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GEOCHEMISTRY AND MICROBIAL DIVERSITY COMPARISON OF TWO NATURAL HYDROCARBON SEEPS ON THE ISLAND OF BARBADOS

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ABSTRACT

This manuscript characterized the relative abundance and diversity of microorganisms present in two natural crude oil and bitumen seeps on the island of Barbados. The two natural macro-seeps, Turner Hall Woods and Conset Bay, are characterized by different environments: siliciclastic versus carbonate, fresh water versus salt water, and crude oil versus bitumen seepage. Samples were collected for microbial diversity assessment and water geochemistry analysis to determine if the possibility of microbial degradation of hydrocarbons has occurred. It was found that the two locations are similar at the phylum level yet differ greatly at the genus level, with the fresh water location being more diverse than the salt water location. Several hydrocarbon degrading bacteria were identified at both locations, making it possible for some degree of degradation to have occurred. Several unclassified species were also detected, thus opening the door for further research into what role each new species may play in the oil seep environments of Barbados. More research

should also be conducted to determine the metabolic activity of the known microbial species found at these oil seeps.

Keywords: geomicrobiology, hydrocarbon microbial communities, microbial diversity, Caribbean

INTRODUCTION

Microbes play a major role in the degradation of petroleum hydrocarbons in marine environments (Rosano-Hernandez et al., 2011). So-called hydrocarbonoclastic microbes hold the potential to be used for bioremediation, a natural “cleanup” process, in the event of an oil spill or in heavily polluted industrial areas. While 79 hydrocarbon-degrading bacterial species have been identified, 9 cyanophyte, 103 fungal, and 14 algal species are also capable of degrading hydrocarbons (Head *et al.*, 2006). Most hydrocarbon-degrading microbial species are capable of degrading only a limited number of organic compounds, and microbes are known to be active/or most effective in consortia (e.g. Vinas *et al.* 2001; Roling *et al.* 2002).

GEOLOGIC BACKGROUND

Barbados is located approximately 125 km east of the Lesser Antilles volcanic arc. Unlike this chain of islands, Barbados formed as an accretionary prism that was tectonically uplifted (e.g., Speed *et al.*, 2012). The oldest strata of the island are Eocene pelagic clastic sediments that have been tectonically deformed, overlain by Miocene chalks and marls, in turn overlain by Pleistocene carbonate terraces that have experienced extensive karstification (Speed 1990; Taylor & Mann 1991; Jones & Banner 2003a, 2003b; Machel *et al.* 2012; Kambesis and Machel 2013).

The oldest strata of Barbados are Eocene deep sea clastic sediments, referred to as the Scotland Formation. This sequence is composed of turbidites and gravity flows (Speed *et al.* 2012), which are extensively faulted and folded due to tectonic compression and mud diapirism, creating many small-scale structural traps for hydrocarbons (Payne *et al.* 1988). Natural seeps occur on the island ranging from gas to highly viscous bitumen (tar), locally known as manjak (Senn 1946; Schombourgh 1948; Parnell *et al.* 1994). A natural gas seep known as ‘Boiling Spring’ existed in this formation and was a curiosity for locals for many years prior to its destruction (Schombourgh 1948). Other natural oil seeps have since been discovered in the area as well. A light oil seep is located nearby in Turner Hall Woods, and a bitumen seep is found at Conset Bay (Figure 1). This natural resource supported bitumen mining in the early 20th century, as well as a small local oil industry that continues to thrive today.

The Scotland Formation, where both study locations are located, is overlain by Miocene chalks and marls, known as the Oceanics Group (Senn 1944). This sequence is hardly deformed except for a locally dense fracture network, which allows for the infiltration and/or passage of natural oil and gas through this otherwise impermeable rock sequence. The Oceanics Group is overlain by three major Pleistocene carbonate terraces, known as the Upper Coral Rock Terrace,

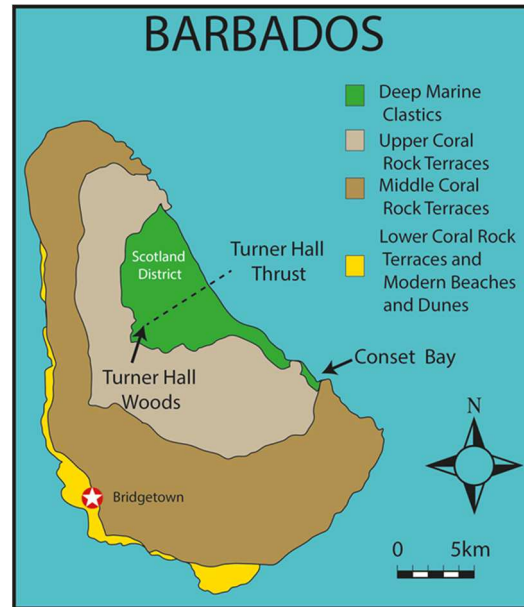


Figure 1 is a simplified geologic map of Barbados with the sampling sites of Turner Hall Woods and Conset Bay. Turner Hall Thrust Fault runs through the Scotland District and terminates at Turner Hall Woods. (Modified from Sumrall *et al.*, 2013)

Middle Coral Rock Terrace, and Lower Coral Rock Terrace (Figure 1), which cover about 70% of the surface of the island today. These terraces formed through tectonic uplift along with sea level rises and falls caused by glacial and interglacial periods (Schellmann and Radtke 2002, 2004). Oil and gas were derived from thermal maturation of organic-rich sediments – chiefly Kerogen III, with minor contributions of Kerogen II – in the accretionary prism (Speed 1987). Based on Sr- and C-isotope data in authigenic carbonates, the hydrocarbons have been seeping from the Eocene source strata into the overlying Oligocene chalks and Pleistocene carbonates since the Late Oligocene, with a spike around 5 m.y. ago (Sumrall *et al.* 2013; Machel *et al.* 2014). Additional information on the geotectonic and sedimentary evolution of the island can be found in Speed (1990, 2012) and Machel (1999, 2011).

The purpose of this study is to identify and compare microbial communities of a natural crude oil and a bitumen seep on Barbados. The oil seep in Turner Hall Woods is associated with a fresh water stream that cuts a deep incised

gully into siliciclastic sediments. The second locality is a bitumen seep in the chalks of Conset Bay located on the Atlantic coast; therefore, this area is influenced by wave action, sea spray, and tidal processes.

SITE DESCRIPTION AND FIELD OBSERVATIONS

There are significant differences in geological setting between the sampling sites of Turner Hall Woods and Conset Bay. The seep in Turner Hall Woods is found within poorly lithified, deep marine clastic sediments (Figure 2A). A fault, Turner Hall Thrust, runs through this area (Figure 1) and may be instrumental in the migration of crude oil to the surface. Several narrow fresh water streams run through this area and many small pools of oil in various consistencies and volatilities (ranging from light crude to cohesive, sticky orange, brown, and black crude oil) (Figure 2B) can be seen floating on top of the water for a considerable length of the streams.



Figure 2. A) Faulted oil stained rocks in Turner Hall Woods. These rocks are poorly lithified sandstones with intermixed clay layers. B) Orange organics growing in small pool of water and oil mixture located along the freshwater stream in Turner Hall Woods, camera cap for scale.

Conset Bay is located along the Atlantic coast and is influenced by seawater and tidal processes. The rocks that host the seep here are chalks of the Oceanics Group, which are nearly

impermeable, although a few beds appear to have sufficient matrix permeability to transmit hydrocarbons. Faulting likely enhanced the infiltration of bitumen (Figure 3A). In stark contrast to the seep at Turner Hall Woods, the hydrocarbons at Conset Bay are a highly viscous, sticky black tar. A shaggy, hunter green, algae-like material covers the bitumen in the intertidal zone (Figure 3B). Deeper into the intertidal zone, the material changes from hunter green to light green and has a rougher, less shaggy texture. At the base of the seep, light brown microbial mats are found.

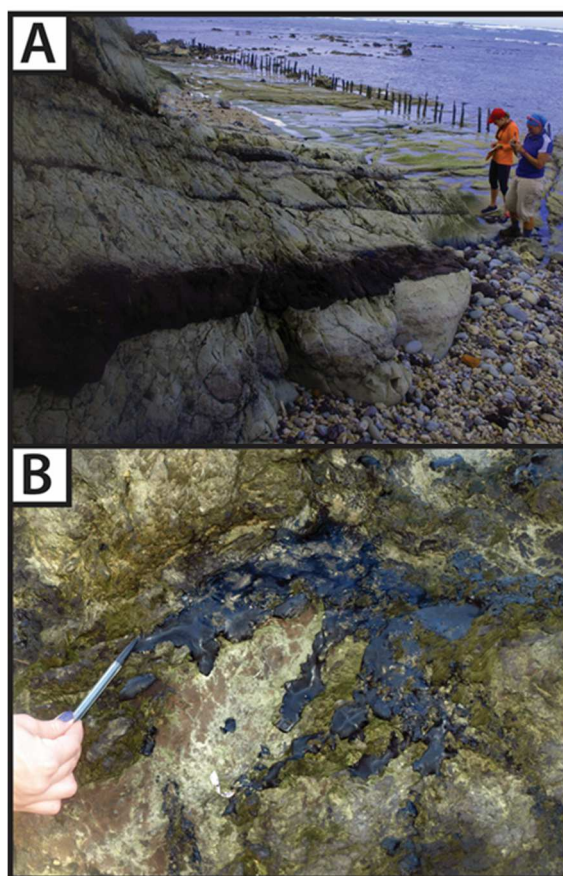
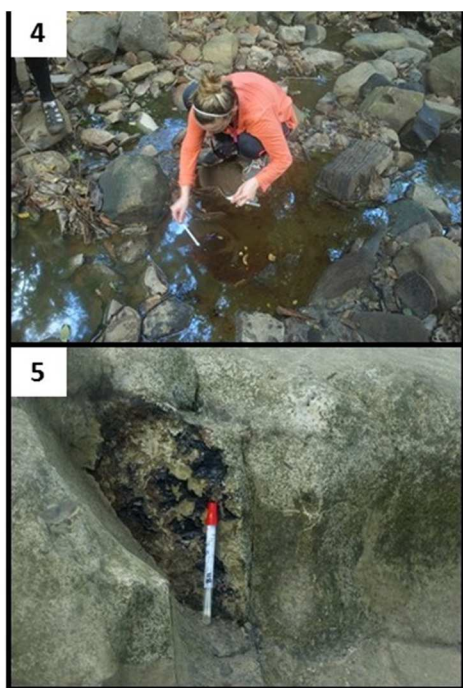


Figure 3. A) The rocks of Conset Bay are chalks. Chalks are typically impermeable and must have been faulted to allow the infiltration of hydrocarbons. B) Crystalline bitumen is pictured with a shaggy, hunter green algae-like growth on and surrounding the bitumen; mechanical pencil for scale.

METHODS

Fieldwork was conducted in late December to early January of 2013-2014. For this pilot study three 50 ml water samples and

five microbial swab samples were collected from separate localities within Turner Hall Woods; the samples were collected from water surrounding small pools of crude oil thought to be affected by microbial communities (Figure 4). Eight microbial swab samples were collected from Conset Bay (Figure 5). These samples were taken where microbes were growing on the bitumen substrate. Water samples were not collected from Conset Bay due to the high energy tidal environment of this area, and because the only water present in and around the outcrop is seawater. Multiple microbial samples were collected from each location to better ensure a successful study, in case of destruction during transport. These locations are difficult to access, and remote. The microbial samples were collected at the same locations as the water samples in progress pools of water around the oil seep.



Figures 4 and 5 depict the sampling process at Turner Hall Woods and Conset Bay, respectively.

The lower the sample number, the closer it was to the oil seep. All samples were collected at the surface from the aerobic zone.

Water Sampling procedure and analysis

In order to better understand the potential source of the water and salinity, water

samples were collected at Turner Hall Woods using unopened, sterilized, Nalgene sampling bottles. The bottles were completely filled with the samples and allowed no head space. They were then capped and wrapped with parafilm to prevent contamination. The bottles were refrigerated at 4° C until analysis. The samples were analyzed within a month of returning from Barbados using Inductively coupled plasma-optical emission spectroscopy (ICP-OES) at the Texas Research Institute of Environmental Studies (TRIES) at Sam Houston State University.

Aliquots (of 25 ml) were acidified for four hours using 0.5 mL concentrated HNO₃ and 1.25 ml concentrated HCl. The volume was then diluted to 25 ml using distilled water before analysis. Samples with oil residue were filtered with a 0.45 micron filter prior to analysis.

Microbial sample processing and sequencing:

Sample processing, 16s rRNA gene amplification and sequencing was performed through the Alkek Center for Metagenomics and Microbiome Research and the Human Genome Sequencing Center (Baylor College of Medicine, Houston, TX) following protocols benchmarked as part of the Human Microbiome Project

(http://www.hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf, PMID 22699609, PMID 22699610). Bacterial genomic DNA was extracted from samples using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). The V4 region of the bacterial genomic DNA was amplified using barcoded primers 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR reaction contained the following: 2uL 4uM barcoded primer stock, 5uL DNA, 2uL Taq Buffer II (Invitrogen), 0.15uL Taq enzyme (Invitrogen), and 10.85uL PCR-grade water. The reactions were amplified in an Eppendorf mastercycler Thermocycler under the following conditions: initial denaturation step for 2 minutes at 95oC, followed by 30 cycles of 20 second denaturation at 95oC, 45 seconds of annealing at 50oC, and 90 second annealing at

72oC. Barcodes allowed pooling of samples for sequencing. All samples were pooled and sequenced on one lane of an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA) at the BCM Human Genome Sequencing Center.

16S rRNA gene data analysis:

Using custom perl scripts, Reads 1 and 2 were pre-quality filtered by trimming the sequences at the first ambiguous base and then joining reads 1 and 2 with a minimum required overlap of 12 bases. Two samples (B7 and B9) did not have any reads and were not included in the subsequent analysis. Sequences were then de-multiplexed and quality trimmed using QIIME version 1.8 (PMID 20383131). Sequences with any ambiguous bases, with a phred quality score less than 20, and with more than 1.5 barcode errors were discarded and not used in further analyses. Quality trimming resulted in 310,826 high quality reads. Sequences were binned into OTUs and assigned taxonomy by clustering sequences using UCLUST against a reference database (the Greengenes May 2013 release) at 97% identity. The OTU table was then filtered to remove any OTUs that composed less than 0.0005% of the entire dataset (roughly the equivalent of singleton OTUs) and rarefied to 5651 sequences per sample (the lowest number of reads associated with any one sample). Diversity was assessed by calculating the Shannon diversity metric and the number of observed species for each sample, from 10 to 5,510 reads per sample (steps of 500 reads per sample). Non-parametric t-tests using Monte Carlo permutations (999) to calculate the p-value (Bonferonni corrected) determined significant differences in diversity between groups. Stacked bar charts were also constructed to visualize the taxa present in each sample and across sample groups.

RESULTS

Water Samples

Samples B2-W, B3-W, and B5-W were collected from a freshwater stream that runs

through Turner Hall Woods to better understand the chemistry of the water in the pools nearest the oil seep. Three samples were taken in the three surrounding pools of water with sample B2-W being the closed pool to the seep and containing large amounts of oil in the water, B3-W was collected from the next closest pool, and B5-W was the furthest from the seep being approximately 5 feet away. Sample B2-W (filtered) contained petroleum and was filtered prior to analysis. The elements found in highest abundance in the three water samples from Turner Hall Woods are listed in Table 1. High values of calcium (>30 ppm), magnesium (>65 ppm), and sodium (>180ppm) were observed for all three water samples. Sample B2-W possesses significantly higher quantities of magnesium, sodium, and sulfur in relation to the other two samples. Sample B3-W is characterized by a higher abundance of aluminum, iron, and silicon when compared to the other samples. Data for sample B5-W is an even distribution between the three samples.

Microbial Swab Samples

The richness and evenness of the sequenced microbial communities sampled were surveyed using the Shannon diversity index. The sequenced microbial communities at Turner Hall Woods are more diverse than those at Conset Bay (Figure 6). When the data were organized to a phylum level, it is clear the two locations differ (Figure 7). Both locations are dominated by Proteobacteria; however, Turner Hall Woods has a higher relative abundance of this phylum than did Conset Bay. Cyanophytes represent an abundant proportion of the sequenced community at Conset Bay, yet not Turner Hall Woods. At the phylum level, both locations are composed of similar relative abundances of Firmicutes, Bacteroidetes, and Actinobacteria.

The differences in sequenced microbial communities between the two sample locations

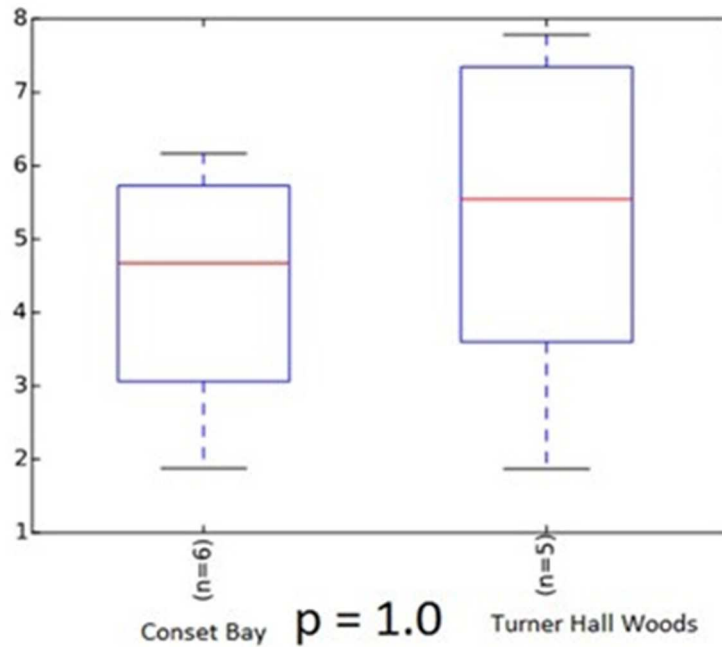


Figure 6- A box plot diagram for the Shannon Diversity Index of Turner Hall Woods and Conset Bay.

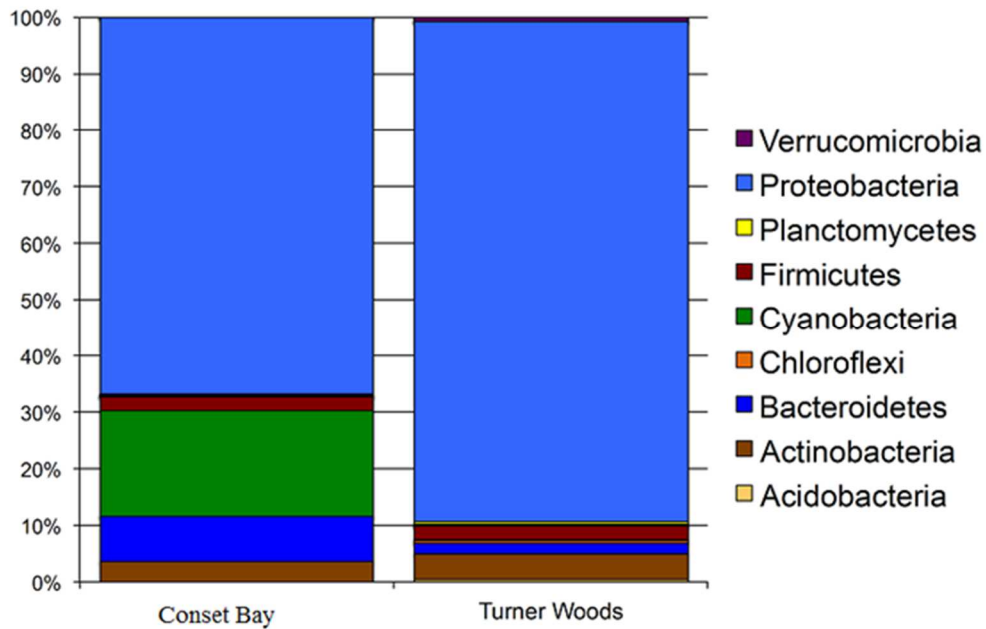


Figure 7. Microbial community data is broken down to the phylum level from Turner Hall Woods and Conset Bay. Both samples are dominated by Proteobacteria. Turner Hall Woods contains roughly 90% Proteobacteria while Conset Bay is characterized by approximately 65%.

becomes more apparent when examined at the genus level (Figure 8). Turner Hall Woods is more diverse at the genus level than Conset Bay, meaning more genera are present at Turner Hall Woods than Conset Bay. The bitumen from

Conset Bay harbors several marine genera including *Pseudoalteromonas*, an unclassified genus of the family Rhodobacteraceae, an unclassified genus of the order Stramenopiles, *Vibrio*, *Rivularia*, an unclassified genus of the

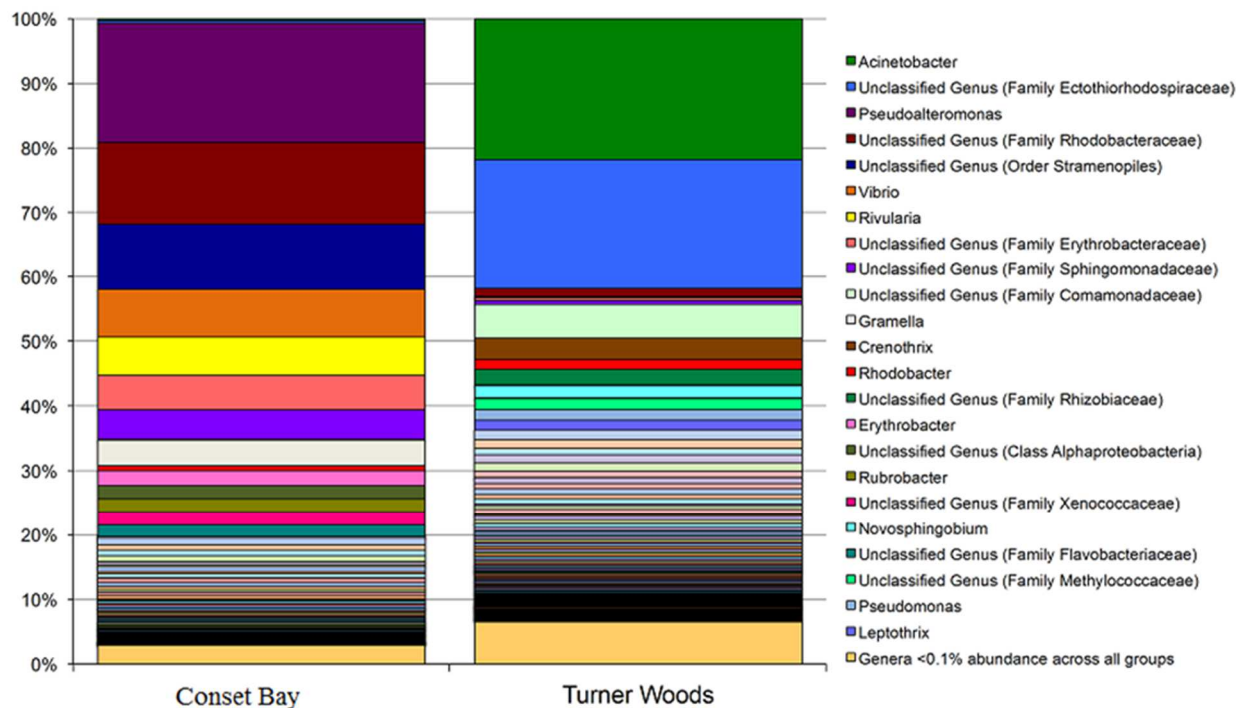


Figure 8. The microbial communities are displayed at the genus level. Turner Hall Woods is mainly dominated by *Acinetobacter* while Conset Bay is characterized by *Pseudoalteromonas*. Each genus is represented by a color and its percentage present is read from the left-hand percentage column.

family Erythrobacteraceae, and *Gramella*. Turner Hall Woods is mainly composed of *Acinetobacter*, an unclassified genus of the family Ectothiorhodospiraceae, an unclassified genus of the family Comamonadaceae, *Crenothrix*, *Rhodobacter*, and an unclassified genus of the family Rhizobiaceae.

These apparent differences are also seen when the data are visualized on a sample-by-sample basis (Figure 9). All microbiome samples from Turner Hall Woods are composed of *Acinetobacter* (4% - 85%), while samples from Conset Bay are composed of only 0% to 3%. All Turner Hall Woods microbiome samples are also dominated by an unclassified genus of the family Ectothiorhodospiraceae (1% - 52%) and an unclassified genus of the family Comamonadaceae (1% to 15%). Conset Bay microbiome samples are dominated by *Pseudoalteromonas* (0% - 86%) and an unclassified genus of the family Rhodobacteraceae (0% to 15%), followed by an

unclassified genus of the family Erythrobacteraceae (0% - 22%).

DISCUSSION

Water Samples

The water samples from Turner Hall Woods have elevated concentrations of calcium, magnesium, and sodium compared to standard (normal) seawater (Table 1). Furthermore, the ratios of the three major ion concentrations in the samples from Turner Hall Woods differs from the same ratios in standard (normal) seawater (Table 1). The higher concentrations of calcium, magnesium, and sodium and accompanying elevated ionic ratios are interpreted to result from inorganic salts and brine carried by the hydrocarbons present at Turner Hall Woods. Inorganic salts are known to be suspended in crude oils or dissolved in associated waters (Kraus 2011).

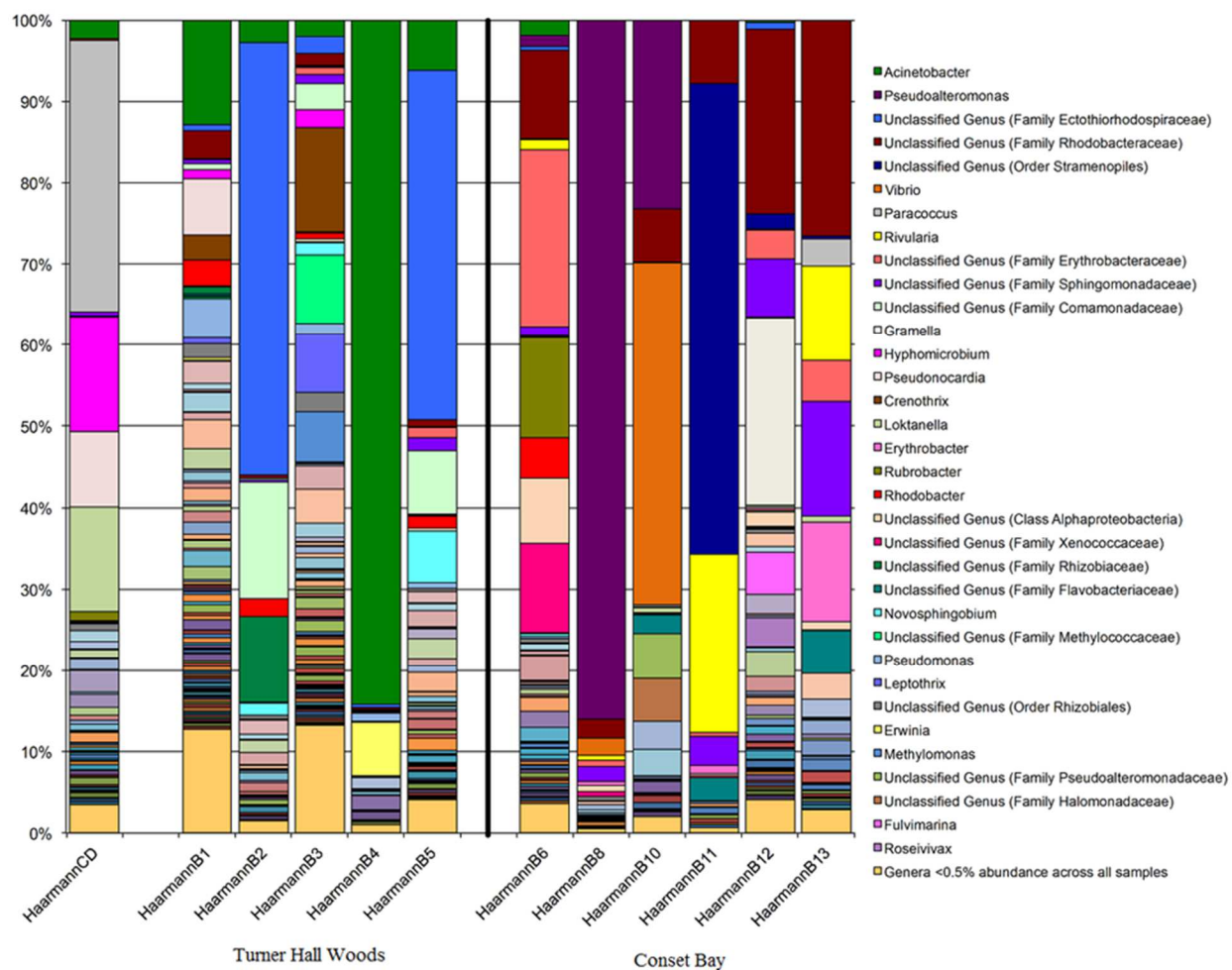


Figure 9. Microbial community data is displayed on a sample-by-sample basis. Each genus is represented by a color and the percentage present is read from the left-hand percentage column. Sample CD data was not included in this study, and Samples B7 and B9 were not analyzed.

	Al (ppm)	Ca (ppm)	Fe (ppm)	K (ppm)	Mg (ppm)	Na (ppm)	S (ppm)	Si (ppm)
B2-W (filtered)	0.3	34.4	0.2	28.0	123.7	345.1	202.7	23.0
B3-W	13.7	32.3	34.7	29.0	68.0	191.5	92.8	66.5
B5-W	0.3	32.0	0.1	25.8	69.0	187.0	99.6	20.8

Table 1. Table of elements found in highest abundance in Barbados water samples. Samples B2-W, B3-W, and B5-W were collected from a freshwater stream that runs through Turner Hall Woods. Elements present are listed as parts per million in columns and sample numbers are listed as rows. Sample B2-W (filtered) contained petroleum and was filtered prior to analysis.

In addition, two water samples from Turner Hall Woods have elevated concentrations of sulfur (B2-W), aluminum (B3-

W), and iron (B3-W). Sample B2-W was collected from an oil-filled ‘pool’ as an oil-water mixture. Thus it is possible, if not likely,

that the higher concentration of sulfur observed in this water sample is derived from the associated oil, either via dissolution of organosulfur compounds in the water, or from microbial sulfate reduction, which can be viewed as a form of anaerobic biodegradation of oil (Machel 2001; Hill and Schenk 2005). The higher concentration of iron in sample B3-W possibly resulted as a by-product of biodegradation of the oil, which may happen aerobically or anaerobically. At Turner Hall Woods, both options are possible, i.e., biodegradation is likely to happen anaerobically some distance from the surface yet aerobically right at the surface, rendering iron predominantly in the ferrous or ferric state, respectively (e.g., Machel 1995). Sample B3-W was collected from a surface pool of petroliferous water that contained an orange mucilaginous microbial community. Genera of iron-oxidizing bacteria such as *Crenothrix* and *Leptothrix* (Cullimore and McCann 1978) are present in the microbial analysis of the same sampling site. A reason for the elevated aluminum concentration is not evident.

Microbial Swab Samples

Turner Hall Woods and Conset Bay differ at the phylum level. Both locations are dominated by Proteobacteria, but the relative abundances of Proteobacteria are higher in the siliclastic sediment environment of Turner Hall Woods. Many well-known species present in hydrocarbon degrading environments are members of the Proteobacteria phylum. It is possible the Proteobacteria present at these locations are functioning as hydrocarbon degraders considering the environment, but more work needs to be done to determine metabolic activity of the specific species sequenced. A study of petroleum influenced beach sediments from a siliclastic and carbonated region in Mexico produced similar results (Rosano-Hernandez 2011). In addition, this study found Alphaproteobacteria and Gammaproteobacteria to be the most abundant classes, and the siliclastic sediments were

more diverse than the carbonate sediments (Rosano-Hernandez 2011).

Cyanobacteria are more abundant at Conset Bay than Turner Hall Woods, which is likely due to the presence of microbial mats at Conset Bay. Cyanobacteria are adapted to withstand low nitrogen environments and can fix nitrogen themselves (Sanchez 2005). Cyanobacteria (Cyanophyte) are unlikely to degrade petroleum compounds, but may play an indirect role in mixed populations and support the growth of those microorganisms that degrade petroleum (Sanchez 2005). Both sites had Firmicutes, Bacteroidetes, and Actinobacteria present at the phylum level. Similar microbes were found in Brazilian oil fields (Silva 2012).

The richness of Turner Hall Woods is greater than Conset Bay at the Genus level (Figure 8). The diversity of microbes within each site, sample compared to sample, (Figure 9) is also notable. Greater diversity at the Genus level is present in the samples closest to the oil seeps. More research could help determine metabolic functions that may lead to a better understanding of these locational differences. Both locations are characterized by common microbes found in hydrocarbon degrading environments. Turner Hall Woods is strongly influenced by *Acinetobacter* and an unclassified genus of family Ectothiorhodospiraceae. *Acinetobacter* is typically associated with aerobic hydrocarbon degradation and is found worldwide (Silva 2012). Conset Bay's microbial community consists of several marine genera such as *Pseudoalteromonas*, *Vibrio*, an unclassified genus of family Rhodobacteraceae, and an unclassified genus of family Erythrobacteraceae. The Erythrobacteraceae are known to metabolize organic substrates, with light enhancing their growth (Röling 2002). This family is also known to play a critical role in the carbon cycle in the ocean (Röling 2002), so its presence at Conset Bay is not surprising. The genera distribution at both locations is somewhat surprising. *Pseudomonas* and *Vibrio* are among the most common hydrocarbon

degraders globally (Rosano-Hernandez 2011), yet *Pseudomonas* are not found in high abundances at either location, and only one sample at Conset Bay contained *Vibrio* (Figure 9).

In marine settings, a highly specialized genera, *Marinobacter*, was found to dominate in hydrocarbon-rich areas (Rosano-Hernandez 2011). Because of its ability to outcompete other microbial competitors, it was surprising that *Marinobacter* was not found within samples from Conset Bay. Certain strains of *Marinobacter* thrive when degrading hydrocarbons in moderate to hypersaline conditions (Al-Mailem *et al.* 2013). The salinity values may play a role in the lack of *Marinobacter* found in Conset Bay. It is also possible the absence of *Marinobacter* at Conset Bay could be due to nature of the highly viscous hydrocarbons found in Conset Bay.

While many of the genera present at the two hydrocarbon seeps in Barbados are known to be present in hydrocarbon degrading environments, several are known for different applications. Many of the genera present are more commonly associated with the medical field and are human pathogens. While these pathogenic bacteria typically use a human host as a carbon source, they could now be adapting to thrive off hydrocarbons. Higher petroleum concentration often produces lower microbial diversity (Rosano-Hernandez 2011). Our samples from Turner Hall Woods and Conset Bay also contain several unclassified genera. With bacteria known for different functions as well as several unclassified genera, further research into what role each microbe plays in the degradation of hydrocarbons from Barbados is necessary. The role each organism plays in biodegradation and their abundances have the potential to be applied to recreate consortiums aiding in the remediation of oil spills. The two oil seeps from vastly different environments were analyzed, allowing for the availability of a broader spectrum of microbial consortiums. Habitats in need of bioremediation vary geochemically as well as biologically. This study characterizes the microbes of specific

marine-influenced bitumen seeps and a lighter oil seep. This could be beneficial when designing future bioremediation communities for similar geochemical or biological settings. Further data collection and analysis efforts in Barbados including in-depth water, oil, and substrate sampling will enable greater understanding of these unclassified genera and their potential usage.

CONCLUSIONS

The two natural hydrocarbon seeps on Barbados offer a unique opportunity to observe and compare contrasting seep environments. Turner Hall Woods and Conset Bay differ in the environments they are characterized by as well as the microbial communities that reside at each location. Turner Hall Woods is more diverse than Conset Bay and both areas are mostly characterized by well-known hydrocarbon degraders. The presence of these hydrocarbonoclastic bacteria indicate the possibility of hydrocarbon biodegradation. Further research focused on the role these microbes play in the degree of biodegradation and metabolic activities should be pursued, as well as their potential use in bioremediation.

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