communities in coastal seawater samples with and without associated sediment that might serve as an inoculant for the seawater-compensated fuel ballast tanks were compared. In addition, the same seawater was incubated under strict anaerobic conditions in both the presence and absence of ULSD. The same ULSD characterized in Figure 1 was used. Representative findings are compared for the seawater with and without ULSD (Figure 2). At the start of the incubation, the three most abundant bacterial groups in seawater were affiliated with the Alphaproteobacteria, Bacteriodetes, and Gammaproteobacteria. In many cases the highest similarity was the cloned sequences of samples from coral reefs, the Atlantic Ocean, Arabian Sea, and other marine environments. Such a community distribution can be considered typical for pristine aerobic coastal seawater samples. Not surprisingly, the Deltaproteobacteria were in very low abundance in this assessment of pristine aerobic coastal seawater. This finding is not synonymous with the absence of these microorganisms in seawater. Rather, they were clearly not the dominant phylotypes in this small clone library (72 sequences). The inclusion of seawater sediments (Seawater/Sediment Replicate 1 and Replicate 2) shows primarily a decrease in the proportion of Bacteroidetes

![Figure 2](image-url)
and an increase in Firmicutes (anaerobic clostridia) and Deltaproteobacteria.

In contrast, the incubation of seawater/sediments under strictly anaerobic conditions (‘Fuel unamended’) resulted in an extreme change in the microbial community profile (Figure 2) relative to the initial inoculum. There was an increase in the proportion of Deltaproteobacteria and other sequences not strongly affiliated to any described bacterial lineage (‘Unclassified’). In addition, the proportion of Alphaproteobacteria and Gammaproteobacteria decreased substantially as a fraction of the total microbial community. Comparable changes were found when the seawater was incubated in the presence of ULSD (Figure 2). Thus, the high proportion of Deltaproteobacteria and unclassified microorganisms in the fuel-unamended incubation suggested that anaerobiosis alone was responsible for the striking differences in the microbial community profile.

The microbial communities in seawater, whether at the beginning of the incubation or after exposure to anaerobic conditions, were quite different from those in the fuels. Figure 3 illustrates the extreme divergence among those bacteria present in fuels, vs those in seawater or seawater/sediment, and those exposed to anaerobic conditions (anaerobic incubation; eg fuel unamended and anaerobic ULSD). Note especially (see Table S1) [Supplementary material is available via a multimedia link on the online article webpage] that none of the sequences found in the fuels were present in the seawater or in the anaerobic incubation samples, and none of the seawater/anaerobic incubation sequences were detected in the fuel samples. Minor members of the seawater community were able to proliferate under strict anaerobic conditions and become dominant members of the resulting community. Moreover, the microbial successional events that occurred during the course of the strict anaerobic incubations must have been similar to result in a comparable relative enrichment of the Deltaproteobacteria and ‘unclassified’, at least at a rudimentary level of similarity. However, a more refined comparison of the types of Deltaproteobacteria (detected as operational taxonomic units (OTUs) at the 97% level of nucleotide sequence similarity (roughly the equivalent of species) shows two out of three OTUs in the fuel-unamended sample were also found in the ULSD sample (Desulfoarcina, 99.6% confidence level; Desulfovibrio, 99.9% confidence level). Eight other OTUs (all single sequences affiliated with the Desulfobacteraceae) were found only in the ULSD and 10 other sequences (affiliated with the Desulfobacteraceae, Desulfococcaceae, Desulfuromonadaceae, Sulfurovulaceae, Syntrophaceae, and Desulfovibrioaceae) were found only in the fuel-unamended sample (Tables S1 and S2) [Supplementary material is available via a multimedia link on the online article webpage]. Similarly, none of the ‘unclassified’ OTUs were found in both samples. However, the most abundant OTU in the seawater and seawater incubations occurred in both the anaerobic enrichment with ULSD (six sequences) and in one of the seawater/sediment samples (one sequence). It was most similar to an uncultivated bacterium obtained from a stratified lagoon in the North Pacific (Galand et al. 2012). While the number of sequences in each clone library was small, these findings suggest that the imposition of anaerobic conditions enriched a large number of different anaerobic species.

Discussion

Fuels that were obtained from refineries as opposed to commercial suppliers differed in their microbiological profile. Fuels obtained directly from a cooperating refinery (BD and ULSD) were completely devoid of extractable DNA. Presumably the high temperatures and pressures typically associated with refining processes destroyed bacteria, resting structures and even traces of DNA.

![Figure 3. Dendrogram showing similarity among fuel samples (Canelina-JPS, ULSD, Algal-F76 and F76), seawater and seawater sediment samples (Seawater, Seawater/sediment rep1, Seawater/sediment rep2) and anaerobic incubations of seawater without fuel (Fuel unamended) and amended with ULSD (anaerobic ULSD) based on a measurement of community structure ($\theta_{VC}$) (Yue and Clayton 2005). Note the fuel communities show some similarity with each other, particularly the Canelina-JPS, ULSD and Algal-F76, but that the seawater and the anaerobic incubations cluster separately from the fuels, reflecting the absence of sequences in common.](image-url)
However, it is reasonable to presume that downstream operations in the fuel infrastructure are not sterile and fuels obtained from commercial suppliers harbored a significant microflora (Figure 1). In a study by Lee et al. (2010), a series of experiments were carried out with alternative diesel fuels mixed with distilled water to assess microbiological contamination and corrosion. They observed that all the fuels supported biofilm formation and the microorganisms in the original fuels were dominant at the end of the incubation periods.

Sequencing revealed that the fuels contained a number of Gram-positive and Gram-negative bacteria. Many of the microorganisms are at least capable of forming resting structures that could conceivably survive exposure to fuel hydrocarbons. However, not all bacteria form resting structures and questions arise as to how microorganisms in general tolerate the exposure to hydrophobic liquids. The topic of solvent-tolerant microorganisms has been the subject of intense investigation. Literature reports indicate that both Gram-positive and Gram-negative microorganisms have unique strategies of solvent resistance, but also likely share some common mechanisms. The latter include energy-dependent efflux pumps that serve to export solvents to the external medium, cis-to-trans isomerization of unsaturated membrane fatty acids as well as modifications in the membrane phospholipid structure, the formation of vesicles loaded with toxic solvents, and changes in the biosynthesis rate of phospholipids to accelerate repair processes (Torres et al. 2011). Organisms or their enzymes that function in the non-aqueous phase are frequently sought out for biotechnological purposes (Gupta and Khare 2009).

Despite their ability to tolerate or even survive exposure to fuels, the question arises whether the fuel microflora has any particular role in the biocorrosion of the carbon steel infrastructure of a seawater compensated ballast tanks. Most of the microorganisms detected are only known to resist with oxygen as a terminal electron acceptor. To the authors’ knowledge, fuel bacteria detected in this study that may also survive as facultative microorganisms are not known to be associated with metal biocorrosion processes. Results from previous investigations demonstrate that coastal seawaters, including the samples assayed in this study, become oxygen-depleted, often within 24 h (personal communication J.S. Lee and B. Little) when exposed to fuels. It is also acknowledged that sulphide accumulation, resulting from microbial metabolic activities, represents the predominant biocorrosion mechanism for carbon steel in anaerobic seawater environments (Lee et al. 2006). Anoxic conditions and sulphide accumulation within marine biofilm/metal interfaces are independent of the bulk seawater oxygen concentration (Hamilton and Maxwell 1986).

However, strict anaerobes, most often associated with metal biocorrosion in seawater/fuel environments, were not found in the fuel per se. Further, the assay of these microorganisms in the initially pristine aerobic seawater samples were also negative and would lead to false confidence that these microorganisms were absent. It is noted that the lack of detection of particular groups at low frequency is especially likely when small numbers of sequences are used to survey microbial communities, as in this study. The mere incubation of seawater under anaerobic conditions was enough to cause major community shifts in the seawater microflora (Figure 2). Under these conditions, the Deltaproteobacteria, Gram-negative microorganisms that include a wide variety of anaerobic sulfate reducing prokaryotes, were clearly evident. This result occurred in either the presence or absence of ULSD. However, there was less phylogenetic diversity of delproteobacteria in the ULSD incubated with seawater, in that most sequences were affiliated with the Desulfo bacteraceae. Thus, the most reasonable conclusion would be that problematic anaerobes come from the seawater sediments where their initial population abundance was low, and that particular strains or species are selected by incubation with fuels under anaerobic conditions.

Quantitative-PCR was used to estimate the abundance of the bacterial population in fuels. Given that, 16S rRNA gene copies can vary by a log-fold on a per cell basis depending on the species of bacteria (Klappenbach et al. 2001), the q-PCR analysis revealed that the estimated numbers per ml of bacteria found in these fuel samples ranged from 10 to 100 (camelina-JP5) to 10^3 to 10^4 (F76). Exactly how these fuels accumulated cells to this extent is not known. As many of the detected sequences are most closely allied to microorganisms that are typical soil inhabitants, it may be that there was some inadvertent exposure of the fuel samples to soil at some point in their handling after the refinery. It is also possible that the DNA recovered and sequenced from the fuels may have originated from dead or dormant cells. Nevertheless, some exposure of the fuels to a contamination source would seem entirely likely.

**Conclusion and recommendations**

Traditional and next generation biofuels are not sterile and can harbor a rich microflora. Most likely, the fuels were contaminated sometime after refining and prior to their point of use. The microorganisms in these fuels are likely not those specifically responsible for fuel decomposition, sulfide formation or metal corrosion in
seawater systems, but are representative of microorganisms that tolerate extreme environmental conditions. It should also be pointed out that the assay of only six different fuels does not constitute an exhaustive evaluation of the subject. It would be a mistake to extrapolate these findings to conclude that concern over the microbiological contamination of fuels is unwarranted. For instance, perhaps the microbial contaminants could become more important for fuel deterioration or corrosion in non-marine systems. Like any analytical technique, the modern tools of molecular biology have inherent advantages and limitations. Nonetheless, this study shows that such procedures can be used to effectively track microorganisms through the fuel infrastructure.

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