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**METABOLIC ACTIVITIES AND DIVERSITY OF MICROBIAL COMMUNITIES
ASSOCIATED WITH ANAEROBIC DEGRADATION:
IMPLICATIONS FOR BIOREMEDIATION AND BIOENERGY**

by

VERLIN RYAN PERRY

Under the Direction of Dr. Kuk-Jeong Chin

ABSTRACT

Sulfate- and Fe(III)-reducing, and methanogenic prokaryotes (SRP, FRP, MGP) are key players in metabolic pathways involved in anaerobic biodegradation processes. Understanding the metabolic activity of these microbes in environments can enhance microbe-mediated processes such as oil spill bioremediation and methane biogas production. In this study, anaerobic microbial activities in Deepwater Horizon oil spill-impacted salt marsh sediments, and in methanogenic coal bed production water enrichment cultures amended with trace elements (TE), were elucidated by employing an approach combining methods in molecular biology and geochemistry. In situ metabolic activity of SRP, FRP and MGP were monitored seasonally and

metabolically-active communities were identified in oil-impacted sediments using quantitative real time Reverse Transcription -PCR and clone library analysis of key functional genes: Dissimilatory (bi)sulfite reductase (*dsrAB*), Geobacteraceae-specific citrate synthase (*gltA*), methyl coenzyme M reductase (*mcrA*), and benzyl succinate synthase (*bssA*). In situ application of montmorillonite clay was assessed for its potential at accelerating PHC degradation by stimulating microbial activities. Levels of *dsrA*, *gltA* and *bssA* transcripts suggested that PHC-oxidizing SRP are more active in summer while FRP are more active in winter, indicating their activities linked to the seasonal changes of redox potential and vegetation. BssA gene expression peaked in winter, and was highest at more highly oil-impacted sites. Expression of all genes was higher in clay-amended sites. *bssA* transcript level and Fe(II) production were highest in clay-amended microcosm. Total petroleum hydrocarbon (TPH) levels were lower in oil and clay-amended microcosm incubation than one with oil only amendment, suggesting enhanced TPH degradation by clay amendment. Pyrosequencing analysis 16S rRNA gene in clay-amended microcosms demonstrated the highest percentage abundance of groups closely related to known anaerobic aromatic degraders. Levels of *mcrA* transcripts correlated with methane production rates in TE-amended coal bed production water enrichments. The findings of the present study clearly support the advantage of gene expression analyses for estimating microbial activity. To the best of our knowledge, this is the first in situ study which employs key functional gene markers as molecular proxies for metabolic activity and diversity assessments in anaerobic oil-contaminated salt marsh sediment and also elucidates clay-enhanced in situ TPH degradation.

INDEX WORDS: Anaerobic, Gene Expression, Metabolic Activities, RNA, Bioremediation, Biodegradation, Petroleum Hydrocarbons, Oil Spill, Deepwater Horizon, Salt Marsh, Methane, Biogas, Coal Bed Methane, Microbial Ecology, Environmental Microbiology

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Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2014

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Verlin Ryan Perry
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DEDICATION

This work is dedicated to Mother Earth.

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1 INTRODUCTION

In diverse environments where molecular oxygen is limited or absent, microorganisms capable of utilizing alternative terminal electron acceptors thrive by the process of anaerobic respiration. Specific terminal electron acceptors such as nitrate, sulfate, Fe(III), or carbon dioxide vary depending on redox conditions within a particular environment and physiological adaptations of resident microorganisms (Canfield, 2005; Madigan *et al.*, 2008). As the fields of microbial ecology, geomicrobiology, and biogeochemistry evolve, it has been well known that these anaerobic, microbial respiratory processes have significant influence on the geochemistry of natural environments (Lovley and Phillips, 1994; Lovley and Coates, 2000; Roden, 2006). Of particular importance is the relationship between microbial metabolic activities and carbon turnover, inorganic nutrient cycling, and- perhaps most relevant in light of ongoing, worldwide environmental degradation- contaminant remediation and renewable bioenergy production (Phelps *et al.*, 1994; Lovley, 1995; Lovley and Coates, 2000; Lovley, 2003; Meckenstoch *et al.*, 2004; Madsen, 2005; Ünal *et al.* 2012). The present study aims to estimate *in situ* microbial activities involved in anaerobic respiration in respect to two scenarios: (1) anaerobic petroleum hydrocarbon bioremediation in an oil spill impacted Louisiana salt marsh, and (2) trace element enhanced coal bed methane biogas production in production water enrichment cultures.

1.2 Anaerobic petroleum hydrocarbon degradation

Aliphatic and aromatic hydrocarbons are ubiquitous in the environment due to natural biogeochemical processes that have persisted for millennia. Although the introduction of such materials into the environment is not uniquely attributed to human activities, the processes of petroleum extraction, refinement, transport, and combustion have drastically increased their accumulation (Widdel and Rabus, 2001). The deleterious consequences of environmental contamination by petroleum hydrocarbons has been a driving force of the study of eco-friendly and efficient remediation strategies of petroleum-impacted sites (Lovely, 2003). Aerobic hydrocarbon degradation by microorganisms has been studied in depth for many decades. However, even oxygenated environments often quickly become anaerobic due to accelerated microbial respiration coupled to contaminant oxidation. Moreover, diverse terrestrial and marine environments susceptible to petroleum contamination are temporally or permanently anaerobic (Coates and Achenbach, 2001).

In-depth study of anaerobic hydrocarbon biodegradation has shed light on the dominant microbial groups and their respective terminal electron acceptors that operate in various anaerobic environments. Isolation, cultivation and characterization of microorganisms capable of hydrocarbon degradation coupled to various anaerobic respiratory processes have been well reported with a focus on dissimilatory nitrate-, iron(III)-, and sulfate-reduction (Fukui *et al.*, 1999; Meckenstock, 1999; So and Young, 1999; Coates *et al.*, 2001; Chakraborty and Coates, 2004; Callaghan *et al.*, 2006; Prakash *et al.*, 2010; Callagan *et al.*, 2012; Kanak *et al.*, unpublished; ;). Meckenstock (1999) reported the

first example of anaerobic toluene oxidation by a dissimilatory iron-reducing bacterium in syntrophic association with nitrate- or fumarate-reducing bacteria. Furthermore, the study described the isolation of a syntrophic, dissimilatory sulfate-reducing bacterium capable of complete toluene oxidation. Coates and colleagues (2001) demonstrated complete benzene oxidation by pure cultures of nitrate-reducing strains of *Dechloromonas* species. A dissimilatory iron(III)- and uranium(VI)-reducing bacterium in *Geobacter daltonii* strain FRC-32 has been isolated from an aquifer contaminated by both radionuclides and hydrocarbons. This strain exhibits tremendous metabolic versatility and has been demonstrated to completely degrade various aromatic hydrocarbons (Prakash *et al.*, 2010; Kanak *et al.*, unpublished). Isolates capable of alkane degradation coupled to dissimilatory sulfate and iron reduction have also been reported along with their respective genetic basis for initial anaerobic alkane oxidation (So and Young, 1999; Callaghan *et al.*, 2006; Callaghan *et al.*, 2012). These studies on pure cultures have led to the elucidation of metabolic pathways, including their genes and gene products, that are responsible for such processes and provide invaluable information used to predict and model these activities both in novel pure culture isolates and within mixed communities (Chakraborty and Coates, 2004; Callaghan *et al.*, 2012). Novel strains capable of growth on a variety of petroleum hydrocarbon substrates, including polycyclic aromatic hydrocarbons, and associated compounds are being cultivated with increased frequency.

In addition to studies on pure cultures, considerable efforts to characterize microbial community responses to and effects on petroleum hydrocarbon contamination have been carried out by numerous research groups (Watanabe *et al.*, 2000; Yagi *et al.*,

2010). Both dissimilatory sulfate- and iron-reducing activities by metabolic consortia have been shown to anaerobically degrade benzene in petroleum-contaminated harbor sediments (Coates *et al.*, 1997) and mixed communities concurrently utilizing several terminal electron acceptors have been investigated for their metabolic potential at polycyclic aromatic hydrocarbon bioremediation in terrestrial aquifers (Yagi *et al.*, 2009). Though a pure culture isolate is lacking, anaerobic petroleum hydrocarbon degradation by methanogenic archaeal communities is now understood to occur (Jones *et al.*, 2008). Through interdisciplinary study of pure cultures and microbial community in environmental samples, a generalized understanding of the energy yields associated with hydrocarbon degradation coupled to reduction of nitrate, Fe(III), and sulfate and methanogenesis has been established. Moreover, comprehensive studies with pure cultures, enrichment cultures, and environmental matrices have provided the physiological knowledge base for the development of molecular proxies for the study of both anaerobic reductive and oxidative respiratory processes *in situ* (Chin *et al.*, 2008; Akob *et al.*, 2012; Ünal *et al.*, 2012).

One of the early steps of alkane and aromatic hydrocarbon degradation is assumed to involve the activation of a methyl group by the addition of a fumarate group to the alkyl carbon (Biegert *et al.*, 1996; Beller and Spormann, 1997; Beller *et al.*, 2002; Washer and Edwards, 2007). The gene products responsible for fumarate addition are best characterized in the alkane-degrading, sulfate-reducing bacterial strain AK-01 (Callaghan *et al.*, 2008) and in toluene-degrading nitrate-, sulfate- and iron-reducing strains (Rabus and Heider, 1998; Beller and Spormann, 1997). Genome mining,

bioinformatics analysis, and growth experiments suggest that 2-methylnaphthalene undergoes a homologous activation step in sulfate-reducing enrichment cultures and by strain N47 (Musat *et al.*, 2009; DiDonato *et al.*, 2010; Selesi *et al.*, 2010). The resulting intermediates formed from fumarate addition as a co-substrate are (n)alkylsuccinate, benzylsuccinate, and naphthylmethylsuccinate. The operons encoding each glycy radical transferase share significant sequence homology, particularly in within the alpha-subunit (alkylsuccinate synthase A, *assA*; benzylsuccinate synthase A, *bssA*; and naphthonethylsuccinate synthase A, *nmsA*) and, therefore, can serve as an useful metabolic biomarker for monitoring petroleum hydrocarbon-degrading activities *in situ* by groups of metabolically diverse microorganisms (Acosta-Gonzalez *et al.*, 2013). Preliminary evidence suggests that anaerobic benzene degradation by hydrocarbon-degrading *G. daltonii* strain FRC-32 might be initiated by the methylation of benzene to toluene, thus utilizing the *bss* pathway for further catabolism (Kanak *et al.*, unpublished). This information indicates that *bssA* genes may be significant candidate biomarker for anaerobic petroleum hydrocarbon degradation (Figure 1.1).

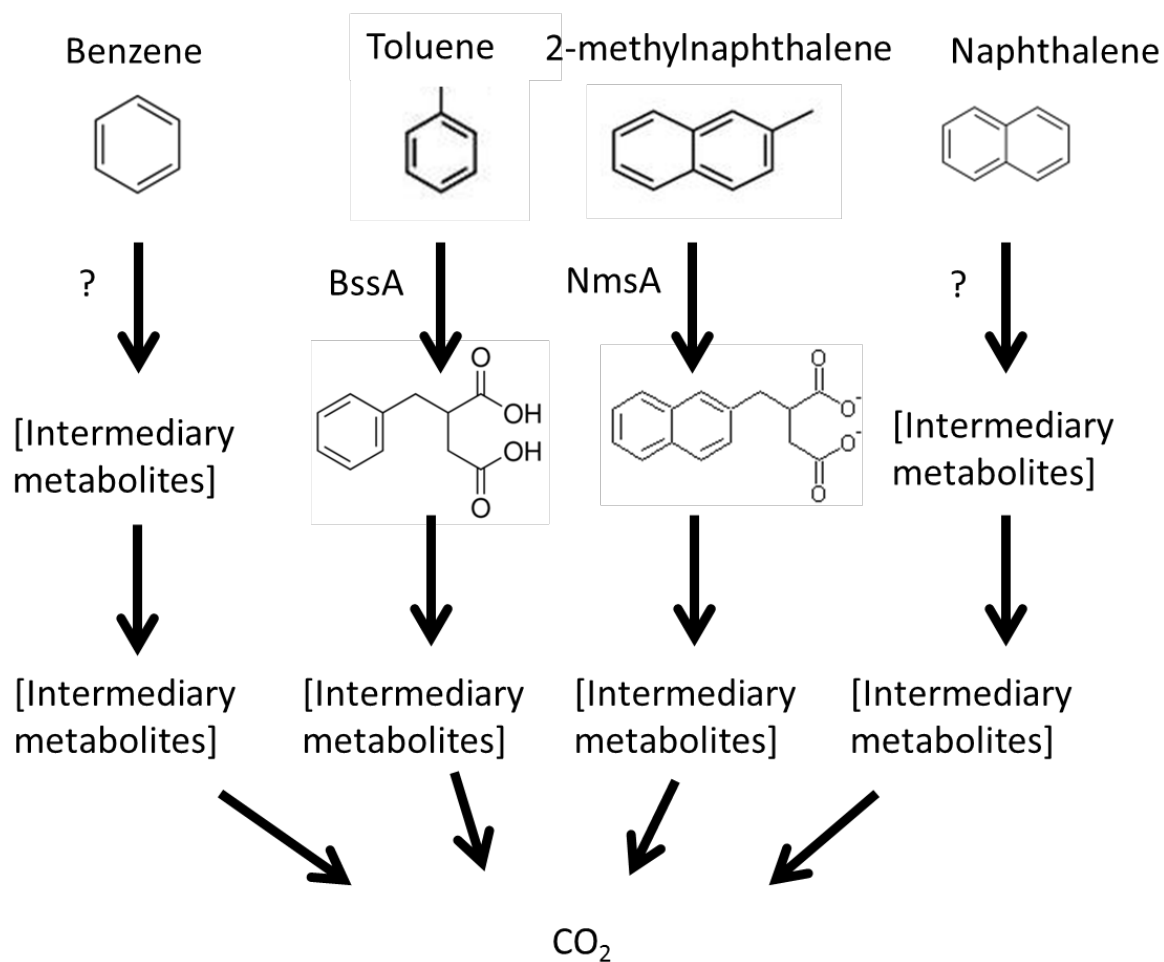


Figure 1.1. Generalized proposed pathways for anaerobic alkane, monoaromatic and polycyclic aromatic hydrocarbon degradation. Intermediary metabolites are largely uncharacterized for the various pathways.

1.3 Methodology of microbial metabolic activity analysis

Biogeochemical modeling of processes, including rates of anaerobic respiration, in environments of interest have traditionally relied on complex methodologies such as measuring the disappearance of added substrates or tracking the transformation of radiolabeled tracers (Jin and Bethke, 2002; Murphy and Schramke, 1998). These techniques are limited by the feasibility of substrate addition or collection of radiotracers in a complex, open system and also suffer from bias due to perturbations of the system by exogenous substrate addition resulting in overestimation of carbon turnover (Phelps *et al.*, 1994). While the geochemical dimension of microbe-mediated environmental processes is paramount for establishing working models, the key to understanding the role of microorganisms in such processes lies in direct assessment of the community structure and rates of metabolic activities *in situ* (Murphy and Schramke, 1998). Advances in molecular biology offer methodologies capable of such assessments without the challenges presented from geochemical manipulations. Sequencing of reverse-transcription PCR (RT-PCR) amplicons and quantitative real-time PCR (qPCR) amplification of functional gene transcripts of biogeochemical interest can provide a significant insight into microbe-mediated processes of biogeochemical importance (Akob *et al.*, 2009; Akob *et al.*, 2012; Chin *et al.*, 2004; Chin *et al.* 2008; Ünal *et al.*, 2012).

Anaerobic sulfate-reducing activities by dissimilatory sulfate-reducing microbial communities in petroleum-contaminated marine harbor sediments have been shown to

correlate with contaminant degradation (Coates *et al.* 2001; Chin *et al.*, 2008). The dissimilatory (bi)-sulfite reductase (*dsr*) gene has shown promise as a metabolic biomarker for phylogenetic and activity assessment within environments of interest, being highly conserved amongst sulfate-reducing prokaryotes and integral to the final electron transferring step of sulfate respiration (Wagner *et al.*, 1998; Wagner *et al.*, 2005; Chin *et al.* 2008).

Dissimilatory Fe(III) reduction is an important anaerobic respiratory process of relevance to mineral transformations and petroleum hydrocarbon degradation in subsurface marine environments (Lovely *et al.*, 1995a; Lovley, 1995; Roden, 2006; Zhang *et al.*, 2010; Zhang *et al.*, 2012). Several iron-reducing strains capable of hydrocarbon degradation, including benzene and toluene, have been grown in pure culture allowing for the determination of critical enzymatic steps in anaerobic hydrocarbon oxidation pathways (Zhang *et al.*, 2012). The dissimilatory iron-reducing species of the delta-Proteobacterial family *Geobacteraceae* appear to dominate iron-reducing activities in most environments and possess a unique citrate synthase gene (*gltA*) that is critical for their central metabolism (Wilkins *et al.*, 2011). The *Geobacteraceae*-specific *gltA* has been used as a means to assess phylogenetic diversity within iron-reducing environments and as a means to directly measure metabolic activity of iron-reducing populations *in situ* (Chin *et al.*, 2005; Akob *et al.*, 2012).

Methanogenesis is an anaerobic metabolic process of global biogeochemical significance (Ferry and Lessner, 2008). Members of methanogenic *Archaea* are known

to participate in syntrophic relationships with other anaerobic respiratory guilds and directly contribute to carbon turnover in subsurface, terrestrial and marine environments (Mesle *et al.*, 2013; Salminen *et al.*, 2004; Ferry and Lessner, 2008). Assessment of methanogenic activity is, therefore, of interest to biodegradation studies within environments where methanogenesis may be the dominant form of anaerobic metabolism (Wiedemeier *et al.*, 1996; Chin *et al.*, 1999; Jones *et al.*, 2008; Ünal *et al.*, 2012). Additionally, biological methane production may be exploited as a sustainable source for natural gas (Jones *et al.*, 2010; Thielemann *et al.*, 2004; Ünal *et al.*, 2012).

In order to enhance petroleum hydrocarbon bioremediation and methane biogas production, the assessment of *in situ* methanogenic activities is of interest. A highly conserved gene encoding the alpha-subunit of methylcoenzyme M reductase, *mcrA*, has been used for characterization of methanogenic community structure, determination of phylogenetic relationships between communities, and assessment of activities within artificial and natural environments (Steinberg and Regan, 2008; Pereyra *et al.*, 2010). Further information concerning *mcrA* and its application for studies in methane biogas production are presented in chapter 5.

1.4 Aims of the present study

The present study aims to elucidate the structure and function of anaerobic microbial communities, who are key players in anaerobic petroleum hydrocarbon degradation and methane biogas production by applying a polyphasic approach combining methods in molecular biology and geochemistry. Specific aims are as follows:

- To determine the metabolically-active microbial community structure of dominant dissimilatory sulfate- and iron-reducing, and methanogenic metabolic guilds involved in anaerobic petroleum hydrocarbon degradation in *Deepwater Horizon* crude oil spill-impacted salt marsh sediments from study sites in Bay Jimmy, Louisiana, USA.
- To elucidate the respective respiratory and catabolic activities of each metabolic guilds in response to *in situ* seasonal changes, site-specific environmental characteristics, and biodegradation-promoting clay amendment.
- To investigate the effects of trace element addition on methanogenic activities and overall biomass of methane biogas producing-enrichment cultures obtained from the production water of coal beds in the Powder River Basin, Wyoming, USA.

2 MATERIALS AND METHODS

2.1 Chemicals and gases

All chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO), Acros Organics (New Jersey, US) and Thermo Fisher Scientific (Waltham, MA). Gases including N₂ (99.999%), CO₂ (99.995%), N₂/CO₂ (80:20 [vol:vol]) and H₂ (99.999%) were supplied by NexAir (Marietta, GA).

2.2 Field site selection, monitoring, sediment sampling and clay application

Two sampling and experimental sites, referred to as Bay Jimmy 1 and Bay Jimmy 3 (BJ1 and BJ3, respectively), were selected within a *Deepwater Horizon* oil contaminated saltmarsh in Barataria Bay, Louisiana (Figures 1.1 and 1.2). Barataria bay was formed by postglacial sedimentation from the Mississippi River and is isolated from the Gulf of Mexico by barrier islands (Folger, 1972). Bay Jimmy is a small bay partially enclosed by salt marsh within the larger Barataria Bay. The environment is estuarine and is partially influenced by marine tidal action. Due to extensive levee construction, minimal Mississippi River fresh water and sediment in-put is received from constructed distributaries of the Mississippi River at Naomi and West Pointe a la Hache, LA, though precipitation is the primary source of fresh water (CWPPRA, 1990).

BJ1 was dominated by *Spartina* sp. with a visible oil slick in surface pore waters. BJ3 was in a state of active erosion and devoid of vegetation with a weathered oil crust on the dry surface sediments. Bulk surface sediment was obtained by hand auger and stored in sterile glass jars on ice and later transferred to a 4°C cold box for long-term storage. Sediment samples used for RNA extraction and pore water chemistry analysis were separately obtained from the first 3 cm of sediment beneath the sediment-water interface and stored in sterile glass jars on ice. For long-term storage, these samples were transferred into sterile, nuclease-free 50mL centrifuge tubes and stored at -80°C until further use. *In situ* measurements of pH, dissolved oxygen, temperature, and conductivity were taken in overlying marsh water and the open bay using a Vernier® LabQuest handheld instrument (Vernier, Beaverton, OR). *In situ* measurements of pH, dissolved oxygen, temperature, and conductivity were taken in overlying marsh water and the open bay using a Vernier® LabQuest handheld instrument. Approximately 4.5 kg of sodium bentonite (Lintech Internationals, Macon, GA, sourced from Wyoming) were mixed with marsh water and applied to the sediment surface of each site in September 2010. Non-clay control areas were selected within 10 m of each site of clay application. Subsequent sampling and site assessment occurred approximately every 60 days from October 2010 through September 2011. Nucleic acid extractions were performed on the first 2 cm of cored sediment.

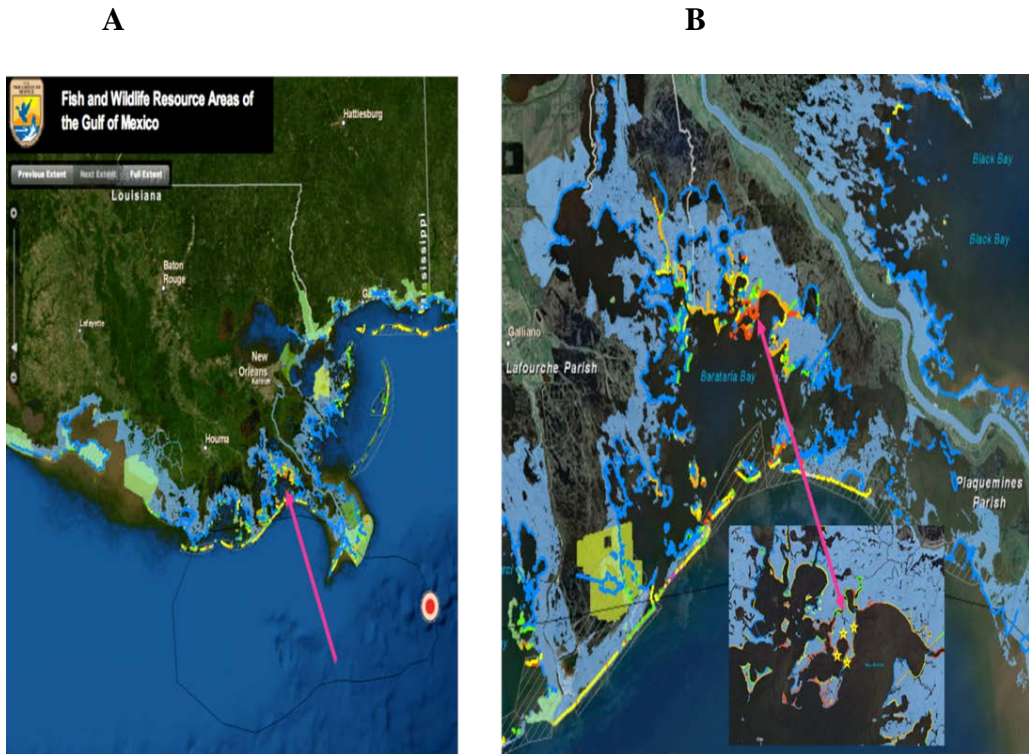


Figure 2.1. Field experimental sites of the study. (A) Area map showing the study sites (Arrow indicates the study sites) impacted by 2010 Deepwater Horizon oil spill (USFWS Resource areas of the Gulf of Mexico Mapper, 2010); (B) View of sampling points in the area of Bay Jimmy and Bay Batiste located in northern Barataria Bay, LA.

**A****B**

Figure 2.2. Field experimental sites in Bay Jimmy. (A) Picture showing a study site BJ1 dominated *Spartina* and *Phragmites* vegetation Bay Jimmy 3 (B) Picture showing a study site BJ3 with significant wrack accumulation due to a high degree of crude oil impact.

2.3 Microcosm design and sampling

A microcosm incubation experiment was performed to assess microbial metabolic activity, community composition and petroleum degradation rates using bulk surface sediments obtained from BJ1 in May 2011. BJ1 sediments were chosen due to their overall higher moisture content which allowed for easy homogenization of the bulk sediment *ex situ*. Microcosm preparation was carried out in a vinyl anaerobic chamber (Coy Laboratory Products, Inc.). Bulk surface sediments were homogenized in a sterile glass beaker with a sterile spatula. Five grams sediment and 10 mL marsh water (previously bubbled for 20 min with N₂ gas) combined in 64 mL serum bottles. Bottles were then divided into three separate treatments: control (N); oil (O); and clay + oil (O+C). Microcosms in the O group were sealed with a butyl stopper and injected with 0.5% (w/v) light, sweet Louisiana crude oil. Microcosms in the O+C group were amended with 1 g Ca-rich montmorillonite (Ca_{0.33}(Al,Mg)₂(Si₄O₁₀)(OH)₂ nH₂O; STx-1b, Clay Minerals Society, Columbia, MO, sourced from Gonzales County, TX) in addition to crude oil to simulate experimental field site conditions. Microcosms for each treatment were prepared for 16 time points in triplicate.

The entire contents of three microcosm bottles per treatment were used for gene expression and terminal electron acceptor analysis at each of 16 time points (T_{0 hrs} – T_{1500 hrs}). Two separate bottles of O and O+C groups for select time points were immediately frozen and stored at -20°C for total petroleum hydrocarbon analysis.

Sediments used for RNA extraction were immediately transferred to sterile, exonuclease-free 2.0mL screw-cap tubes and stored at -80°C.

2.4. Coal bed methane production water enrichment

Long-term, methanogenic enrichment cultures were established by Unal and colleagues at the University of Massachusetts-Amherst in the laboratory of Dr, Klaus Nusslein. 10% (v/v) of actively growing log phase enrichments were amended with 1X trace element solution additions were provided for the present study. Enrichments were incubated at 30°C and aseptically sampled at regular intervals for RNA extraction, RT-PCR and gene expression quantification.

2.5 Total RNA extraction and mRNA enrichment

A protocol for total RNA extraction was modified from Chin *et al.* (2008). The complete procedure was performed at 2°C or on ice. All materials were either certified RNase-free or baked for minimum of 8 hours at 200°C. Extractions for surface sediments, microcosm slurries, and enrichment cultures were conducted in a similar manner. Surface sediment, microcosm slurry, or enrichment culture aliquots (0.5 g, 0.6 mL, 0.6 mL, respectively) were added to 2.0 mL reaction tubes containing 0.5 g of 0.1 mm diameter glass beads, 0.6 mL TPM buffer (50 mM Tris-HCl, 1.7% polyvinylpyrrolidone, 20 mM MgCl₂ x 6 H₂O, in DEPC-treated water) and 2 µL yeast tRNA (mRNA carrier). Cells were lysed by bead beating at 4200 rpm for 1 min in a

Biospec Mini-bead beater. After 5 min of centrifugation at 14,000 rpm at 2°C, the supernatant was transferred to a chilled 2.0mL reaction tube containing 0.6 mL phenol 1 µL of Ribolock RNase inhibitor (Life Technologies-Ambion). The sediments were then retreated with TNS-Phenol buffer (50 mM Tris-HCl, 50mM EDTA, 1% SDS, and 6% water-saturated phenol) for further RNA extraction, and bead-beaten as in the previous step, followed by centrifugation for 5 min at 14,000 rpm at 2°C. The supernatant was transferred to the tube containing the previously transferred supernatant from the first extraction, phenol, and RNase inhibitor. The pooled supernatants underwent extraction with phenol, phenol-chloroform-isoamylalcohol (25:24:1), chloroform-isoamylalcohol (24:1, for phenol removal). Total nucleic acids were precipitated with 1/10 volume 3M sodium acetate (pH 5.2), 2uL linear acrylamide, then brought to 2.0 mL by 100% ethanol (cold), incubated for 30 min at -80°C and centrifuged for 30 min at 14,000 rpm at 2°C. Nucleic acid pellets were then washed with 0.5mL 70% ethanol (-80°C) and centrifuged for 10 min at 14,000 rpm at 2°C. After drying in a desiccation compartment filled with silica-gel, pellets were resuspended in 40 or 20uL RNase-free water and pooled from 8 or 10 reaction tubes. Treatment with DNase and further purification with 1/10 volume sodium acetate, 4 volumes of 100% ethanol (-80°C) and centrifuged for 20 min at 14,000 rpm at 2°C, followed by the addition of 1mL cold 70% ethanol (stored at -80°C) and centrifugation for 10 min at 14,000 rpm at 2°C. After drying the pellets were resuspended in 20uL RNase-free water and stored at -80°C for mRNA enrichment. For mRNA enrichment using a metal bead hybridization method and rRNA and residual small RNA removal, MICROB Express Bacterial mRNA purification kit was used (Life Technologies, TX).

DNA contamination was checked with agarose gel electrophoresis following PCR amplification using citrate synthase (*gltA*) primers CS375nF and CS598nR. RNA concentration was determined by absorbtion at 260 nm with a Biophotometer (Eppendorf, Hamburg, Germany). All RNA was stored at -80°C until further use.

2.6 Environmental DNA extraction

Total DNA Extractions for surface sediments and microcosm slurries were conducted in a similar manner. 0.25 g Sample aliquots were added to 2.0 mL reaction tubes containing 0.1 g of 0.1 mm diameter glass beads, 0.6 mL Sodium phosphate buffer (0.1 M NaH₂PO₄, 0.1 M Na₂HPO₄ in nuclease free water (Sigma-Aldrich, Saint Louis, MO, USA). Cells were lysed by bead beating at 4200 rpm for 1 min in a Biospec Mini-bead beater. After 5 min of centrifugation at 14,000 rpm at room temperature, the supernatant was transferred to a chilled 2.0mL reaction tube containing 0.6 mL phenol-chloroform-isoamylalcohol (25:24:1) and centrifuged for 3 minutes at 14,000 rpm. The supernatant was transferred to another chilled tube containing 0.6 mL chloroform-isoamylalcohol (24:1, for phenol removal) and centrifuged for 3 min at 14,000 rpm. The supernatant was transferred to a clean 0.2 mL reaction tube. Total nucleic acids were precipitated with 1/10 volume 3M sodium acetate (pH 5.2) and then brought to 2.0 mL by 100% ethanol (cold), incubated for 30 min at -20°C and centrifuged for 30 min at 14,000. Nucleic acid pellets were then washed with 0.5mL 70% ethanol (-80°C) and centrifuged for 10 min at 14,000 rpm at 2°C. After drying in a desiccation

compartment filled with silica-gel, pellets were resuspended in 40 or 20uL nuclease-free water and pooled from 8 or 10 reaction tubes.

2.7 PCR primer design

The primers used for amplification of *mcrA* transcripts, MLAS and *mcrArev*, were described by Steinberg and Regan (2008). The primer sets used for the amplification of a 1.9kb transcript of *dsrAB*, were DSR1F (Wagner *et al.*, 1998) and DSR4RdegN. The primer DSR4RdegN was modified from a primer described in Leloup *et al.* (2006). For nested PCR with the DSR1F-DSR4RdegN amplicon, *dsrB* was amplified using the forward primer DSR2060F with DSR4RdegN yielding a 450 bp product. The reverse primer, DSRQP3R (Akob *et al.*, 2012) was used with DSR1F for obtaining a 116 bp amplicon for qPCR. For *gltA* transcript amplification, the following two primer pairs previously described by Akob *et al.* (2012) were used for amplification of long fragments for phylogenetic analysis (893 bp and 803 bp, respectively): CS1Fdeg and CS2Rdeg; CS18nF and CS821nR. For qPCR of *gltA* transcripts, primer pair CS375nF and CS598nR were used to yield a 224 bp amplicon (Akob *et al.*, 2008). SRP-specific 16S rRNA primers, *dsv230F* and *dsv838R* were used to generate a 610 bp amplicon as described by Daly *et al.* (2000). Degenerate primers targeting the *bssA* gene with phylogenetic coverage for SRP, FRP and denitrifiers (*bssA*DEGf and *bssA*DEGr) were designed by sequence alignment for *Desulfobacula toluolica* (EF123663.1), *Desulfovibrio desulfuricans* (NC_011883.1), *Geobacter metallireducens* strain GS-15

(AF441130.1), *Geobacter daltonii* strain FRC-32 (CP001390.1), and *Aromatoleum aromaticum* strain EbN1 (NC_006513.1) obtained from NCBI database using the BLAST program. SRP-specific *bssA/nmsA* primers (*bssA/nmsADTNf* and *bssA/nmsADTNr*) were designed using *bssA* sequences for *D. toluolica* and NaphS2. *Geobacteraceae*-specific *bssA* primers (*bssAGEOf* and *bssAGEOr*) were designed by sequence alignment for *G. daltonii*, *G. metallireducens*, *G. grbiciae* strain DSM 13689 (EF123664.1) and *Geobacter* sp. TMJ1 (EF123666.1). Detailed information for all primers and primer sequences used in this study are described in Table 1.

2.8 Primer validation

Pure cultures of the following microorganisms were grown in the laboratory and their genomic DNAs were used as positive controls for primer validation by PCR followed by agarose gel-electrophoresis and cloning: *G. metallireducens* GS-15, *G. daltonii* FRC-32, *Desulfovibrio* BEN4, *Methanosarcina barkerii*, and strain NaphS2. Primers were further validated for environmental samples by cloning and sequencing cDNAs synthesised from mRNA isolated from surface salt sediments collected from a *Deepwater Horizon* oil-impacted salt marsh within Bay Jimmy, Louisiana.

Table 2.1. Primers used in this study

Gene target and primer	Sequence (5'-3')	Reference
Methyl coenzyme A reductase		Steinberg and Regan (2008)
mlas	GGTGGTGTMGDDTTCACMCARTA	
mcrA	CGTTCATBGCCTAGTTVGGRTAGT	
<i>Geobacteraceae</i> citrate synthase		Akob <i>et al.</i> (2012)
CS1Fdeg	CCGYGACATYCGCWGCCT	
CS2Rdeg	GGAGTSTTSWGGCAGAACTC	
CS18nF	CTCGCGRCATYCGCAGTCT	
CS821nR	TGRCCGGCRTTCAGGGTAT	
CS375nF	AACAAGATGRMGCCTGGG	Akob <i>et al.</i> (2008)
CS598nR	TCR TGGTCGGARTGGAGAAT	
Dissimilatory (bi) sulfite reductase A DSR1F	ACSCACTGGAAGCACG	Wagner <i>et al.</i> (1998)
DSR2060F	CAACATCGTYCAYACCCAGGG	Humphys (2009)
DSR4RdegN	GTRTARCAGTTDCCRCA	Leloup <i>et al.</i> (2006)
DSRQP3R	CGCATGGTRTGGAARTG	Akob <i>et al.</i> (2012)
<i>Geobacteraceae</i> outer membrane cytochrome C reductase		This study
omcB75F	CGAKTCGCCSCACRWA	
ombB492F	GTGGCAGTCGAGGCASA	
omcB824R	TGGCAGTTGGTGCAGATGTT	
BamA		This study
BamAGeoF1	TGATCGACYGYGAAGTKGAG	
BamAGeoF2	ACCGGCGGCAAYACCAAGGA	
BamAGeoR1	CCTTGGTRTTGCCGCCGGT	
BamAGeoR2	GAAGTCGCAGGCCATGCCGAT	

Table 2.1 Continued.

Gene target and primer	Sequence (5'-3')	Reference
Benzoylsuccinate synthase A & naphthylmethylsuccinate synthase A		This study
BssAGeoF1	CAGTATGCCTACGYSACGAG	
BssAGeoF2	TCGTTGCCGCCAACTCCCTG	
BssAGeoR1	CAGGGAGTTGGCGGCAACGA	
BssAGeoR2	ATGTARAGKCCGACCGCCTG	
BssADegF1	CTGRTYTWYGAMGARAAGAAG	
BssADegF2	AATGGVCCRACBGCVGTRCT	
BssADegR1	AGYACBGCVGTYGGBCCATT	
BssADegR2	ACSMMGTTTTRACTGSACRTG	
BssA/NmsADeg F1	CTGRTYTWYGAMGADAAGAAR	
BssA/NmsADeg F2	AAWGGVCCRACBGCVGTRYT	
BssA/NmsADeg R1	ARYACBGCVGTYGGBCCWTT	
BssA/NmsADeg R2	ACSM MRTTTRACTGSACRTG	
NmsADegF1	CTKGTMTTTGACGAYAAGAAA	
NmsADegF2	AAAGGYCCYACBGCRCGYTT	
NmsADegR1 NmsADegR2	AARACYGCVGTRGGRCCTTT ACSACATTRACTGAACATG	

Table 2.1 Continued.

Gene target and primer	Sequence (5'-3')	Reference
SRB- BssA/NmsAF1	AGAAGACCCGKWCCGARGG	This study
SRB- BssA/NmsAF2	CCTKGATGAYGSCTGYATGGA	
SRB- BssA/NmsAR1	TCCATRCAGSCRTCATCMAGG	
SRB- BssA/NmsAR2	TTGCCCCACTTRGGMGCC	
16S rRNA		
27f	AGAGTTTGTATCMTGGCTCAG	Lane (1991)
331f	TCCTACGGGAGGCAGCAGT	Nadkarni <i>et al.</i> (2002)
338f	ACTCCTACGGGAGGCAGCAG	Lane (1991)
349f	GYGCASCAGKCGMGAAW	Takai and Horikoshi (2000)
515f	GTGCCAGCMGCCGCGGTAA	Caporaso <i>et al.</i> (2012)
797r	GGACTACCAGGTATCTAATCCTGTT	Nadkarni <i>et al.</i> (2002)
806r	GGACTACVSGGGTATCTAA	Caporaso <i>et al.</i> (2012)
907r	GGTGACCTTGTTACGACTT	Lane (1991)
1492r	GGTTACCTTGTTACGACTT	Stackebrandt <i>et al.</i> , 1993)
<i>Desulfovibriales</i>		
16S rRNA		
dsv230f	GRGYCYGCGTYYCATTAGC	Daly <i>et al.</i> (2000)
dsv838r	SYCCGRCA YCTAGYRTYCATC	
<i>Desulfobacteriales</i>		
16S rRNA		
dsb127f	GATAATCTGCCTTCAAGCCTGG	
dsb1273r	CYYYYYGCRRA GTCGSTGCCCT	

2.9 Reverse Transcription-Polymerase Chain Reaction and PCR optimization

cDNA of the mRNA for *mcrA*, *dsrAB*, *bssA*, *bssA/nmsA* and *gltA*, and of rRNA for SRB-specific 16S rRNA was generated with respective reverse primers with 0.5 µg mRNA as template. Multiscribe Reverse Transcriptase™ (250 U; Life Technologies-Applied Biosystems) was used according to manufacturer's instructions for 20 µL reactions in 0.2 mL thin walled tubes. The cDNA synthesis program conditions were as follows: initial incubation at 25°C for 10 min, incubation at 37°C for 120 min, enzyme inactivation at 85°C for 5 sec, followed by rapid cooling to 4°C. cDNA samples were stored at -20°C until use. Subsequent end-point PCR amplification of gene transcript-specific cDNA was performed in 20 µL of optimized PCR master mix using iProof High Fidelity DNA polymerase and 5X High Fidelity reaction buffer (BIO-RAD, Hercules, CA, USA), 0.2 mM each deoxynucleoside triphosphate, 1X bovine serum albumin, and 0.8 mM each primer for its respective gene transcript target. PCR conditions were optimized for each primer set using pure-culture genomic DNA.

2.10 Cloning, sequencing, and phylogenetic analysis

Appropriate size RT-PCR amplicons were visualized by 1.2 – 1.5% agarose gel electrophoresis and either gel-excised and purified or PCR purified using GeneJet Purification kits (Life Technologies-Fermentas, Pittsburgh, PA, USA). Product was ligated to pCR2.1-TOPO, pCR4-TOPO, or pJET1.2 cloning vector and transformed into TOP10 or Mach1 chemically

competent *E.coli* cells. Purified plasmids were sequenced for respective inserts. Clone sequences were then analysed and compared to reference sequences using BLAST analysis.

2.11 Real-time Reverse Transcription-PCR quantification

Quantitative real-time PCR of *mcrA*, *dsrA*, *gltA*, *Geobacteraceae*-specific *bssA* and SRP-specific *bssA/nmsA* transcripts, and 16S rRNA were performed for transcript or rRNA quantification using an Applied Biosystems 7500 Real Time PCR system and Sequence Detection Software. Precision and reproducibility were carefully optimized and confirmed by performing all reactions with three replicates for each unknown sample and duplicates for standards. Duplicate or triplicate separate runs were performed as needed. Correct PCR amplicon length (bp) and specificity were checked by end-point PCR using assay primers followed by band visualization with agarose gel electrophoresis. Dilution series of purified RT-PCR products were used as calibration standards for qPCR. End-point PCR products were obtained using the above method and purified using GeneJET PCR Purification Kits (Thermo Fisher Scientific-Fermentas, Waltham, MA, USA). Purified RT-PCR products were quantified by A_{260} measurement with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The concentration for numbers of molecules per microliter was calculated for each gene standard. Standards were then serially diluted from 10^{10} to 10^1 target molecules per reaction and stored at -20°C until use.

qPCR was performed with 96-well optical reaction plates and sealed with MicroAmp Optical Adhesive Film (Life Technologies-Applied Biosystems, Carlsbad, CA, USA) and run on the

7500 Real Time PCR system. A 2 μ L sample of standard RT-PCR amplicon or unknown cDNA template was added to 18 μ L of master mix containing 1X High Fidelity PCR Buffer (BIO-RAD, Hercules, CA, USA), iProof High Fidelity DNA Polymerase, 0.2mM each dNTP, 1X bovine serum albumin, 0.5mM each primer, and 1X SYBR Green PCR Buffer (Life Technologies-Applied Biosystems, Carlsbad, CA, USA). The temperature profile began with an initial polymerase activation step of 98°C for 1 minute; followed by 40 cycles of denaturation at 98°C for 10 seconds; 52°C (*dsrA* and *gltA*), 55°C (*mcrA*), 58°C (*bssA*), or 58.9°C (*bssA/nmsA*); annealing at 65°C for 10 seconds; and a final elongation at 65°C for 10 minutes. 16S rRNA cDNA was quantified similarly with 2 μ L of template cDNA added to 18 μ L of SYBR Green Master Mix (Life Technologies-Applied Biosystems, Carlsbad, CA, USA) with 0.5 mM each assay primer. The temperature profile was the same for 16S rRNA cDNA quantification except for the annealing temperature which was 51°C and 56°C for eubacteria-specific and archaea-specific primer sets, respectively. Each run was followed by a dissociation step for verification of accurate peak (amplification of target) detection.

2.12 Petroleum hydrocarbon and terminal electron acceptor analyses

Gas chromatography mass spectrometry (GC/MS) was used for alkane and aromatic hydrocarbon analysis from sediments obtained in September 2010. Anaerobic terminal electron acceptors were measured in lab on a Dionex anion mode ion chromatograph for sulphate and by colorimetric method with ferrozine by a UV spectrophotometer for iron(II) and total iron using previously described methods (Kostka and Luther, 1994; Lovely and Phillips, 1994). Total petroleum hydrocarbon (TPH) analysis was performed using the gravimetric method

(EPA Method 1664A) with hexadecane as the internal standard. Polycyclic aromatic hydrocarbon (PAH) analysis was conducted using EPA Method 8310 with 1,4-Dichlorobenzene-d4, Acenaphthene-d10, Naphthalene-d8, Perylene-d12, and Phenanthrene-d10 as internal standards.

2.13 Pyrosequencing of 16S rRNA and 16S rRNA gene

Next generation sequencing offers an advantage over more traditional Sanger sequencing of cloned plasmids in that genomic DNA or cDNA can be directly sequenced using primers without the limitations of cloning. The high-through put nature of such technologies permits for the rapid sequencing of thousands of operational taxonomic units (OTUs) simultaneously (Quail *et al.*, 2012).

Ion Torrent™ Personal Genome Machine (PGM) sequencing of high-quality DNA isolated from microcosm sediments was carried out at The University of Illinois at Chicago's DNA Services facility. Wide-coverage, degenerate 16S rRNA primers 515f and 806r were used to amplify a ~300bp amplicon of the bacterial and archaeal small subunit 16S rRNA gene (Caporaso *et al.*, 2012). High-quality DNA was isolated and checked for quality by end-point PCR and 1.5% agarose gel electrophoresis. *In situ* sediment samples chosen for pyrosequencing were BJ1-Control-May 2011, BJ1-Clay-May 2011, BJ1-Control-February 2011, and BJ3-Control-May 2011. These particular site and time point samples were chosen for pyrosequencing in order to compare microbial molecular diversity between time points of low and high metabolic activity at BJ1, between control and experimental plots at BJ1, and

between vegetated (BJ1) and non-vegetated (BJ3) sites both at times of overall high activity (verified by gene expression analysis, Figures 3.4 and 3.5).

In addition to this also 454 sequencing of 16S rRNA cDNA amplicon using GS Junior Titanium Series sequencer (Roche) was performed in order to elucidate active microbial community composition in in situ Bay Jimmy sediment. High quality of total RNA was isolated from BJ3 sediment collected in October, 2010, and cDNA was synthesized using 16S rRNA eubacterial-specific primer1492r. Then 16S rRNA cDNA amplicon obtained using 338f and 907r primer set was used for 454 sequencing.

3 ***IN SITU* METABOLIC ACTIVITIES OF ANAEROBIC MICROBIAL COMMUNITIES IN *DEEPWATER HORIZON* OIL SPILL-IMPACTED LOUISIANA SALT MARSH SEDIMENTS**

3.1 **Introduction**

Coastal crude oil contamination resulting from the explosion of *Deepwater Horizon* drilling rig on April 20, 2010 is of particular concern in the oxygen-limited salt marshes that surround many of the highly productive fishing bays of southern Louisiana (Jackson and Pardue, 1997; Kastner *et al.*, 1998; Wilcke, 2000; Williams, 2004; Guitart *et al.*, 2008; Hazen *et al.*, 2010; Kerr *et al.*, 2010). While aerobic degradation of petroleum hydrocarbon is well known to occur rapidly via the dioxygenase pathway (Atlas, 1981), anaerobic aromatic hydrocarbon degradation coupled to reduction of dissimilatory sulfate, iron and nitrate, and methanogenesis is known to be an important set of sequential processes in oxygen-depleted environments (Coates *et al.*, 1997; Shin *et al.*, 2000; Chang *et al.*, 2002; Meckenstock *et al.*, 2004; Hallberg and Johnson, 2005; Foght, 2008; Carmona *et al.*, 2009; Zhang *et al.*, 2010). Indeed, anaerobic biodegradation of petroleum hydrocarbons may be chiefly responsible for the natural attenuation of crude oil-contaminated Louisiana salt marsh sediments (Jackson and Pardue 1997; Williams, 2004; Zhu *et al.*, 2004; Gallego *et al.*, 2006; Andreoni and Gianfreda, 2007; Vega *et al.*, 2009).

Complete oxidation of mixed petroleum hydrocarbons is likely accomplished by a community of functionally diverse, yet complementary microbial metabolic guilds (Meckenstock *et al.*, 2004; Foti *et al.*, 2007; Cardenas *et al.*, 2008; Pereyra *et al.*, 2010; Carmona *et al.*, 2009; Zhang

et al., 2010). Knowledge of the structure and function of the microbiota within a polluted environment is fundamental to the understanding of *in situ* contaminant degradation. Monitoring petroleum hydrocarbon degradation in open environmental systems presents many challenges, including but not limited to the difficulty of extracting and measuring contaminants over time. Instead, molecular approaches used to ascertain microbial diversity, phylogeny and metabolic activity have been used to monitor bioremediation in a range of natural and constructed environments (Achenbach and Coates, 2000; Beller *et al.*, 2002; Lovley and Hayes, 2002; Ibekwe *et al.*, 2003; Lovley, 2003; Heijs *et al.*, 2007; Beller *et al.*, 2008; Chin *et al.*, 2008).

The discovery of the 16S rRNA gene and its use as a molecular proxy for phylogenetic assessment was a milestone in the fields of microbial ecology and environmental microbiology and continues to be used as the *de facto* molecular target in respective studies (Achenbach and Coates, 2000). The high degree of sequence similarity that is observed across many distantly related lineages within sediments presents problems when attempting to derive functional information from 16S rRNA gene sequence analysis (Achenbach and Coates, 2000; Luton *et al.*, 2002; Heijs *et al.*, 2007; Pereyra *et al.*, 2010). Instead, a more precise method for the study of microbial communities in complex environments is the use of functional gene markers as proxies for specific metabolic processes. Functional genes for sulfate reduction (dissimilatory (bi) sulfite reductase, *dsrAB*), methanogenesis (methyl-coenzyme M reductase, *mcrA*) and central metabolism (eukaryotic-like *Geobacteraceae*-specific citrate synthase, *gltA*) among others have been successfully targeted in a range of pristine and contaminated anaerobic environments for microbial diversity and activity assessment (Wagner *et al.*, 1998; Beller, *et*

al., 2002; Luton *et al.*, 2002; Chin *et al.*, 2004; Friedrich, 2005; Holmes *et al.*, 2005; Nercessian *et al.*, 2005; Perez- Jimenez and Kerkhof, 2005; Song and Ward, 2005; Wagner *et al.*, 2005; Akob *et al.*, 2008; Beller *et al.*, 2008; Chin *et al.*, 2008; Pereyra *et al.*, 2010; Akob *et al.*, 2012). Specific to anaerobic aromatic hydrocarbon biodegradation, the catabolic gene target benzylsuccinate synthase (*bssA*) for anaerobic toluene degradation has served as useful biomarker for studying *in situ* bioremediation activities of ecologically relevant microorganisms (Beller *et al.*, 2002; Song and Ward, 2005; Beller *et al.*, 2008; Kuntze *et al.*, 2009; Selesi, 2010). The gene homologue to *bssA* for 2-methylnaphthalene degradation, *nmsA*, is introduced for environmental RT-PCR application in this study to provide more extensive metabolic and phylogenetic coverage considering the potential for polycyclic aromatic hydrocarbon degradation.

In addition to microbial community structure and phylogenetic assessment, activity rates might be estimated by quantifying transcript levels of functional genes of interest using quantitative real-time polymerase chain reaction (qPCR). The goal of such studies is to correlate gene expression levels to *in situ* rates of microbe-mediated biogeochemical and biodegradation activities (Akob *et al.*, 2008; Chin *et al.*, 2008; Pereyra *et al.*, 2010; Akob *et al.*, 2012), thus providing a straightforward molecular method for monitoring bioremediation in the field.

For the present study, degenerate primers for the aforementioned genes have been designed in order to amplify both long (for phylogenetic analysis) and short (for qPCR) gene transcripts to encompass sequences from cultured strains and environmental clones belonging to sulfate- and iron-reducing and methanogenic prokaryotes. To the best of this researcher's knowledge, this

is the first study employing such an approach to gain a complete understanding and assessment of all relevant functional respiratory microbial processes involved in anaerobic petroleum hydrocarbon degradation within a crude oil-contaminated estuarine salt marsh environment.

As well as providing a seasonal microbiological survey of the environment in question, the molecular assays developed for this study will be applied to test the effects of applying high surface area, high surface charge montmorillonite clay to the surface of petroleum impacted sediments on anaerobic hydrocarbon degrading and respiratory activities of indigenous microorganisms (Carmichael and Pfaender, 1997; Chaerun *et al.*, 2005; Kinner *et al.*, 2003; Warr *et al.*, 2009; Witthuhn *et al.*, 2006). Previous studies have already yielded preliminary evidence that crude-oil degradation may be significantly accelerated by the addition of montmorillonite. It has been hypothesized that clay-mineral-seawater cation interactions provide a suitable electrochemical microenvironment allowing for increased adsorption of environmental contaminants and/or microbial cell envelopes at the mineral surface (Chaerun *et al.*, 2005a; Chaerun *et al.*, 2005b; Warr *et al.*, 2009; Witthuhn *et al.*, 2006). Warr *et al.*, (2009) propose that cations serve as a bridge between the negatively charged clays and the surfaces of microbial cell membranes or biofilms, thus increasing the stability of clay-microbe complexes. Aromatic hydrocarbons weakly bound to the clay surface become more easily accessible to attached microbes resulting in accelerated petroleum biodegradation rates. The present study will hopefully contribute to the understanding of microbe-clay interactions of relevance to *in situ* anaerobic petroleum hydrocarbon bioremediation.

3.2 Results

3.2.1 *Pore water chemistry in Bay Jimmy study sites*

The pore water chemistry was analysed approximately 1cm below the sediment-surface water interface in no clay-amended sites (control) and clay-amended sites at BJ1 and BJ3. At both BJ1 and BJ3, clay-amended sediments had a slightly lower pH on average. The pH of BJ3 pore water was slightly lower, overall. Average yearly temperature of BJ1 sediment pore water was 26.3°C with a lowest temperature in February 2011 of 26.1°C and a highest temperature in September 2010 at 28.9°C (Table 1). Average yearly temperature of BJ3 sediment pore water was 26.5°C with a lowest temperature in February 2011 at 26.1 and a highest temperature in May 2011 at 28.1°C. No significant difference in temperature was found at either BJ1 or BJ3 between control and clay-amended sediments (Table 3.1). Dissolved oxygen was constant at 0.8 mg L⁻¹ for all pore waters except for September 2010 at BJ1 which measured 3.0 mg L⁻¹ (Table 3.1). Salinity of the bay water was 11 ppt which is slightly lower than the range of 18 to 30 ppt that is typical of salt marshes (Conner and Day, 1987). There was a pronounced seasonal variation in pore water sulfate with the highest overall sulfate content at both BJ1 and BJ3 in the winter months with a peak in March 2011 at BJ1 and in February 2011 measuring 19.4 mM and 39.8 mM, respectively (Figure 3.1). Total iron and Fe(II) were measured at both sites for all time points. There was no significant seasonal variation in total iron or Fe(II) content for either site, though BJ1 sediments contained considerable more total iron (36.2 μM g⁻¹) and Fe(II) (13.5 μM g⁻¹). Average iron content for BJ3 sediments were 14.7 μM g⁻¹ total iron and 9.6 μM g⁻¹ Fe(II) (Figure 3.2).

Table 3.1 Pore water chemistry in study sites BJ1 and BJ3 at all sampling dates

	BJ1			BJ3		
	T ^a (C°)	pH	DO ^b (mg/L)	T ^a (C°)	pH	DO ^b (mg/L)
September-10	28.9	6.3	1.4	n/a	n/a	n/a
October-10	28.1	6.1	0.8	26.4	6	0.8
December-10	No data	No data	No data	No data	No data	No data
February-11	26.1	6.2	0.8	26	6.1	0.8
March-11	26.7	6.4	0.8	26.9	6.1	0.8
May-11	26.4	6.7	0.8	27.7	6.2	0.8
September-11	27.9	6.9	0.8	28.1	6.0	0.8

^a T indicates temperature; ^b DO indicates dissolved oxygen

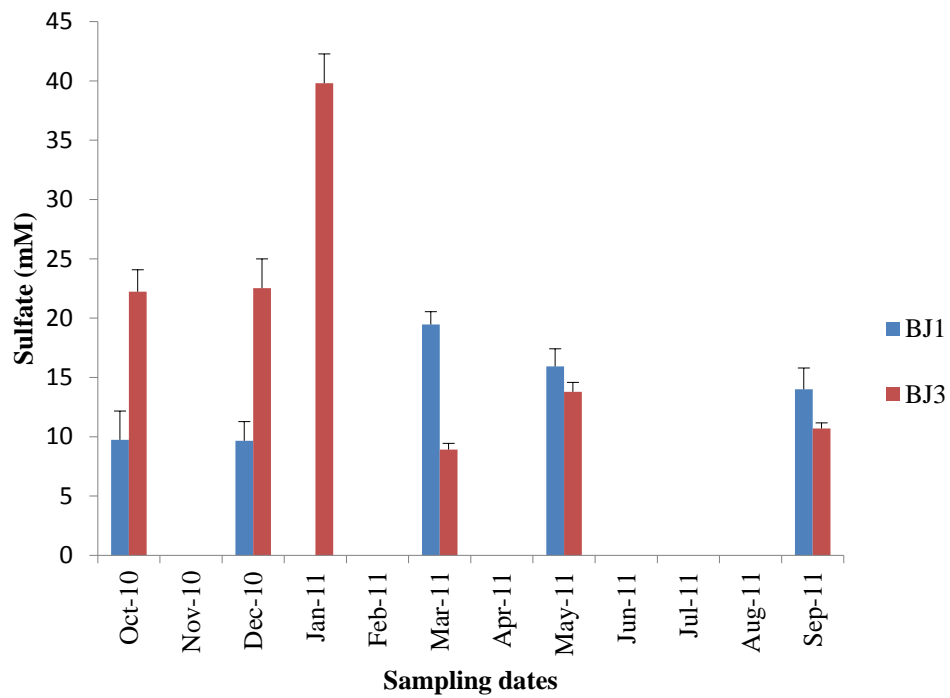


Figure 3.1. Sulfate concentrations in pore water of sites BJ1 and BJ3.

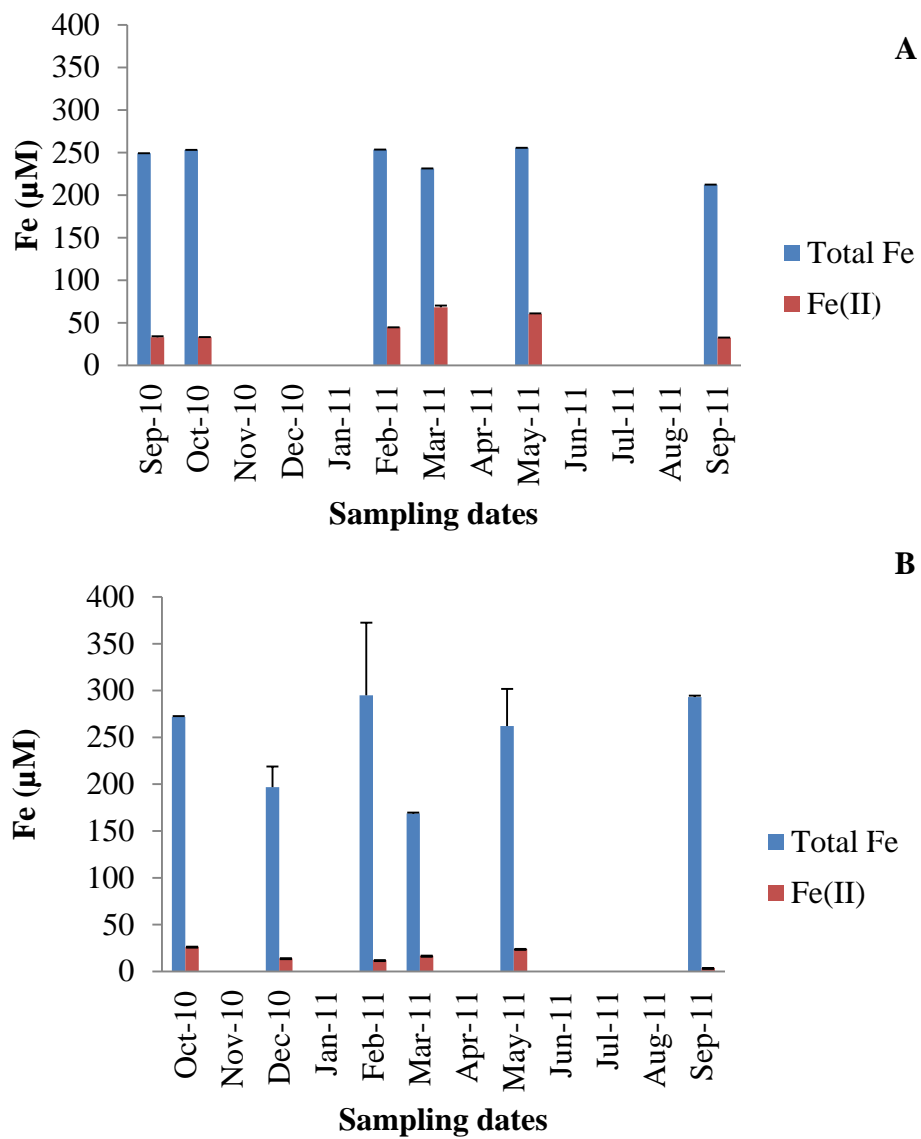


Figure 3.2. Total iron and Fe(II) concentrations in pore water of sites BJ1 (A) and BJ3

(B).

3.2.2 *Primer development, PCR optimization and sequence analysis of metabolically-active microbial communities*

Primers for *dsrAB*, *gltA* and *mcrA* were previously designed and have well documented application for environmental RT-PCR in the literature (Chin *et al.*, 2008; Humphrys, 2009; Akob *et al.*, 2008 & 2012; Ünal *et al.*, 2012). Primer sets for highly degenerate, *Geobacteraceae*-, and SRP-specific *bssA* were designed for this study based on *in silico* specificity with multiple aligned sequences from various microbial lineages using ClustalW. The annealing temperatures and other PCR conditions were optimized for each primer set individually. Two primer sets for each degenerate or group-specific *bssA* target were validated for reference strain specificity using purified genomic DNA and for experimental use on environmental samples using RT-PCR generated cDNAs for each reverse primer. On the basis of 1.2 – 1.5% agarose gel visualization, target specificity of RT-PCR amplicons were further evaluated by cloning for the following primer sets: *bssA*DEGf2-*bssA*DEGr2, *bssA*GEOf2-*bssA*GEOGr2 and *bssA*DTNf2-*bssA*DTNGr2. Target specificity and range was also evaluated for previously designed *dsrAB*, *gltA* and *mcrA* primer sets by 1.2 – 1.5% agarose gel electrophoresis and cloning of environmental RT-PCR amplicons.

RT-PCR products obtained using mRNAs isolated from control and clay-amended sediments were used for clone library construction. Sequences retrieved from randomly selected clones (total 7, 10, 8, 3, 3, and 3 clones for *dsrAB*, *gltA*, *mcrA*, degenerate *bssA*, *Geobacteraceae*-targeted *bssA*, and SRP-specific *bssA*, respectively) were further analyzed. All primer sets were confirmed to exclusively amplify the desired gene targets and demonstrated sufficiently broad

coverage (Table 3.2). Analyses of all *dsr* mRNA clone sequences retrieved from oil-contaminated sediments in Bay Jimmy studysites(1.3 kb length for *dsrAB* and ca. 450 bp length for *dsrB*) demonstrated that groups closely related to uncultivated SRPs from various marine and terrestrial environments are metabolically active and abundant. All the *dsrA* mRNA clone sequences (116 bp length) were most closely related to an uncultured SRP from a PAH-contaminated marine environment. All *gltA* mRNA clone sequences (819bp length) were most closely related to known aromatic-oxidizers *Geobacter daltonii* strain FRC-32, *G. metallireducens* strain GS-15 and *G. bemidjiensis* suggesting metabolic activity and high abundance of anaerobic aromatic-oxidizing and Fe(III)-reducing bacteria in oil-contaminated Bay Jimmy salt marsh sediments. Also, analyses of all *mcrA* mRNA clone sequences (496 bp length) demonstrated that members of the order *Methanosarcinales* are metabolically active and dominant amongst methanogenic Archaea.

Although the degenerate primer sets were designed to target all *bssA* within denitrifying, SRP and FRP groups, all the *bssA* mRNA clones sequences (180 bp length) retrieved from *in situ* Bay Jimmy salt marsh sediments were only closely related to *G. metallireducens*. Therefore, two further primer sets were designed to specifically target members of the family *Geobacteraceae* and SRPs, respectively. All the *Geobacteraceae*-specific *bssA* mRNA clone sequences (212 bp length) were most closely related to *G. daltonii* FRC-32 and *G. grbiciae*. All the SRP-specific *bssA/nmsA* mRNA clone sequences (220 bp length) were most closely related to *Marinobacter hydrocarbonoclasticus*, which has been demonstrated to anaerobically degrade toluene (Gauthier *et al.*, 1992).

Table 3.2A. Phylogenetic assignment of *dsrAB*, *dsrB*, and *dsrA* mRNA clone sequences retrieved from Bay Jimmy sediments using BLAST analysis

Clone	Accession	Closest BLAST matches	E value	Max identity
BJ1_DSR1F-n3R_1	AB124917.1	Uncultured sulfate-reducing bacterium <i>dsrA</i>	1.00E-77	69%
	EU350969.2	Uncultured prokaryote clone DB <i>dsrA</i>	1.00E-71	69%
	FM212312.1	Uncultured sulfate-reducing bacterium partial <i>dsrA</i>	4.00E-71	69%
BJ1_DSR2060F-4RDeg_1	FJ948556.1	Uncultured bacterium clone ev219 srb9 <i>dsrA</i>	2.00E-108	87%
	EF065071.1	Uncultured sulfate-reducing bacterium clone LGWK18 <i>dsrA</i>	2.00E-108	87%
	DQ415720.1	Uncultured Desulfotomaculum sp. clone DSR_srb9 DsrA-like gene	2.00E-108	87%
BJ1_DSR2060F-4RDeg_2	AB631445.1	Uncultured bacterium <i>dsrB</i>	3.00E-98	86%
	AY753109.1	Uncultured bacterium clone ng1d124 <i>dsrB</i>	1.00E-96	84%
	AB631514.1	Uncultured bacterium <i>dsrB</i>	3.00E-92	86%
BJ1_DSR2060F-4RDeg_3	AB631110.1	Uncultured bacterium <i>dsrB</i>	2.00E-101	87%
	GU395939.1	Uncultured prokaryote clone D40D04_08 <i>dsrB</i>	1.00E-97	84%
	AB263127.1	Uncultured sulfate-reducing bacterium <i>dsrA</i>	1.00E-96	83%
BJ1_DSR2060F-4RDeg_4	AY337169.1	Uncultured sulfate-reducing bacterium clone W301112 <i>dsrAB</i>	4.00E-91	84%
	AY337165.1	Uncultured sulfate-reducing bacterium clone M300141 <i>dsrAB</i>	2.00E-88	84%
	AY337174.1	Uncultured sulfate-reducing bacterium clone W306388 <i>dsrAB</i>	7.00E-88	83%
BJ1_DSR1F-QP3R_1	FN554563.1	Uncultured bacterium partial <i>dsrA</i>	3.00E-34	90%
	FJ403696.1	Uncultured sulfate-reducing bacterium clone 25H-0D-13 <i>dsrA</i>	3.00E-34	90%
	AM408821.1	Uncultured sulfate-reducing bacterium partial <i>dsrAB</i>	3.00E-34	90%
BJ1_DSR1F-QP3R_2		Uncultured sulfate-reducing bacterium clone PuertoRico.mCR018 <i>dsrA</i>	3.00E-35	91%
	AY367658.1			
				84%
	EF065033.1	Uncultured sulfate-reducing bacterium clone LGWG23 <i>dsrAB</i>	5.00E-26	
	AF327315.1	Uncultured phenanthrene mineralizing bacterium clone Phe03 <i>dsrB</i>	5.00E-19	80%

Table 3.2B. Phylogenetic assignment of *gltA* clone sequences retrieved from Bay Jimmy sediments using BLAST analysis

Clone	Accession	Closest BLAST matches	E value	Max identity
BJ1_gltA_1	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	84%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
BJ1_gltA_2	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>GltA</i> <i>gltA</i>	0	84%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
BJ1_gltA_3	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	98%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	83%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
BJ1_gltA_4	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	84%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
BJ3_gltA_1	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	84%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
	CP002479.1	<i>Geobacter</i> sp. M18, complete genome	0	80%
BJ3_gltA_2	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	84%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
	CP002479.1	<i>Geobacter</i> sp. M18, complete genome	0	80%
BJ3_gltA_3	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	98%
	EF414562.1	Uncultured <i>Geobacter</i> sp. clone PLYCITSYN1 <i>gltA</i>	0	83%
	CP000698.1	<i>Geobacter uraniireducens</i> Rf4, complete genome	0	80%
	CP002479.1	<i>Geobacter</i> sp. M18, complete genome	0	79%
BJ3_gltA_4	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	0	99%
	CP001124.1	<i>Geobacter bemidjensis</i> Bem, complete genome	0	79%
	CP000148.1	<i>Geobacter metallireducens</i> GS-15, complete genome	0	78%

Table 3.2C. Phylogenetic assignment of *mcrA* mRNA clone sequences retrieved from Bay Jimmy sediments using BLAST analysis

Clone	Accession	Closest BLAST match	E value	Max identity
BJ1_mcrA_1	AY436550.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME73121 <i>mcrA</i>	0	99%
	AY436547.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME73118 <i>mcrA</i>	0	99%
	AY436548.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME73218 <i>mcrA</i>	0	98%
BJ1_mcrA_2	AY436552.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME7311 <i>mcrA</i>	0	99%
	AY436551.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME73391 <i>mcrA</i>	0	99%
	AY436550.1	Uncultured <i>Methanosarcinales</i> archaeon clone NANK-ME73121 <i>mcrA</i>	0	99%
BJ1_mcrA_3	AB679171.1	<i>Methanosaeta harundinacea mcrA</i>	0.004	78%
	JN315289.1	Uncultured archaeon clone C4_1 <i>mcrA</i>	0.004	100%
	CP003117.1	<i>Methanosaeta harundinacea</i> 6Ac, complete genome	0.004	78%
BJ3_mcrA_1	HM153600.1	Uncultured archaeon clone McrA.c23.25 <i>mcrA</i>	5.00E-94	99%
	EF452663.1	<i>Methanosarcina mazei mcrA</i>	9.00E-78	100%
	AY125632.1	Uncultured <i>Methanosarcinaceae</i> archaeon MRR-mcr38 <i>mcrA</i>	1.00E-79	100%
BJ3_mcrA_2	HM153600.1	Uncultured archaeon clone McrA.c23.25 <i>mcrA</i>	2.00E-93	99%
	EF452663.1	<i>Methanosarcina mazei mcrA</i>	4.00E-77	100%
	Y00158.1	<i>Methanosarcina barkeri mcrBCDGA</i>	2.00E-72	100%
BJ3_mcrA_3	HM153605.1	Uncultured archaeon clone McrA.c23.30 <i>mcrA</i>	5.00E-106	99%
	AM746774.1	Uncultured methanogenic archaeon partial <i>mcrA</i>	5.00E-105	100%
	AY125632.1	Uncultured <i>Methanosarcinaceae</i> archaeon MRR-mcr38 <i>mcrA</i>	4.00E-104	100%

Table 3.2D. Phylogenetic assignment of *Geobacteraceae*-specific *bssA* clone clone sequences retrieved from Bay Jimmy sediments using BLAST analysis.

Clone	Accession	Closest BLAST matches	E value	Max identity
BJ1_bssADEG _1	CP000148.1	<i>Geobacter metallireducens</i> GS-15, complete genome	5.00E-94	95%
	AE017180.2	<i>Geobacter sulfurreducens</i> PCA, complete genome	8.00E-41	100%
	CP002031.1	<i>Geobacter sulfurreducens</i> KN400, complete genome	5.00E-37	100%
BJ1_bssADEG _2	CP000148.1	<i>Geobacter metallireducens</i> GS-15, complete genome	7.00E-35	99%
	AE017180.2	<i>Geobacter sulfurreducens</i> PCA, complete genome	1.00E-31	100%
	CP002031.1	<i>Geobacter sulfurreducens</i> KN400, complete genome	1.00E-45	100%
BJ1_bssADEG _3	CP000148.1	<i>Geobacter metallireducens</i> GS-15, complete genome	5.00E-43	99%
	AM420293.1	<i>Saccharopolyspora erythraea</i> NRRL2338 complete genome	5.00E-43	93%
	CP002343.1	<i>Intrasporangium calvum</i> DSM 43043, complete genome	7.00E-92	90%
BJ1_BssAGE O_1	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	5.00E-94	99%
	FN564097.1	Uncultured bacterium partial <i>bssA</i>	8.00E-41	80%
	EF123666.1	<i>Geobacter</i> sp. TMJ1 <i>bssA</i>	5.00E-37	78%
	AB167725.1	<i>Magnetospirillum</i> sp. TS-6 <i>bssDCABE</i>	7.00E-35	78%
	EF134966.1	Bacterium <i>bssA</i> -1 <i>bssADCABE</i>	1.00E-31	77%
BJ1_BssAGE O_2	EF123664.1	<i>Geobacter grbiciae</i> strain DSM 13689 <i>bssA</i>	1.00E-45	96%
	CP000148.1	<i>Geobacter metallireducens</i> GS-15, complete genome	5.00E-43	94%
	AF441130.1	<i>Geobacter metallireducens</i> <i>bssAB</i>	5.00E-43	94%
BJ1_BssAGE O_3	CP001390.1	<i>Geobacter</i> sp. FRC-32, complete genome	7.00E-92	98%
	FN564097.1	Uncultured bacterium partial <i>bssA</i>	3.00E-39	79%
	EF123666.1	<i>Geobacter</i> sp. TMJ1 <i>bssA</i>	9.00E-34	77%
	AB167725.1	<i>Magnetospirillum</i> sp. TS-6 <i>bssDCABE</i>	1.00E-31	77%

Table 3.2E. Phylogenetic assignment of SRB-specific *bssA/nmsA* clone sequences retrieved from Bay Jimmy sediments using BLAST analysis

Clone	Accession	Closest BLAST matches	E value	Max identity
bssA/nmsa_1	FO203363.1	<i>Marinobacter hydrocarbonoclasticus</i> str. ATCC 49840 chromosome, complete genome	2.10E+00	96%
	CP000821.1	<i>Shewanella sediminis</i> HAW-EB3, complete genome	7.40E+00	92%
	CP002379.1	<i>Arthrobacter phenanthrenivorans</i> Sphe3, complete genome	7.40E+00	85%

3.2.3 *Validation of quantitative real time RT-PCR*

Selected previously designed primer sets for *dsrA*, *gltA*, and *mcrA* genes; and *Geobacter*sp.-targeted and SRP-specific *bssA/nmsA* gene primers designed for this study were used for qPCR in order to quantify the abundance of transcripts for key functional genes in *Deepwater Horizon* oil-impacted salt marsh sediments from Bay Jimmy, Louisiana, USA. Dilution series of purified environmental RT-PCR products were used for making standard curves for each gene. All functional gene transcripts were successfully quantified in both control and clay-amended surface sediment samples from study site BJ1 with high quality of standard curves.

3.2.4 *In situ metabolic activity of SRP, FRP and MGP*

DsrA transcripts were detected in all samples from BJ1. qPCR of *dsrA* cDNA reveals a seasonal variation in SRP activity with significantly increased activity during the summer months and lower expression during winter months, though sulfate concentrations are notably higher in winter months. Despite this seasonal trend noticed from February through September 2011, lowest *dsrA* expression overall is observed in September 2010. This is thought to be due to the significant increase in fresh water released from the opening of the Atchafalaya River levees that was intended to retard the migration of oil through the marshes (personal communication, Deocampo). Much higher *dsrA* expression is observed in surface sediments amended with montmorillonite clay, indicating that the clay stimulates dissimilatory sulfate reduction *in situ*. Most significantly higher *dsrA* expression in clay-amended sediments vs. control sediments is observed in May 2011 (Figure 3.3a). Although seasonal trends in *dsrA* expression were similar between sites BJ1 and BJ3, levels of *dsrA* transcript were overall

higher at the highly vegetated BJ1 site (Figure 3.4a). There was an overall multifold increase in gene expression for all assayed functional genes except for SRP-specific *bssA/nmsA* in clay-amended sediments compared to the control plots, especially in the winter months. *GltA* and Geobacteraceae *bssA* expression were most increased by clay amendment (Figure 3.5).

GltA and *mcrA* expression have been used as a proxies for assessing FRP activity and methanogenesis, respectively, in previous studies (Chin *et al.*, 2004; Akob *et al.*, 2008 & 2012; Ünal *et al.*, 2012). *GltA* transcripts were detected in all samples from BJ1 with significantly highest expression in February 2011 within clay-amended sediments. Overall, expression in clay-amended sediments is higher than in control sediments, indicating that the clay stimulates the metabolic activity of the family *Geobacteraceae in situ* (Figure 3.3b). Expression of *mcrA* did not correlate to any seasonal patterns, nor was there any indication that clay-amendment had any influence on expression levels. Expression was uniform within an order of magnitude for all time points and for both clay-amended and control sediments (Figure 3.3c). Seasonal trends in *gltA* and *mcrA* expression were similar at both BJ1 and BJ3. However, *mcrA* expression was overall highest at BJ1 whereas *gltA* expression was significantly higher in non-vegetated BJ3 sediments during the winter months (Figure 3.4b & c).

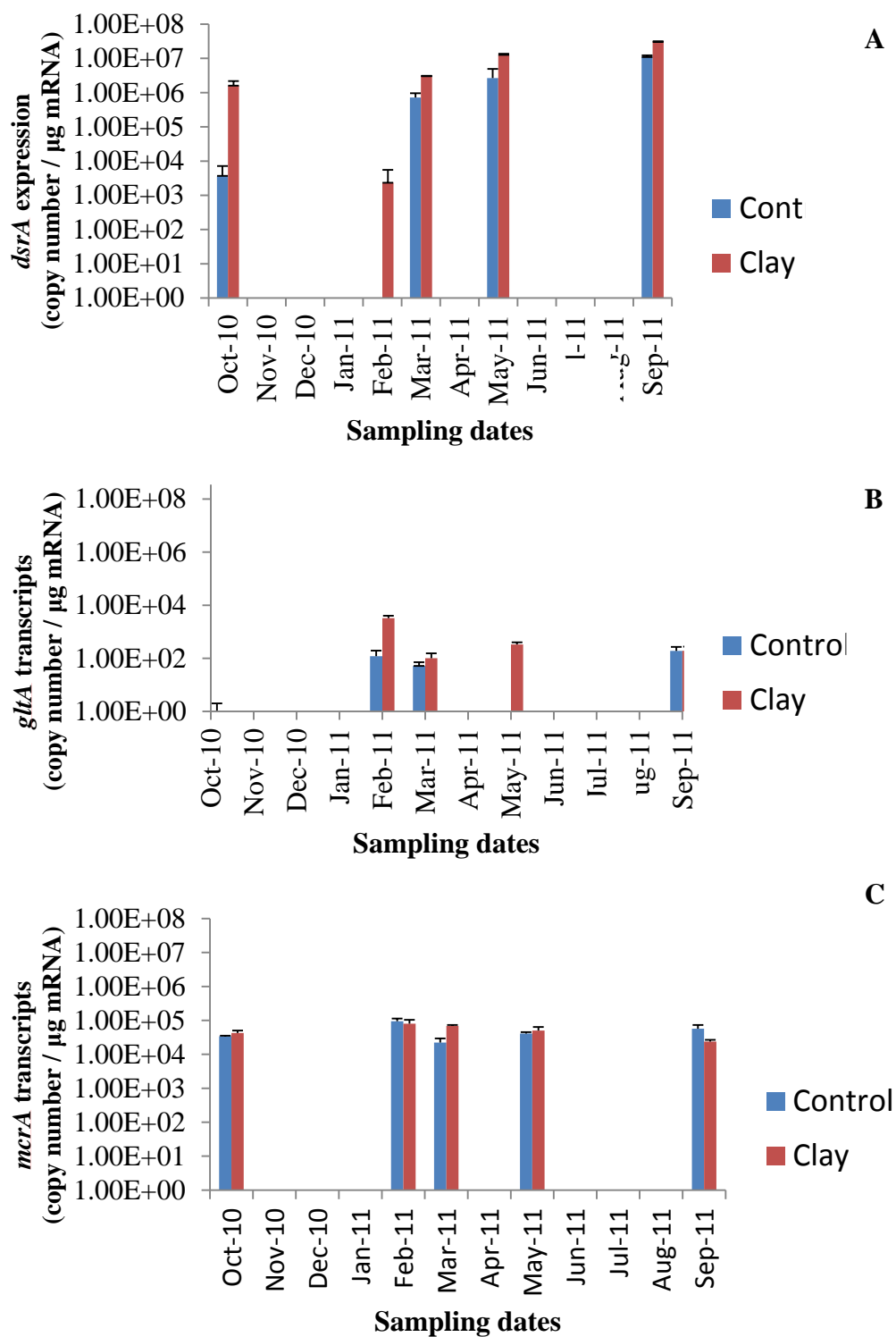


Figure 3.3. Expression of *dsrA* (A), *gltA* (B), and *mcrA* (C) in sediments from *in situ* Control and Clay-amended plots at study site BJ1.

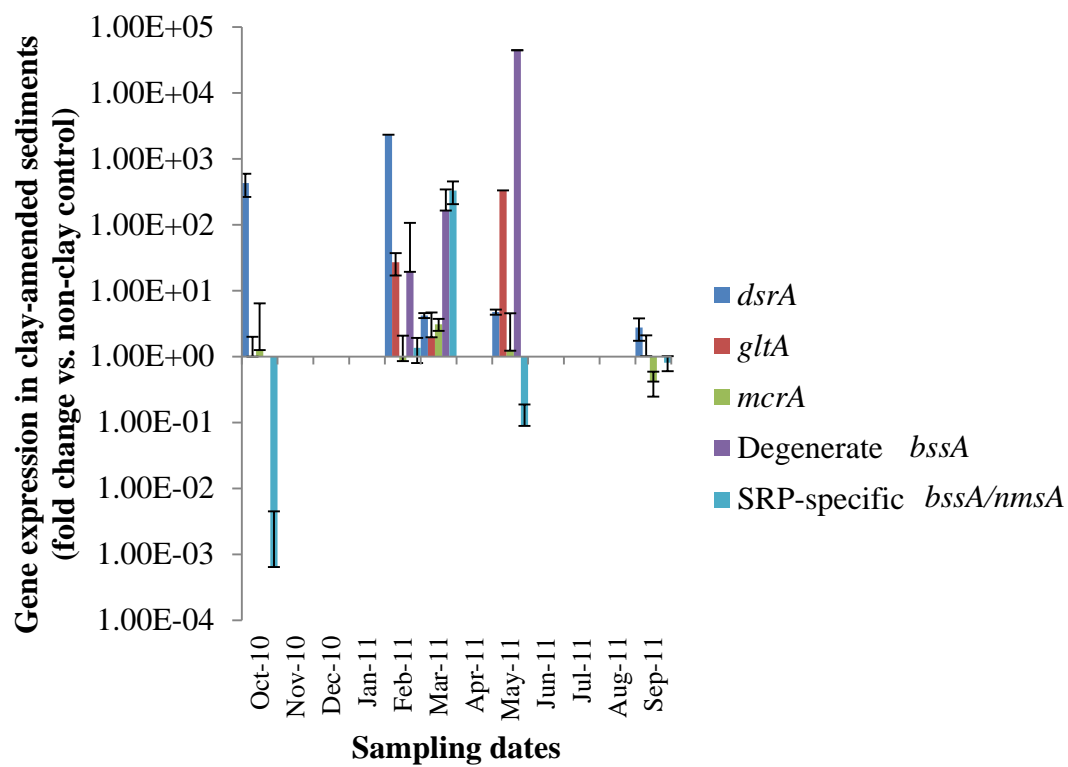


Figure 3.4. Fold change in functional gene expression in Clay-amended plots vs. control plots at study site BJ1.

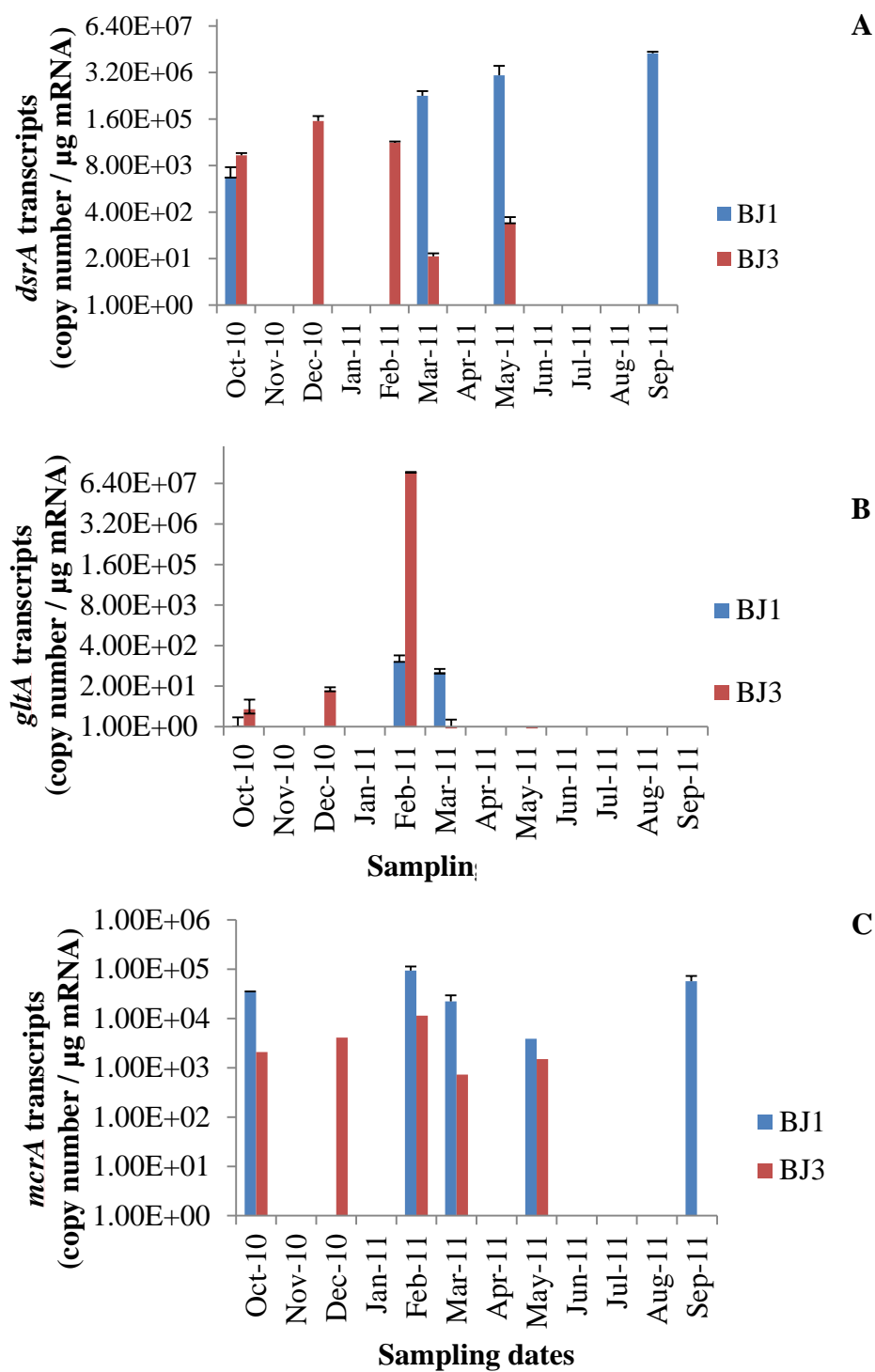


Figure 3.5. Expression of *dsrA* (A), *gltA* (B), and *mcrA* (C) in sediments from *in situ* control plots at BJ1 and BJ3 study sites

3.2.5 In situ metabolic activity associated with anaerobic aromatic hydrocarbon degradation

Transcripts for the *bssA* and *nmsA* genes were used as molecular proxies for the assessment of *in situ* metabolic activity associated with anaerobic aromatic hydrocarbon degrading activity. *BssA* is known to be utilized for activation of the methyl group of toluene by carboxylation during anaerobic toluene degradation (Beller and Spormann, 1997). 2-methylnaphthalene carboxylation is accomplished in representative sulphate-reducing isolates by the *nmsA* gene which shared considerable nucleotide sequence similarity to *bssA* (Selesi *et al.*, 2010). Transcripts for the *bssA* gene targeted within the *Geobacteraceae* were detected in all sediment samples except for September and October 2010. Significantly highest expression was observed in February 2011 within clay amended sediments. Expression was overall highest in clay-amended sediments (Figure 3.6a). Transcripts for SRP-specific *bssA/nmsA* were detected in all sediment samples except for September 2010. Significantly highest level of expression was observed in clay amended sediments from March 2011 (Figure 3.6b). Overall highest *Geobacteraceae bssA* expression was observed in non-vegetated BJ3 sediments while highest expression of SRP-specific *bssA/nmsA* occurs in highly vegetated BJ1 sediments (Figure 3.7a & 3.7b). In general, *Geobacteraceae*-specific *bssA* and SRP-specific *bssA/nmsA* follow similar seasonal and vegetation-influenced trends as *gltA* and *dsrA*, respectively. Oil-free sediments from Beauregard Island in Barataria Bay were collected for use as a negative control for *bssA* and *bssA/nmsA* expression. As expected, *dsrA*, *gltA*, and *mcrA*, were all highly expressed while *bssA* and *bssA/nmsA* transcripts were undetectable (Figure 3.8).

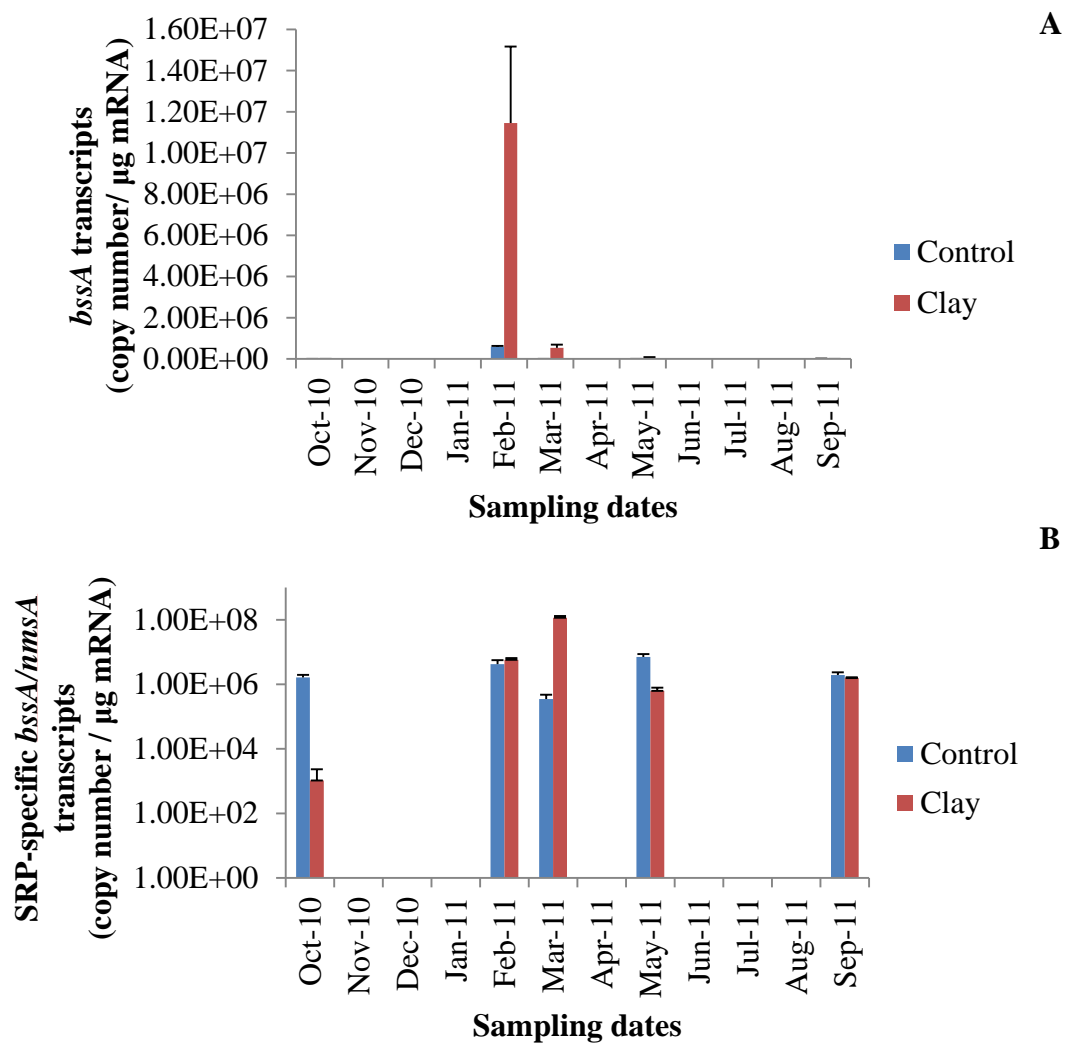


Figure 3.6. Expression of Degenerate, *Geobacteraceae*-specific *bssA* (A) and SRB specific *bssA/nmsA* (B) in sediment from *in situ* Control and Clay-amended plots at site BJ1

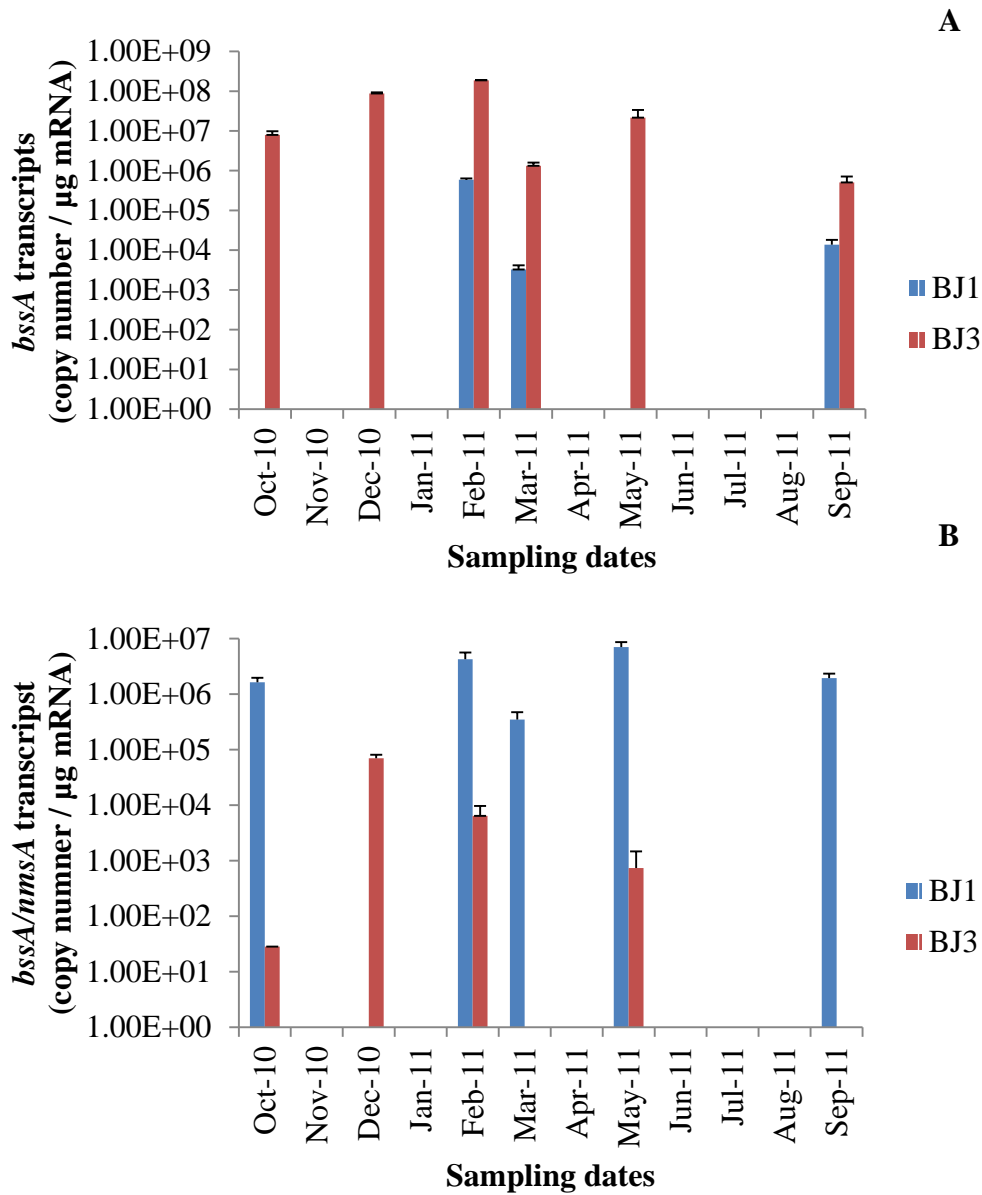


Figure 3.7 Expression of Degenerate, *Geobacteraceae*-specific *bssA* (A) and SRB-specific *bssA/nmsA*(B) in sediment from *in situ* Control plots at sites BJ1 and BJ3.

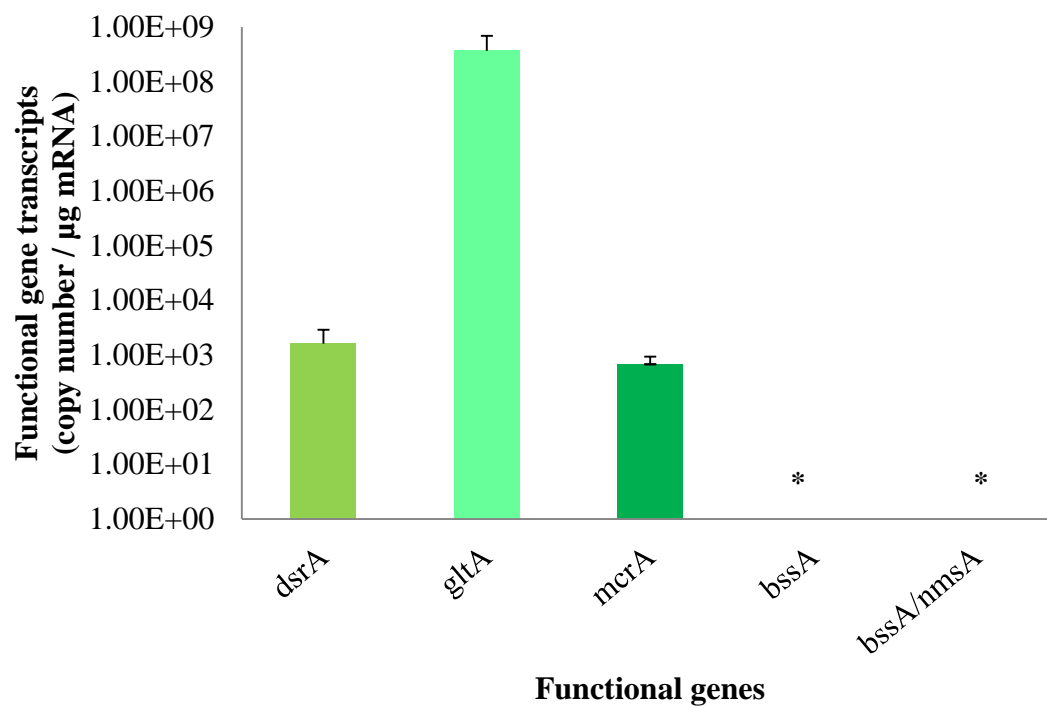


Figure 3.8. Expression of key functional genes in oil-free control sediments from *in situ* plots at Beaugard Island, Barataria Bay, Louisiana.

*indicates that transcripts were not detected

3.2.6 *In situ* field sediment microbial diversity

Phyla were assigned to OTUs recovered from *in situ* samples by Ion Torrent™ pyrosequencing of a 291 bp amplicon of the 16S rRNA gene using degenerate universal primers. *In situ* microbial diversity is site-dependent, with BJ1 Control (February and May 2011) and Clay (May 2011) samples all showing similar patterns in phyla abundance. *Proteobacteria* was the most abundant phylum at BJ1, accounting for 68% of all OTUs from the three BJ1 samples. A higher degree of evenness was observed amongst phyla abundance at BJ3, however, revealing an overall different pattern of diversity. The *Proteobacteria* phylum was still the most abundant (29%), though *Chloroflexi* (18%) and *Euryarchaeota* (13%) were also relatively abundant (Figure 3.9). Within the phylum *Proteobacteria*, differences in abundance of each class were observed. *Epsilonproteobacteria* were most abundant at BJ1-Control-May 2011 (71%), *Gammaproteobacteria* were most abundant at both BJ1-Clay-May 2011 and BJ1-Control-February 2011 (both 41%), and *Deltaproteobacteria* were most abundant at BJ3-Control-May 2011 (60%) (Figure 3.10).

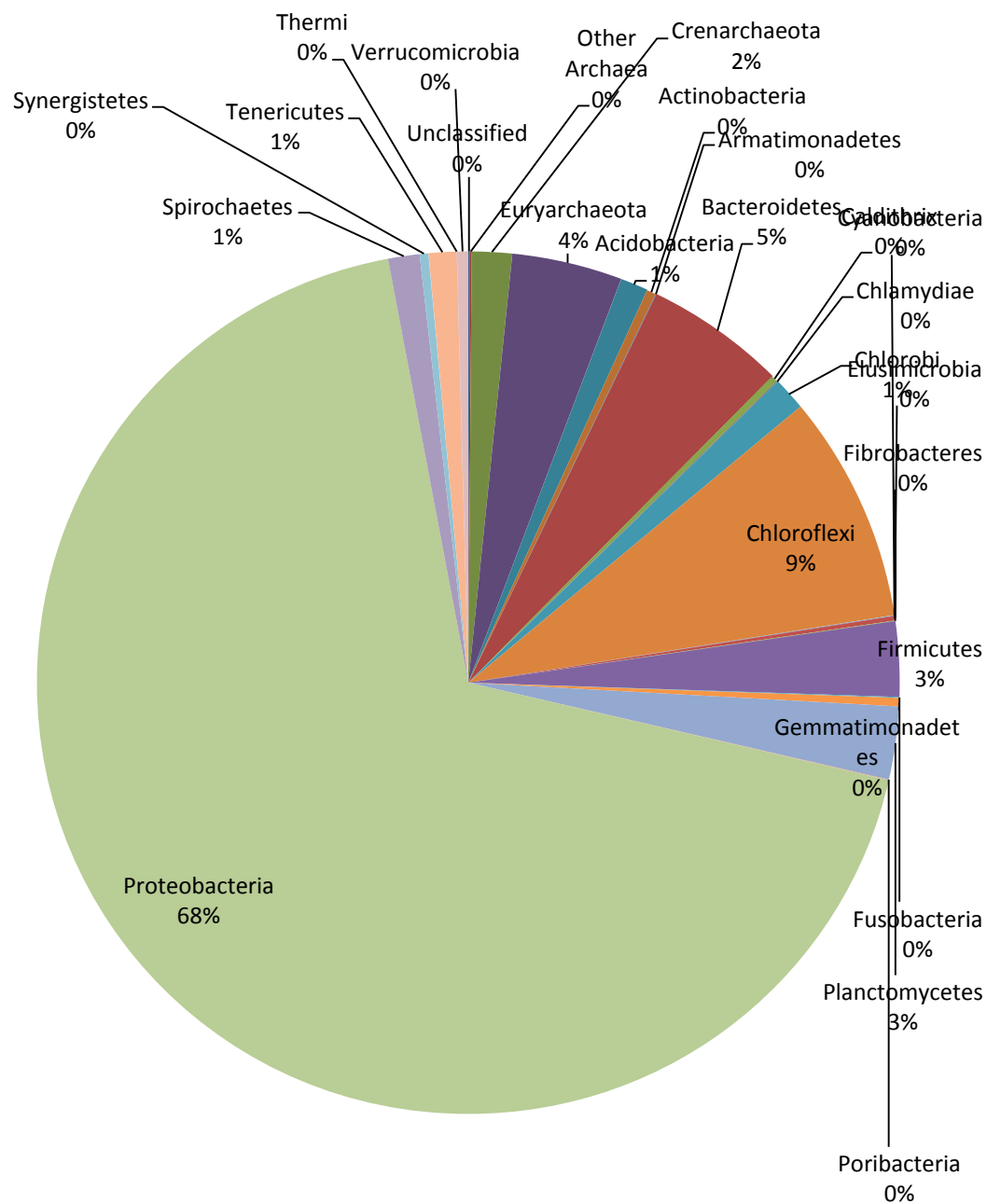


Figure 3.9A. Percentage abundance of archaeal and bacterial 16S rRNA gene sequences by phyla in sediments from the control plot at site BJ1 (May 2011)

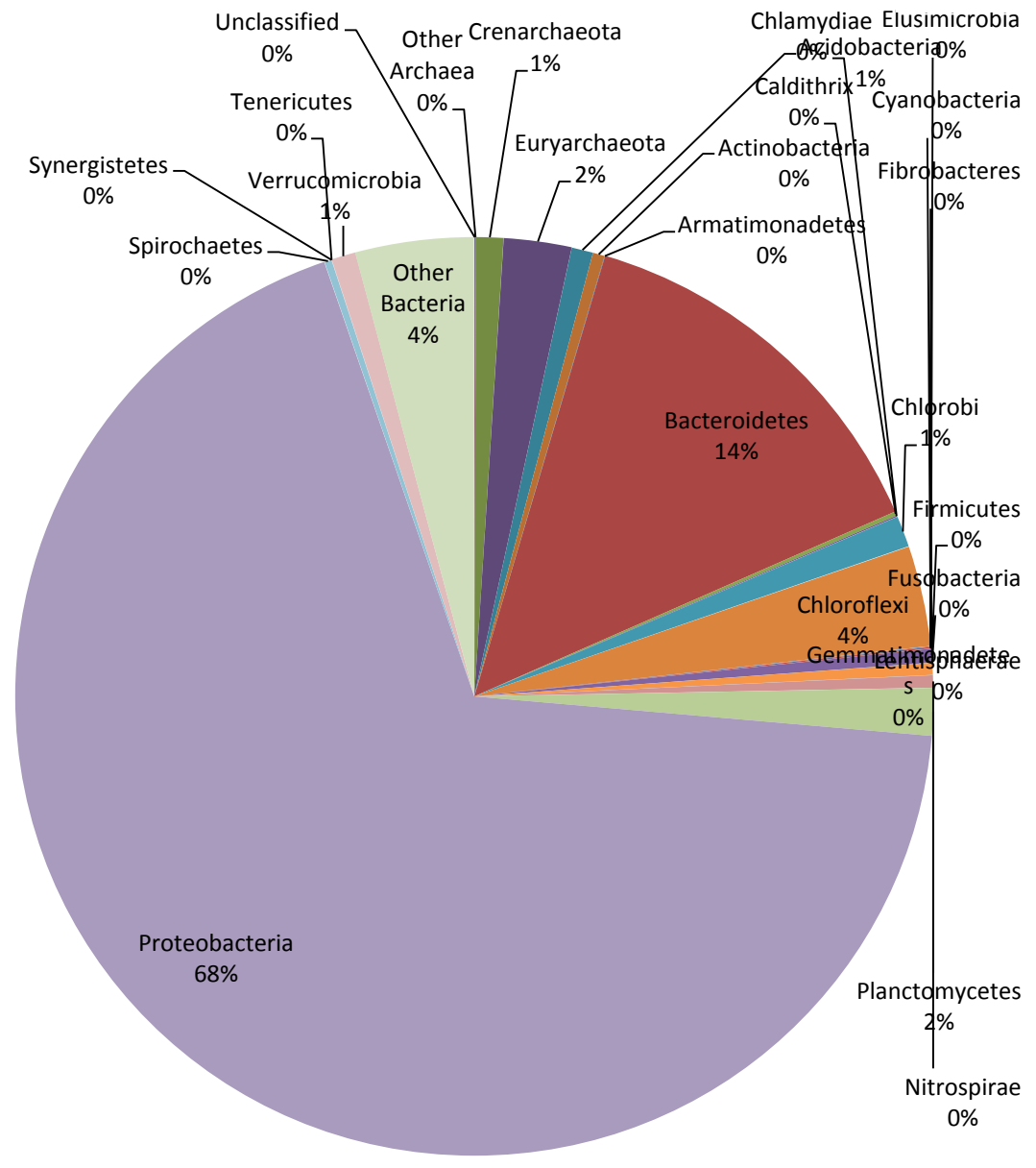


Figure 3.9B. Percentage abundance of archaeal and bacterial 16S rRNA gene sequences by phyla in sediments from the clay-amended plot at site BJ1 (May 2011)

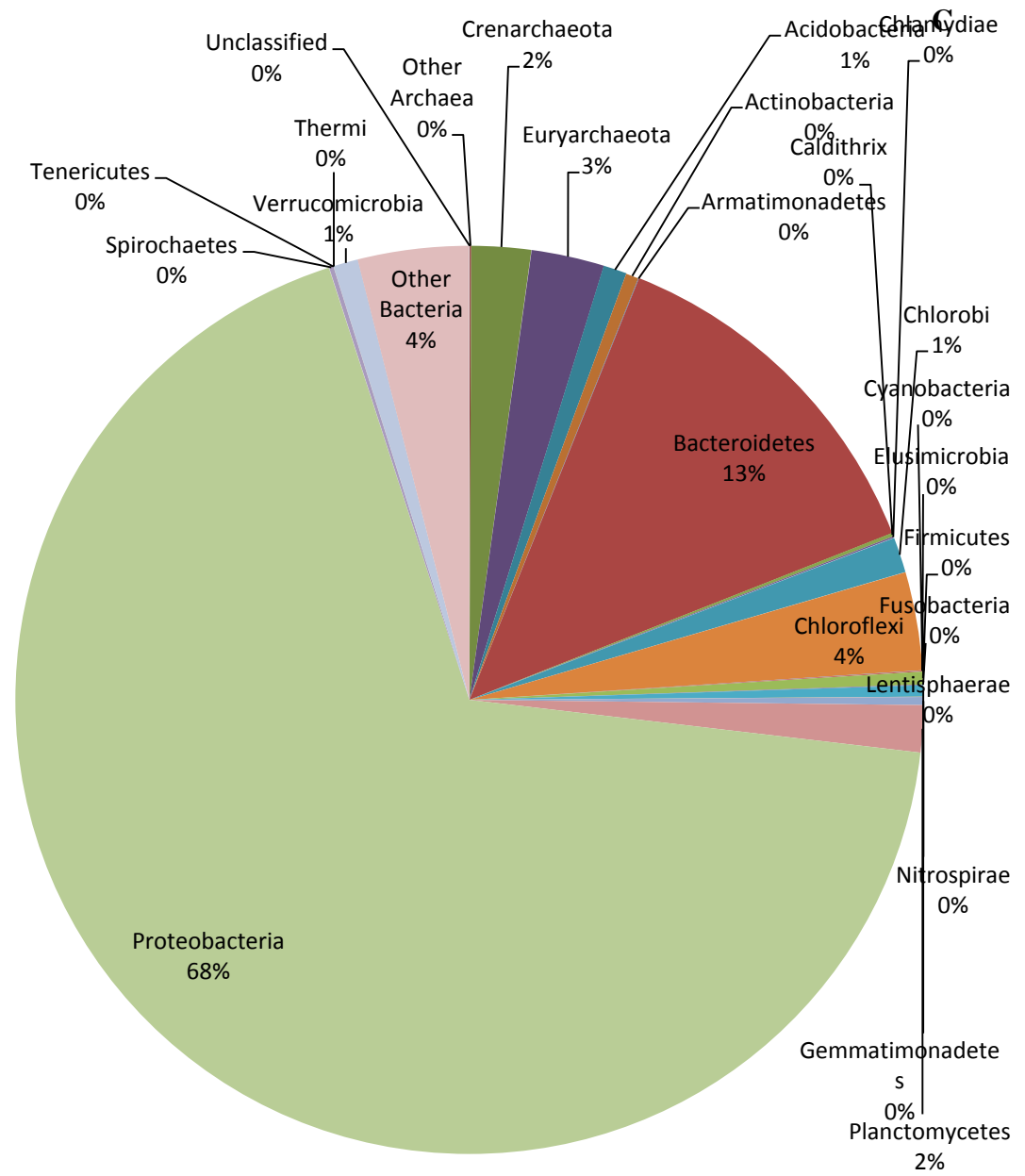


Figure 3.9C. Percentage abundance of archaeal and bacterial 16S rRNA gene sequences by phyla in sediments from the control plots at site BJ1 (February 2011)

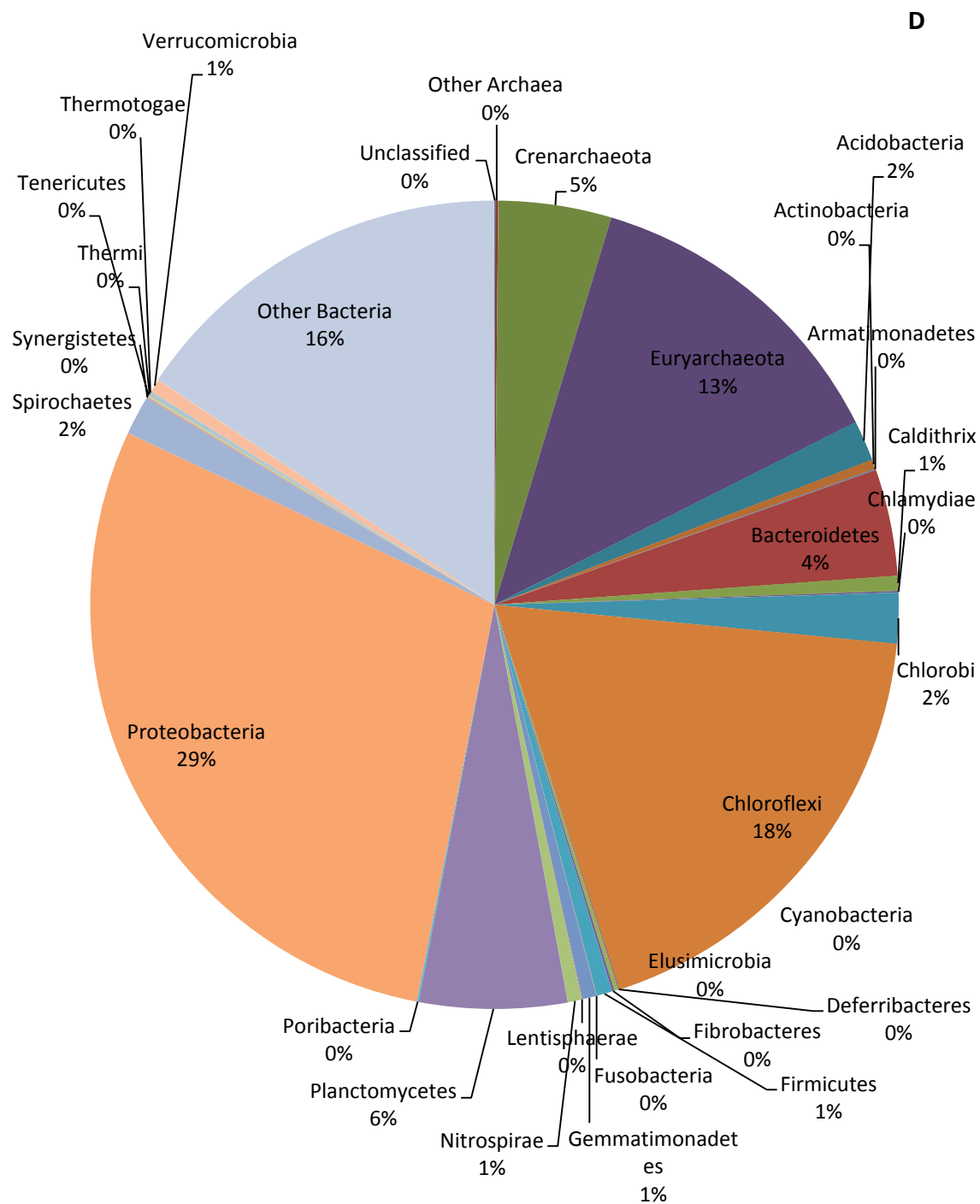


Figure 3.9D Percentage abundance of archaeal and bacterial 16S rRNA gene sequences by phyla in sediments from the Control plot at site BJ3 (May 2011)

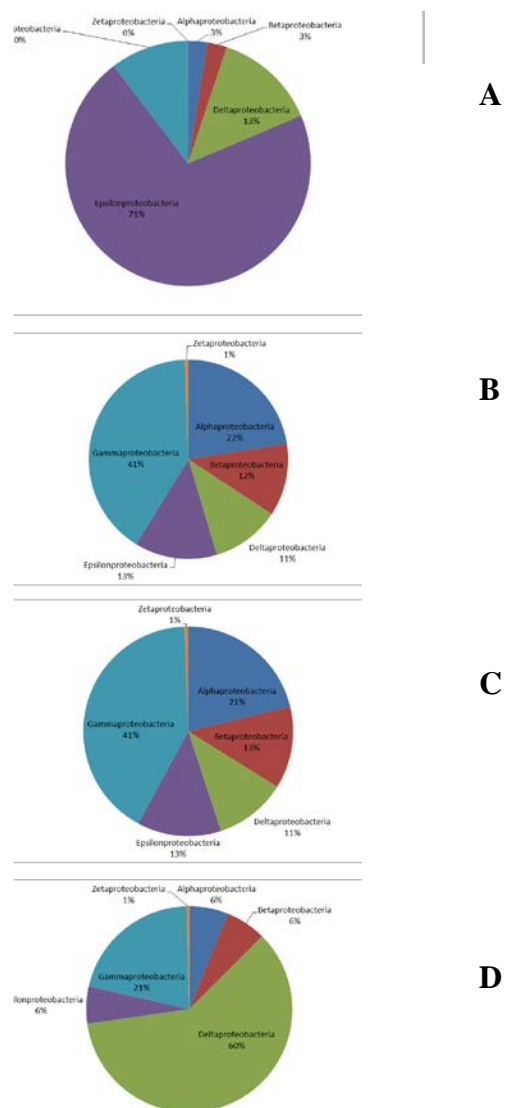


Figure 3.10. Percentage abundance of 16S rRNA sequences by *Proteobacteria* classes in sediments from the control and clay-amended plots at site Bay Jimmy: BJ1-Control-May 2011(A), BJ1-Clay-May 2011(B), BJ1-Control-February 2011(C), and BJ3-Control-May 2011(D).

Understanding that using DNA for sequencing does not reflect activity in the same way as sequencing of 16S rRNA reverse transcripts (cDNA), effort was made to obtain a cDNA amplicon of *in situ* 16S rRNA. A 466 bp RT-PCR product was obtained from BJ3-Clay sediments from October 2010 using primer pair Eub331f-797r and sequenced on a Roche 454 Sequencer at DNA Core Facilities, Georgia State University (Figure 3.11). Though Ion Torrent™ Sequencing using a DNA template from BJ3-Control sediments revealed that *Proteobacteria* was the dominant phylum, RNA results demonstrate that it is also the most metabolically active phylum, accounting for 88% of 16S rRNA RT-PCR amplicons. On the other hand, while *Firmicutes* shows only 1% abundance based on DNA analysis, it accounts for 9% of active phyla based on RT-PCR amplicon sequencing.

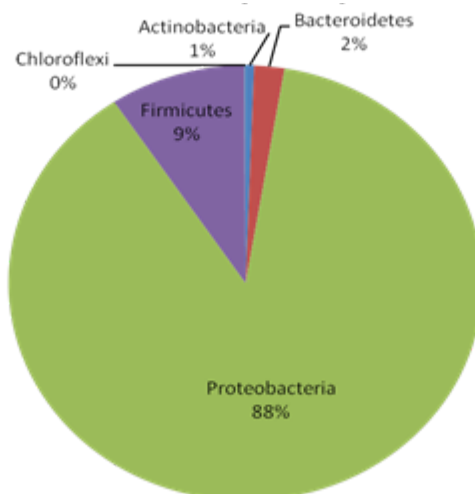


Figure 3.11. Percentage abundance of 16S rRNA sequences showing active bacterial phyla in sediments from the control plots at site BJ3 (October 2010)

3.3 Discussion

3.3.1 *Seasonal and vegetation influences on activity and diversity of anaerobic microbial communities in Deepwater Horizon impacted salt marsh sediments*

Expression levels for *dsrA*, *gltA*, *mcrA* and *bssA* were quantified as proxies for *in situ* dissimilatory sulphate- and iron reduction, methanogenesis, and anaerobic methylaromatic hydrocarbon oxidation, respectively, in *Deepwater Horizon* petroleum impacted salt marsh sediments from Bay Jimmy, LA between September 2010 and September 2011. Transcripts for all targeted functional genes were detected in samples from all time points in the study, though significant seasonal trends were observed for each gene except for *mcrA*. *DsrA* expression is highest in the spring and summer months of 2011, peaking in September of that year. *GltA* expression showed an inverse trend in expression, with highest expression in the winter months of 2010 and 2011, peaking in February 2011. *McrA* expression was highly variable and did not conforming to any seasonal trend. Lower expression levels for all genes were reported for September and October 2010 likely due to a high degree of freshwater released into the salt marsh by the opening of levees on the Atchafalaya River. This increase in hydrologic flow from the river was presumed to retard petroleum infiltration of the estuary (Personal communication, Dr. Daniel Deocampo, Georgia State University).

Degenerate *bssA/nmsA* transcript levels indicated a similar trend to *gltA* expression with highest expression in winter months. This suggests an increase in methylaromatic hydrocarbon degrading activity of *Geobacteraceae* in the winter based upon clone identities for this gene all

closely matching to aromatic-degrading members of the *Geobacteraceae*. SRP-specific *bssA/nmsA* transcripts levels were overall highest in spring and summer 2011, peaking in March. In relation to vegetation cover, highest SRP-specific *bssA.nmsA* expression is observed in the highly vegetated sediments of BJ1 compared to the non-vegetated sediments of BJ3. This trend closely matches to *dsrA* expression levels and suggests that SRP populations generally out-compete FRPs in the summer months when *Spartina* growth rates are highest and decrease in the winter months allowing for higher levels of *Geobacteraceae* activity. Based on transcript copy numbers for each primer pair, methylaromatic hydrocarbon oxidation is dominated throughout the year by SRPs at BJ1 while *Geobacteraceae* may dominate aromatic hydrocarbon degradation in the more heavily oiled and non-vegetated sediments at BJ3.

The expression trends uncovered in this study closely conform to results in prior seasonal studies conducted in salt marshes in which sulphate and iron reduction rates were experimentally assessed (Koretsky *et al.*, 2002; Kostka *et al.*, 2002; Koretsky *et al.*, 2003; Koretsky *et al.*, 2005). These data compared to findings from previous studies support the hypothesis that SRP and FRP populations are most highly active within the rhizosphere of marsh plants compared to bulk sediment and pore water (Isaksen and Finster, 1996), likely owing to short-lived dissolved oxygen that is pumped into the rhizosphere by *Spartina* roots that reoxidizes sulfide and Fe(II) to respirable forms (Mendelssohn *et al.*, 1981; Howes and Teal, 1994; Lee *et al.*, 1999). Interestingly, in this study SRP and FRP activities seem to correspond more to interactions between the dominant SRP population and the *Spartina* growth cycle than sediment terminal electron acceptor abundance. Linking SRP activities to the *Spartina* growth cycle, with sulphate reduction rates increasing in the summer months, has

been reported by Koretsky and colleagues. (2005). Indeed, it has been previously suggested that SRP and FRP populations may coexist due to labile organic substrates originating from plant root exudates into the rhizosphere which may favor certain populations, allowing them to outcompete or coexist with other microbial populations for which *in situ* redox chemistry is more favorable (Lovely and Phillips, 1987).

This is the first study to look at concurrent metabolic group-specific aromatic hydrocarbon degrading activities at the transcriptional level. Based upon gene transcript levels for key respiratory and methylaromatic hydrocarbon oxidation genes selecting for SRP, FRP and methanogenic populations, previously reported hypotheses concerning seasonal variation in these groups' activities are supported by data from the present study. These trends are also manifest in anaerobic aromatic hydrocarbon degrading activities as detected by phylogenetic group-specific cloning and qPCR.

3.3.2 Effects of montmorillonite clay amendment on activity and diversity of anaerobic microbial communities in Deepwater Horizon impacted salt marsh sediments

The clearest trend in expression levels for all genes in this study manifests in the difference in gene expression between control and clay-amended sediments. Genes expression levels of *dsrA* and *gltA* indicate higher rates of dissimilatory sulphate and iron reduction, respectively, in clay-amended sediments over control sediments. Generally higher *dsrA* expression occurred during the spring and summer months of 2011 with the overall highest expression level

detected in clay-amended sediments in March 2011. Similarly, generally higher *gltA* expression was observed in winter months with the highest overall expression level detected in clay-amended sediments in February 2011. While it is argued that cycles in *Spartina* growth plays an important role in the seasonal trends of dissimilatory sulfate and iron reduction rates, and consequently *dsrA* and *gltA* expression, it is clear that the presence of montmorillonite clay amendment significantly positively affects anaerobic respiratory and aromatic hydrocarbon degrading activities *in situ*.

The precise mechanism for this observation remains undetermined, though it has been suggested that the cation exchange between seawater and spaces between the phyllosilicate layers of the montmorillonite provides a favorable electrochemical environment for microbial cell envelope attachment and contaminant sorption (Chaerun *et al.* 2005; Warr *et al.*, 2009; Witthuhn *et al.*, 2006), which would allow for increased cell aggregation within the surface sediments where oxidized forms of electron acceptors (Koretsky *et al.*, 2005) and, in the case of the present study, petroleum-derived substrates are more concentrated. Microbial diversity appears to be site-dependent rather than influenced by clay amendment or seasonal conditions *in situ* (Figure 3.8).

4 ANAEROBIC MICROBIAL METABOLIC ACTIVITIES AND PETROLEUM HYDROCARBON DEGRADATION IN *DEEPWATER* *HORIZON* OIL-SPILL IMPACTED SALT MARSH SEDIMENT MICROCOSMS

4.1 Introduction

As discussed in Chapter 3, anaerobic respiration coupled to petroleum hydrocarbon degradation are the important processes contributing to *in situ* bioremediation of the *Deepwater Horizon* oil spill in the coastal salt marshes of Louisiana (Jackson and Pardue, 1997; Williams, 2004; Gallego *et al.*, 2006; Andreoni and Gianfreda, 2007; Vega *et al.*, 2009). *In situ* gene expression assays, functional gene transcript sequencing and geochemical analyses have provided tremendous insight into the structure and function of anaerobic communities and their metabolic activities in a petroleum impacted salt marsh in Bay Jimmy, LA (Results, Chapter 3).

Sediment microcosm incubations are used in microbial ecology to assess microbial community interactions and microbially mediated processes within a small-scale habitat to provide insight into the behavior of the same processes within the large-scale environment (Chapelle, 2001). Additionally, microcosm experiments provide the researcher with the means to specify modifications to the system and study the effects of such modification in terms of its influence on microbial community structure and metabolic activities. Most significantly, biogeochemical processes mediated by microbial activities may be studied in detail without perturbations to the

system by external variables. Microcosm experiments have contributed to our understanding of microbial metabolic processes in a range of sulfate-reducing, iron-reducing, methanogenic and other anaerobic environments (Edwards *et al.*, 1992; Beller *et al.*, 1997; Edwards *et al.*, 1994; Shi *et al.*, 1999; Hiibel *et al.*, 2008). For instance, Yagi *et al.* (2010) demonstrated the relative influences of sulfate-, nitrate-, and metal-reducing activities on anaerobic naphthalene degradation in contaminated terrestrial groundwater microcosms. Coats *et al.* (1995) investigated aromatic hydrocarbon degradation in coastal marine sediment microcosms in relation to SRP activities. Pereyra *et al.* (2010) employed the use of an assortment of primer pairs targeting genes of metabolic interest for molecular characterization of microbial guilds in several different anaerobic systems. Direct correlation of functional gene expression with microbial processes of geochemical relevance was elegantly demonstrated in microcosm studies by Chin *et al.* (2004 and 2008). Indeed, insights through microcosm experimentation have given rise to many developments in molecular methods applied to *in situ* biogeochemical studies.

The methodology of such microcosm studies often includes the stimulation of certain process, such as aromatic hydrocarbon degradation, by supplying an overabundance of a limiting factor such as alternative electron acceptor, which can provide insight into the catabolic potential of individual respiratory guilds (Yagi *et al.*, 2010). Other studies examine changes to microbial community structure in response to biogeochemical alterations such as nutrient amendment (Kolukirik *et al.*, 2011), nutrient limitation (Medvedeff *et al.*, 2014) or contaminant introduction (Shi *et al.*, 1999).

The present study's aims are to investigate the major anaerobic processes of microbial dissimilatory sulfate- and iron-reduction, methanogenesis, and petroleum hydrocarbon degradation in relation to one another and as influenced by the presence of excess crude oil and/or montmorillonite clay. These respiratory and catabolic activities are assessed by quantitative real-time PCR of functional gene transcripts for genes integral to the specific metabolic pathways in question or for central metabolism. No other nutrients or electron acceptors are added with the intent to most accurately reflect *in situ* conditions. Changes in community structure and phylogeny are assessed by Ion TorrentTM pyrosequencing of the 16S rRNA gene using universal primers. Correlations between montmorillonite addition and rates of petroleum hydrocarbon degradation are also investigated by direct total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) measurements at the beginning and end of the incubation period. The goal of such an experiment is to better understand the metabolic potential of each respiratory guild (SRP, FeRP, and MGP) to contribute to overall crude oil biodegradation independent of environmental variables such as seasonal effects, tidal action, and the growth cycle of salt marsh vegetation. Additionally, direct assessment of hydrocarbon loss is more practical within a closed system as provided by microcosm methodology.

4.2 Results

4.2.1 *Respiratory activity of SRP, FRP and MGP in sediment microcosm*

Transcripts for *dsrA*, *gltA*, and *mcrA* were isolated, reverse-transcribed, PCR amplified and visualized by 1.5% agarose gel electrophoresis for confirmation of primer efficiency and RNA quality. cDNAs for all genes were detected by qPCR. Overall, *dsrA* was more highly expressed in all treatments (Control, Oil, and Oil + Clay; N, O, and O + C, respectively) than both *gltA* and *mcrA* indicating that sulfate-reduction is the dominant the respiratory activity in the microcosm incubations (Figure 4.1a-c). Of the three treatments, N and O+C incubations demonstrated higher sulfate-reducing activities than incubations in the O treatment according to gene expression assays (Figure 4.1a). Similarly, *gltA* expression was overall highest in N and O+C treatments than in the O treatment (Figure 4.1b). Expression of *mcrA* was significantly highest in the O+C treatment at 10 days of incubation, suggesting that the presence of montmorillonite has a more significant influence on methanogenic activities of sediment microbial communities (Figure 4.1c). All gene expression levels began to decrease rapidly between 16 and 21 days of incubation.

Dissimilatory iron-reducing activity was further examined by monitoring Fe(II) production for up to 40 days of incubation. By the end of the incubation period, both the O and O+C treatments resulted in overall highest Fe(II) production, although incubations in the O+C treatment showed higher Fe(II) production rates until day 40 (Figure 4.2). Only approximately

30% of the total iron available to the systems was reduced by the end of the experiment (Figure 4.2).

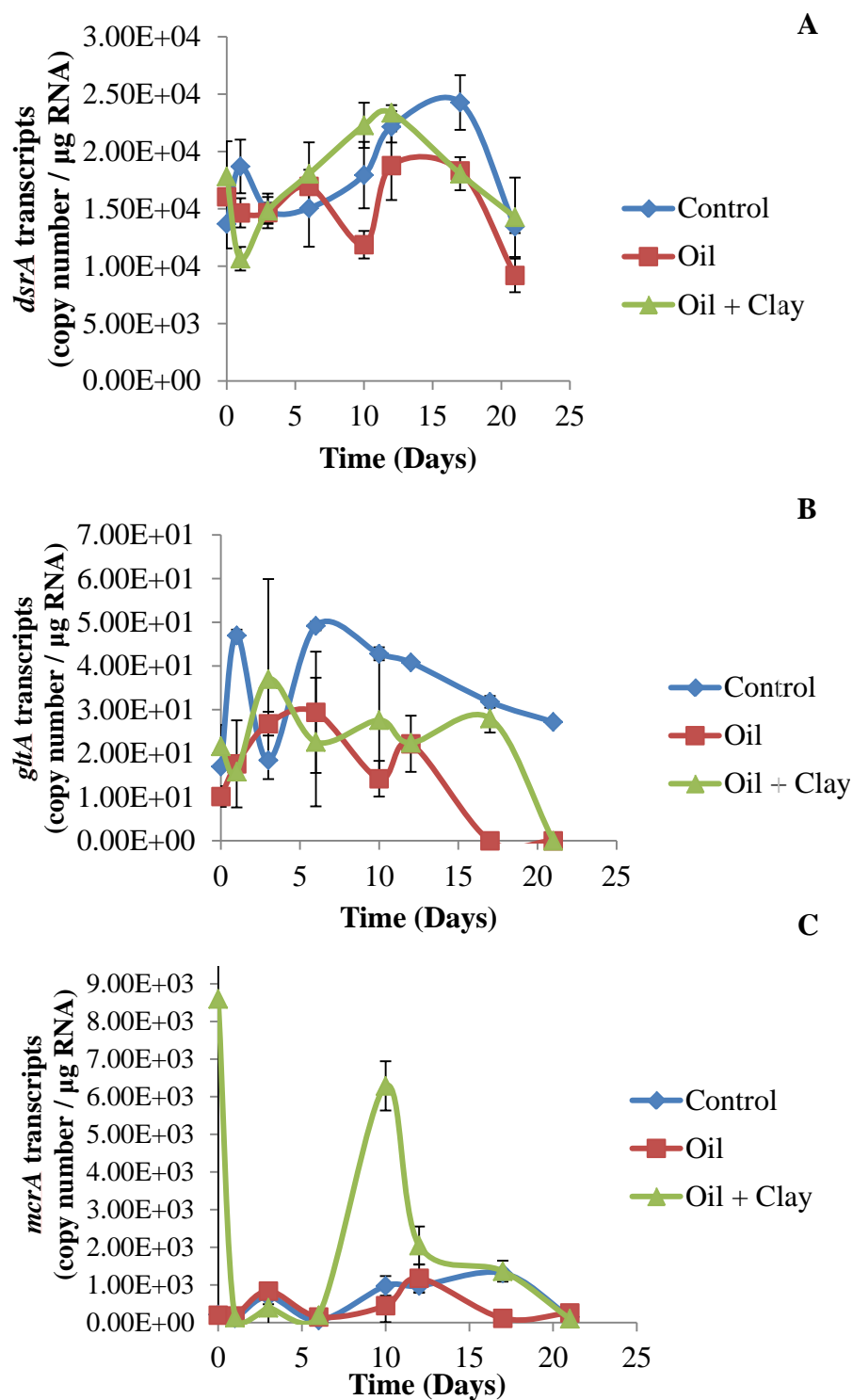


Figure 4.1. Expression of *dsrA* (A), *gltA* (B) and *mcrA* (C) in BJ1 sediment microcosm incubation

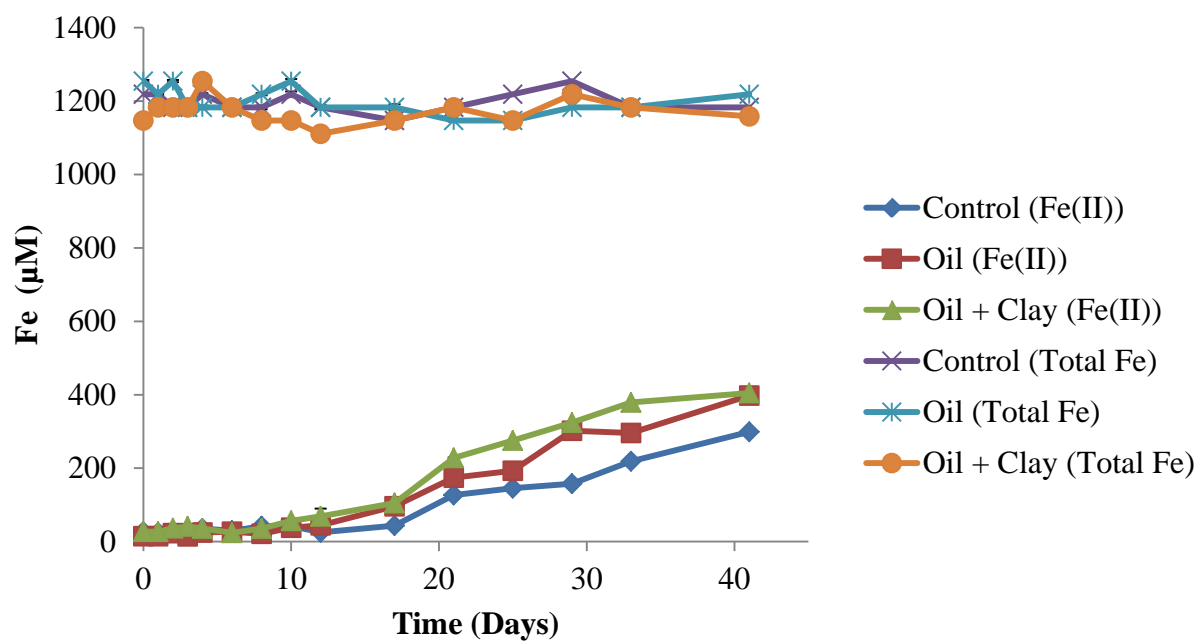


Figure 4.2. Total Fe and Fe(II) concentration in BJI sediment microcosm incubation without any treatment (Control), with oil only and oil and clay amendment

4.2.2 *Anaerobic aromatic hydrocarbon-degrading activity*

Degenerate primers which were designed to target *bssA* and SRP-specific *bssA/nmsA* transcripts were used to estimate anaerobic aromatic hydrocarbon-degrading activity. Although *Geobacteraceae* and SRP-specific transcripts were detected *in situ* at both BJ1 and BJ3 sites, only *bssA* transcripts for the *Geobacteraceae* were detected in BJ1 sediment microcosm incubations. Transcript levels were similar to those observed *in situ*, peaking around 1.5×10^6 copies of transcripts per μg RNA. Highest overall *bssA* expression was observed in microcosm amended with oil and clay with expression peaking at 12 days of incubation. Lowest expression was detected in microcosm amended with oil, though expression levels in Oil-amended microcosm were not significantly lower than microcosms without any treatment (Figure 4.3). These results suggest that montmorillonite clay contributes to overall higher aromatic hydrocarbon-degrading activities in salt marsh sediments when petroleum hydrocarbon levels are elevated. This finding further suggest that in a closed system, anaerobic aromatic hydrocarbon degrading-activities are dominated by members of the family *Geobacteraceae*, while *in situ* activities are dominated by sulfate-reducing prokaryotes (Figures 3.5 and 3.6).

Concentration of total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) were measured in microcosms with oil amendment and oil and clay amendment after 1 and 21 days incubation in order to assess the efficacy of clay amendment at enhancing overall hydrocarbon degradation in crude-oil spiked microcosm incubations. After 21 days of incubation, TPH levels were significantly lower in the microcosms with oil and clay

amendment than microcosm with oil only amendment, suggesting that montmorillonite clay amendment significantly enhanced the rates of TPH degradation (Figure 4.4). Concentration of extractable PAHs slightly increased after 21 days of incubation compared to 1 day incubation (Figure 4.5). These results are consistent with findings based on *in situ* measurements of alkanes and PAHs at sites BJ1 and BJ3 (Ghelerter *et al.*, unpublished).

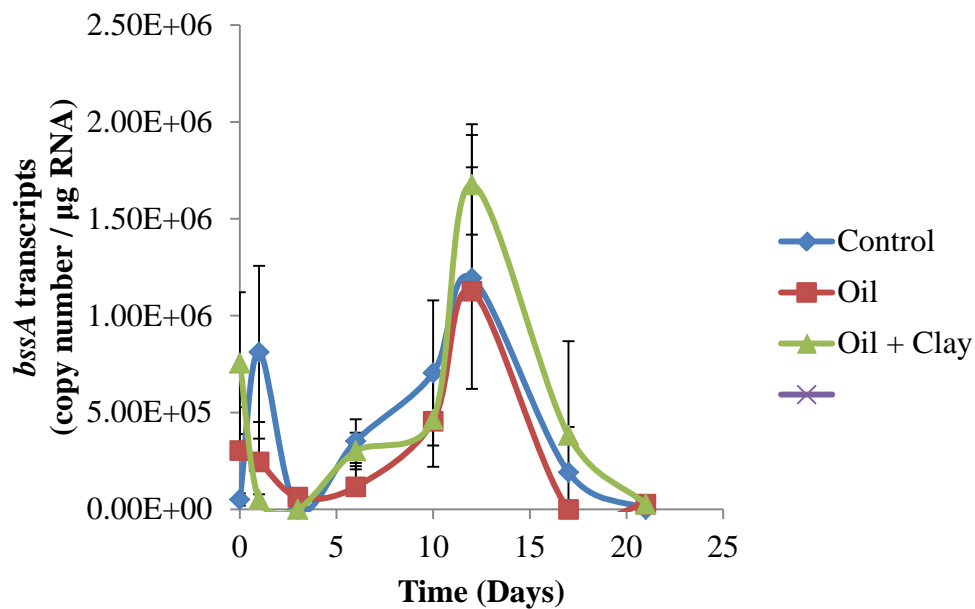


Figure 4.3. Expression of *Geobacteraceae*-specific *bssA* in BJ1 sediment microcosm incubation without any amendment (control), with oil only amendment, and with both oil and clay amendment

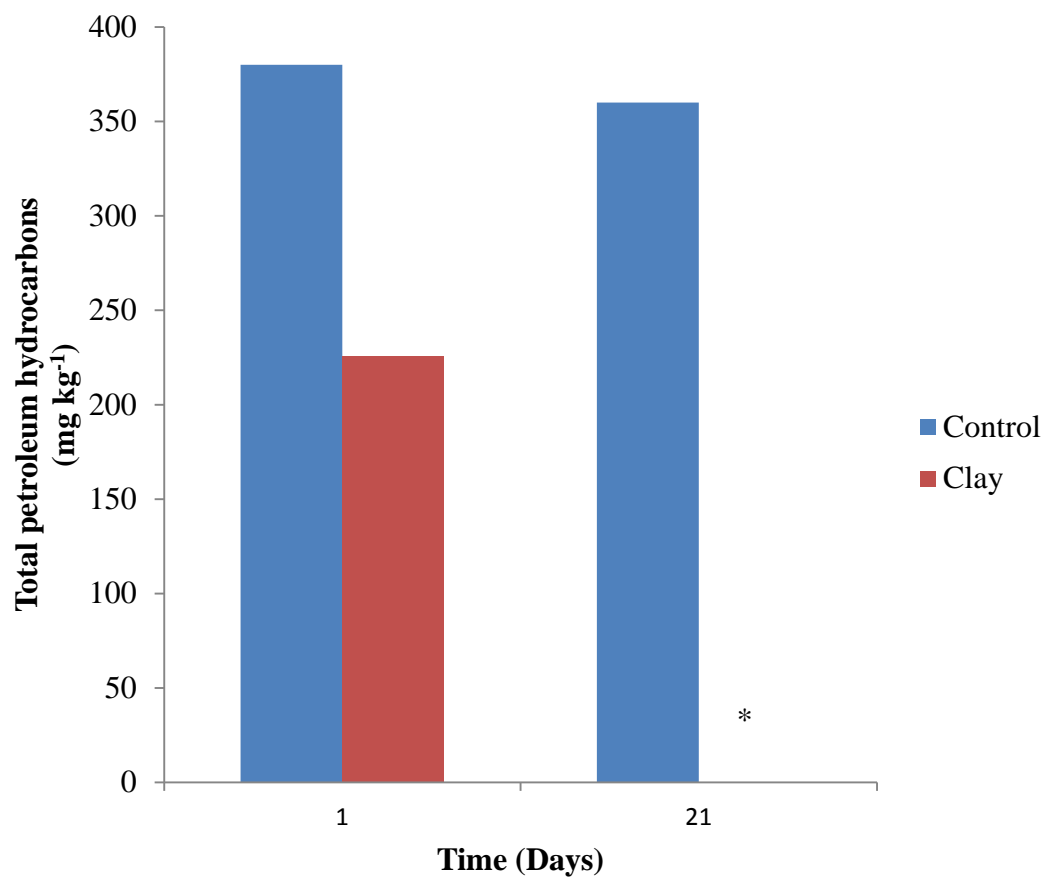


Figure 4.4. Total petroleum hydrocarbon (TPH) concentration in control and clay-amended BJ1 sediment microcosm after 1 and 21 days incubation.

* indicates non-detected

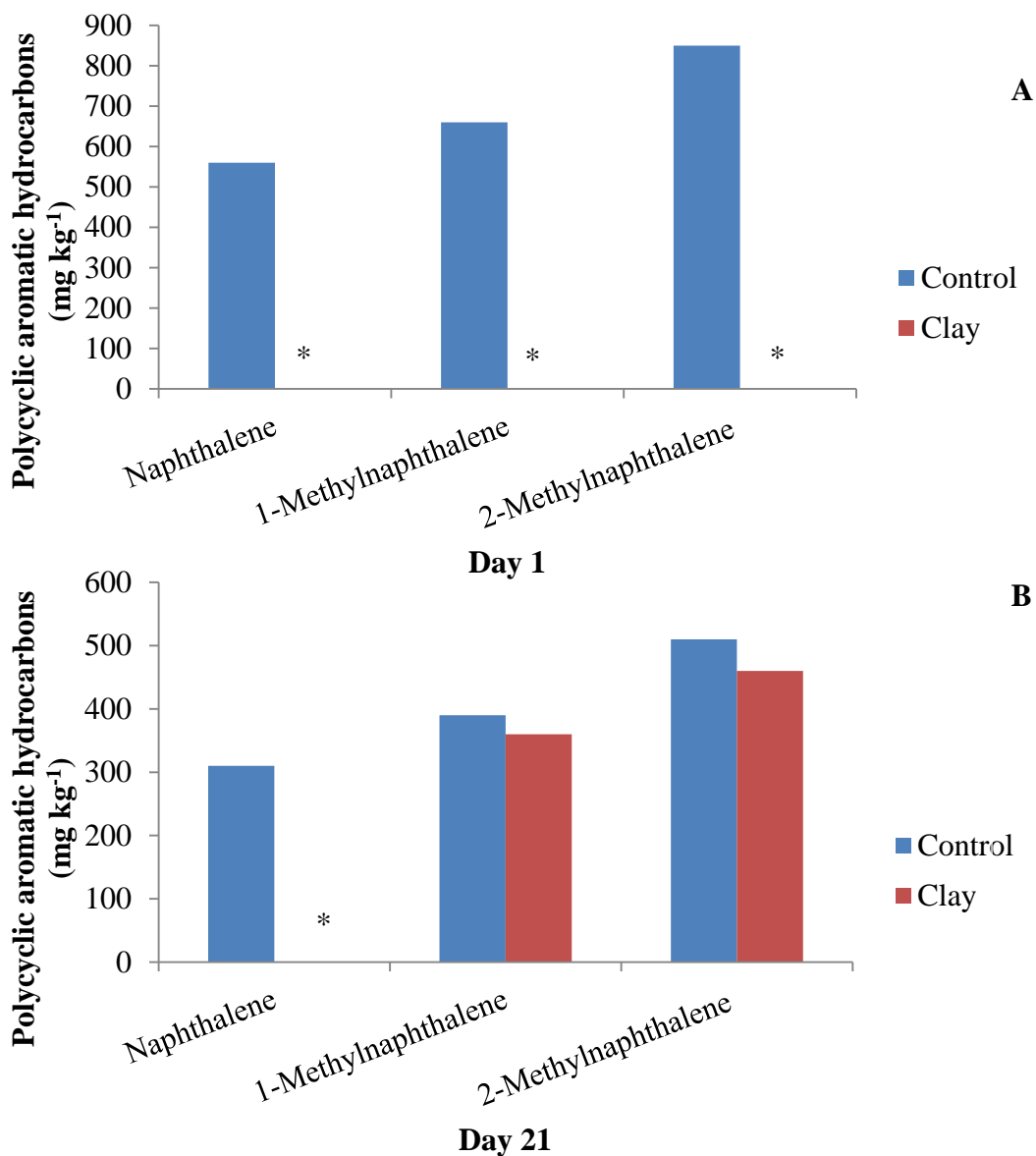


Figure 4.5. Polycyclic aromatic hydrocarbon (PAH) concentration in microcosm without any amendment (control) and clay-amended microcosm after 1 day (A) and 21 days (B) incubation. For all PAHs analyzed, only naphthalene, 1-methylnaphthalene and 2-methylnaphthalene were detected.

* indicates non-detected

4.2.3 Pyrosequencing of 16S rRNA gene from microcosm incubations

Universal primers (515f-806r) were used to amplify a highly conserved 291 bp region of the 16S rRNA gene using high quality DNA extracted from the microcosm sediments after 12 days of incubation. Pyrosequencing using Ion Torrent™ next generation semiconductor sequencing technology demonstrated differences in diversity and abundance of operational taxonomic units (OTUs) in three different set of microcosms. The lowest microbial diversity with 4120 distinct OTUs was observed in sediment microcosms without any amendment (control) while in the microcosm with the oil amendment only slightly higher diversity with 4197 OTUs was observed. In the microcosms amended with both oil and clay the highest microbial diversity with 4420 OTUs was shown. The most abundant OTU was the same in each microcosm accounting for 2.69%, 0.50%, and 1.42% of the total OTUs for the Control, Oil and Oil + Clay treatments, respectively, most closely matched to *Methanococcus* sp. of the phylum *Euryarchaeota*. Of the all microcosms, the results obtained from the microcosm with both oil and clay amendment demonstrated the highest percent abundance of OTUs which are most closely related to members of anaerobic aromatic hydrocarbon degrading bacteria with 7 out of the 10 most abundant OTUs in the class *Deltaproteobacteria* (Figure 4.6).

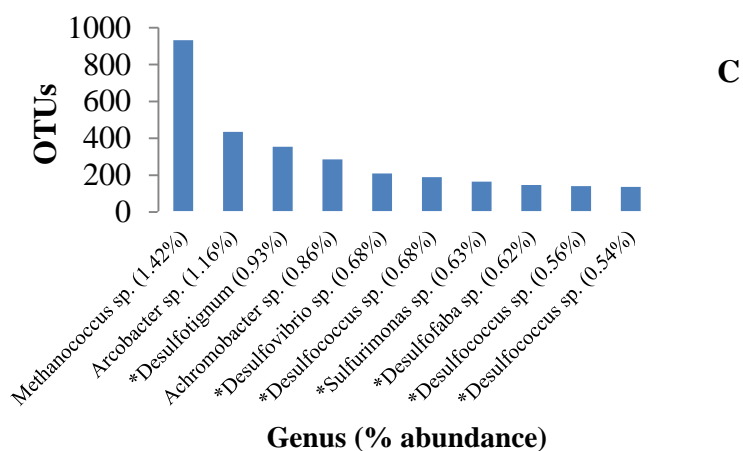
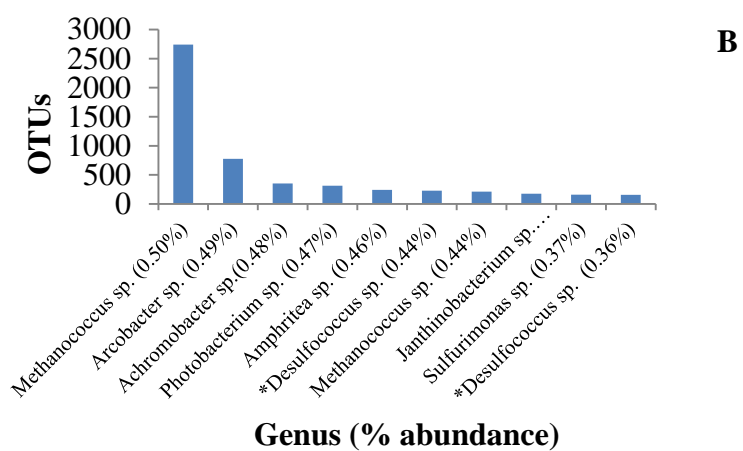
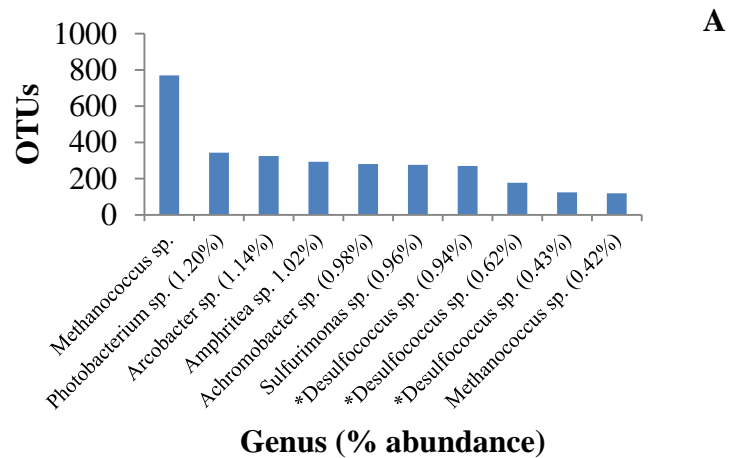


Figure 4.6. Most dominant Operating Taxonomic Units (OTU) in BJI sediment microcosm incubation without any amendment (control, A), with oil only amendment (B), and both oil and clay amendment incubations (C). * indicates the sub-Class *Deltaproteobacteria*

Percent abundance of each archaeal and bacterial phylum that was represented by at an OTU was calculated for each treatment (Figure 4.7). The predominant phylum in all three treatments was the *Proteobacteria*, representing 53%, 48%, and 50% of all OTUs in the Control, Oil, and Oil + Clay treatments, respectively. *Bacteroidetes*, *Euryarchaeoata*, and *Chloroflexi* were the other dominant phyla by percent abundance in each treatment. Among the *Proteobacteria*, the *Deltaproteobacteria* were the most dominant, accounting for 44%, 41%, and 56% of the three treatments' *Proteobacteria* classes (Figure 4.8). It is notable that the clay-amended microcosms showed a 12% increase in *Deltaproteobacteria* abundance over the Controls and 15% increase over the Oil-amended microcosms.

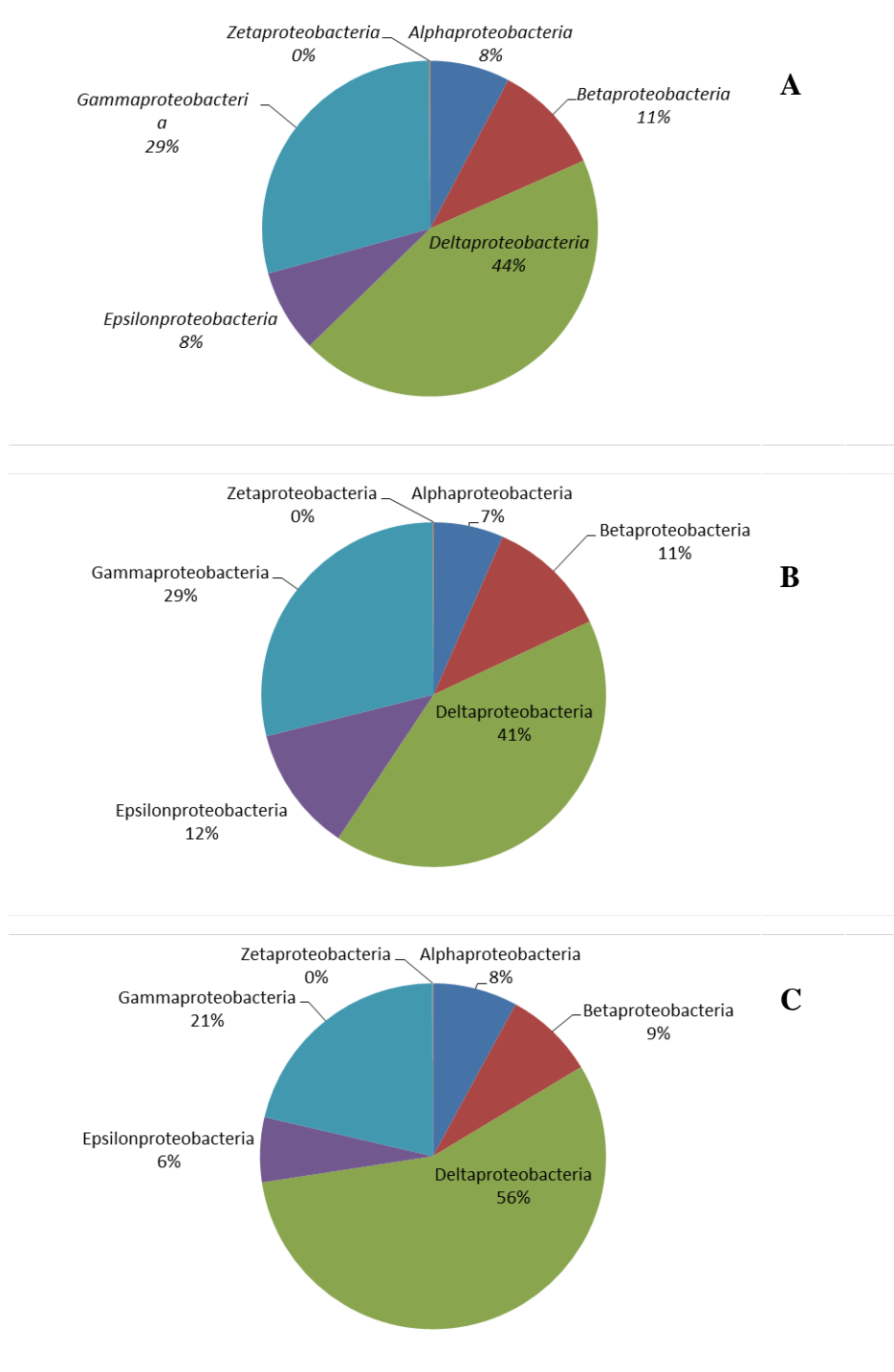


Figure 4.8 Percentage abundance of 16S rRNA gene sequences showing composition members of the *Proteobacteria* class in BJ1 sediment microcosms without any treatment (Control, A), with oil only amendment (B), and with both Oil and Clay amendment (C) after 12 days incubation

4.3 Discussion

The objective of this study was to better understand the interrelations of anaerobic microbial respiratory guilds in crude oil-impacted salt marsh sediments. Microcosm incubations are standard protocol for bioremediation studies in contaminated environments (Phelps *et al.*, 1998). By observing the principle respiratory processes in relation to one another in a closed anaerobic system, the dominant process or processes may be determined without the influence of external environmental perturbations. Additionally, the presence of montmorillonite clay at enhancing anaerobic microbial metabolic activities leading to accelerated biodegradation of petroleum hydrocarbons was investigated with the hopes of ameliorating coastal oil spill bioremediation *in situ*.

Knowing that clay minerals have been shown to positively affect microbial growth under various environmental conditions and, particularly, in the presence of high amounts of crude oil (Chaerun *et al.*, 2005a & 2005b; Warr *et al.*, 2009), it was not surprising to observe a positive correlation between montmorillonite amendment and an increase in microbial anaerobic respiratory activities in crude-oil spiked sediment microcosm incubations. Overall higher expression of key microbial anaerobic respiratory genes was observed in crude oil-spiked microcosm incubations supplemented with montmorillonite than in incubations with just crude oil. Indeed, crude oil-spiked incubations without montmorillonite showed lower sulfate- and the family *Geobacteraceae* and methanogenic gene expression than the control incubations without crude oil. This lends support to the assumption made by Chaerun and

colleagues (2005a) that microbial cells in the presence of high amounts of crude oil and clays are better capable of adapting to the stress of a crude-oil rich environment.

In addition to observations of microbial activities, direct TPH analysis of oil and oil+clay microcosm incubations confirm that the presence of montmorillonite increases the rate of TPH degradation after 21 days of incubation. This is consistent with previous studies on montmorillonite-enhanced crude oil biodegradation both in laboratory (War *et al.*, 2009) and *in situ* (Gelehter *et al.*, unpublished), though the present study is the first to report such findings in strictly anaerobic conditions. Enhanced TPH degradation as revealed by gravimetric analysis in clay-amended microcosms also correlated well with the results of gene expression analysis that demonstrated an increased level of *bssA* transcripts in microcosms with clay and oil amendment. These results further support employing functional gene expression analysis as molecular proxy for estimating microbial activities involved in bioremediation of contaminants in environments.

Ion TorrentTM next generation semiconductor sequencing of 16S rRNA gene amplicons in the three treatments demonstrated significant differences in microbial community structure resulting from the additions of crude oil and oil with montmorillonite clay. Notably, in the clay- and crude oil amended-microcosms the highest diversity of OTUs and, moreover, a greater abundance of *Deltaproteobacteria*, a class of *Bacteria* with many known anaerobic alkane and aromatic hydrocarbon degraders, were observed compared to than the other two treatments. Precise mechanisms for the increase in abundance and activity of petroleum hydrocarbon degrading microbial groups is as yet undetermined, though detoxification of the environment due to the adsorption of organic contaminants to clays may be a possibility

(Sharmasarkar *et al.*, 1999) as well as electrochemical stabilization of the outer membrane of microorganisms due to cation interpolation between phyllosilicate layers (Warr *et al.*, 2009).

5 DETERMINATION OF TRACE ELEMENT ADDITION ON COAL BED METHANE BIOGAS PRODUCTION USING GENE EXPRESSION ANALYSIS

5.1 Introduction

Coal biodegradation to methane is a strictly anaerobic process that is mediated by methanogenic prokaryotes in coal-rich subsurface environments (Thielemann *et al.*, 2004; Flores *et al.*, 2008). Coal bed methane is natural coal gas that is biologically produced deep within boreholes that have been drilled into unworked coal-bearing rocks for the purpose of coal mining (Thielemann *et al.*, 2004). The fact that *in situ* CBM production continues long after the coal mining has ceased allows for prolonged use of the coal bed for energy extraction far beyond its original intended purpose (Gale and Freund, 2001).

At present, the United States is the world's leading supplier of natural gas, of which CBM constitutes 10% of methane produced annually in the United States (Flores *et al.*, 2008). Such methane producing coal beds are abundant in continental North America and therefore present a lucrative source of domestically-produced, renewable natural gas (Thielemann *et al.*, 2004). The prospect of optimizing *in situ* CBM production without further drilling or removal of coal-bearing rock would significantly increase the sustainability of such energy extraction projects and decrease the need for increased natural gas exploration and its consequential environmental degradation (Gale and Freund, 2001). For this reason, there is a general interest in better understanding the microbiological and geochemical dimensions of CBM production (Ünal *et al.*, 2012).

Although the microbial metabolic pathways leading to methane production are diverse, all known pathways converge at a step involving the methylation of Coenzyme M through covalent modifications to acetate, CO₂, or reduced C₁ compounds. Methylcoenzyme M and Coenzyme B are subsequently oxidized to a heterodisulfide compound accompanied by the loss of methane ($\text{CH}_3\text{-S-CoM} + \text{H-S-CoB} \rightarrow \text{CH}_4 + \text{CoM-S-S-CoB}$) (Thauer *et al.*, 1993). The following step, which is an exergonic reaction leading to ADP phosphorylation, is catalyzed by methyl coenzyme M reductase (Thauer, 1993; Christoserdova *et al.*, 1998). The gene for the alpha-subunit of this critical enzyme, *mcrA*, has been used extensively in molecular investigations of methanogenesis within several different environments (Friedrich, 2005; Luton *et al.*, 2002). Its use as a metabolic biomarker for assessing rates of microbe-mediated methanogenesis holds promise for quantifying such processes *in situ* through high-quality mRNA isolation and quantitative real-time PCR (qPCR) with *in situ* environmental samples, microcosms, and enrichment cultures (Juottonen *et al.*, 2008; Yuan *et al.*, 2011; Ünal *et al.*, 2012).

The goal of the present study was to contribute to an on-going investigation of CBM production at a field site within the Powder River Basin coal seam in east-central Wyoming. Stable, sediment free, methanogenic enrichment cultures, which were prepared from coal bed production water at the laboratory of Dr. Klaus Nüsslein at the University of Massachusetts, Amherst, were used for the present study. The effects of several trace elements and combinations thereof were examined for their potential at enhancing metabolic processes leading to optimized methane production. Microbial activity involved in methane production was estimated by quantification of transcripts for *mcrA* and *Eubacterial* and *Archaeal* 16S

rRNA using qPCR assay reported in Materials and Method section (Chapter 2). Gene expression rates were correlated with methane production and reported in Ünal *et al.* (2012).

5.2 Results

The levels of *mcrA* transcripts and archaeal and eubacterial 16S rRNAs were quantified by qPCR in weekly time intervals over a 35-day incubation period. Enrichment cultures amended with all trace elements (All TE) showed an immediate strong response in *mcrA* expression, reaching maximum transcript levels after one week of incubation (2.18×10^7 copy numbers/ μg mRNA) and declining steadily for the remainder of the incubation to just below 1.0×10^4 copy numbers/ μg mRNA. The level of *mcrA* transcripts in no trace element (No TE) amended enrichment cultures reached a maximum copy number of 1.30×10^7 after 14 days of incubation and declined steadily to 1.0×10^4 copies by the end of the incubation period (Figure 5.1).

While results for All TE-amended enrichments and enrichments without TE amendment are to be expected given the physiological requirement for multiple various trace elements, enrichment cultures amended with specific trace elements (Fe, Mo, Ni, Co, and a combination of the four) demonstrated interesting responses in terms of gene expression. The addition of Co and Ni resulted in the overall highest transcription levels of *mcrA* which peaked at 7 days of incubation (3.57×10^7 and 3.44×10^7 copy numbers/ μg mRNA, respectively). All other trace element-amended enrichments showed maximum *mcrA* expression at 7 days with the exception of Fe, which peaked at 14 days of incubation (2.23×10^5 copy numbers/ μg mRNA) (Figure 5.1). Methane production rates for All TE-amended enrichments correlate closely with *mcrA*

expression while there was less correlation seen between *mcrA* expression and enrichment cultures without TD (Figure 5.2).

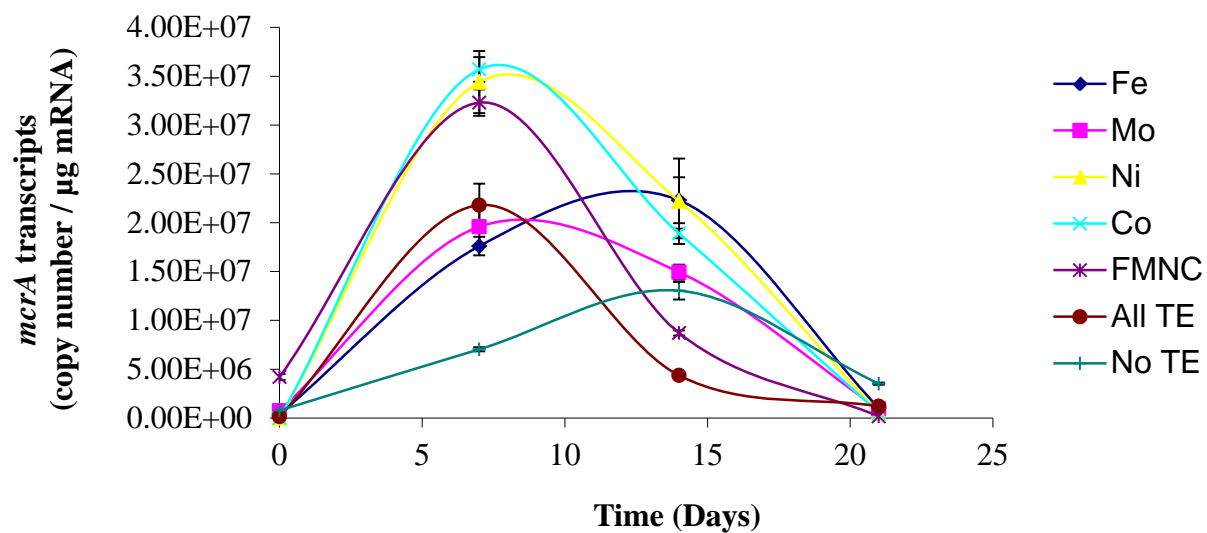


Figure 5.1. Expression of *mcrA* gene in enrichment cultures with coal bed production water ($m = -3.23x + 34.057$ and $r^2 = 0.992$).

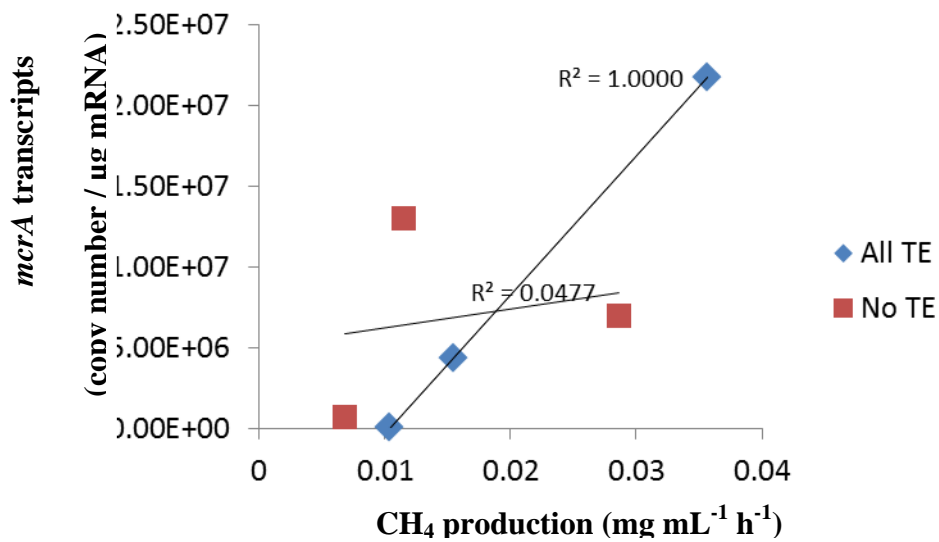


Figure 5.2 Methane production rate correlated with *mcrA* transcript level in All TE and No TE enrichment cultures. Adapted from Ünal *et al.* (2012).

In order to estimate overall microbial productivity in enrichment cultures, 16S rRNA was reverse transcribed and quantified by qPCR using *Eubacteria*- and *Archaea*-specific 16S rRNA primers (Table 2.1). Only All TE and No TE enrichment cultures were used for this analysis per the recommendations of a journal editor. Archaeal 16S rRNA copy number was highest at 21 days of incubation for both All TE and No TE enrichment cultures (3.16×10^{11} and 4.17×10^{11} copy numbers/ μg mRNA, respectively) indicating a slightly higher archaeal biomass in the No TE enrichment media. Eubacterial 16S rRNA copy numbers were also highest at 21 days for All TE enrichment cultures (6.84×10^9 copy numbers/ μg mRNA), while the highest overall copy number was seen in No TE enrichment cultures at 14 days (7.94×10^8 copy numbers/ μg mRNA). Results indicate that while *mcrA* expression was highest between 4 and 14 days, overall archaeal and eubacterial biomass was highest between 14 and 3 weeks for both All TE and No TE enrichments (Figure 5.3).

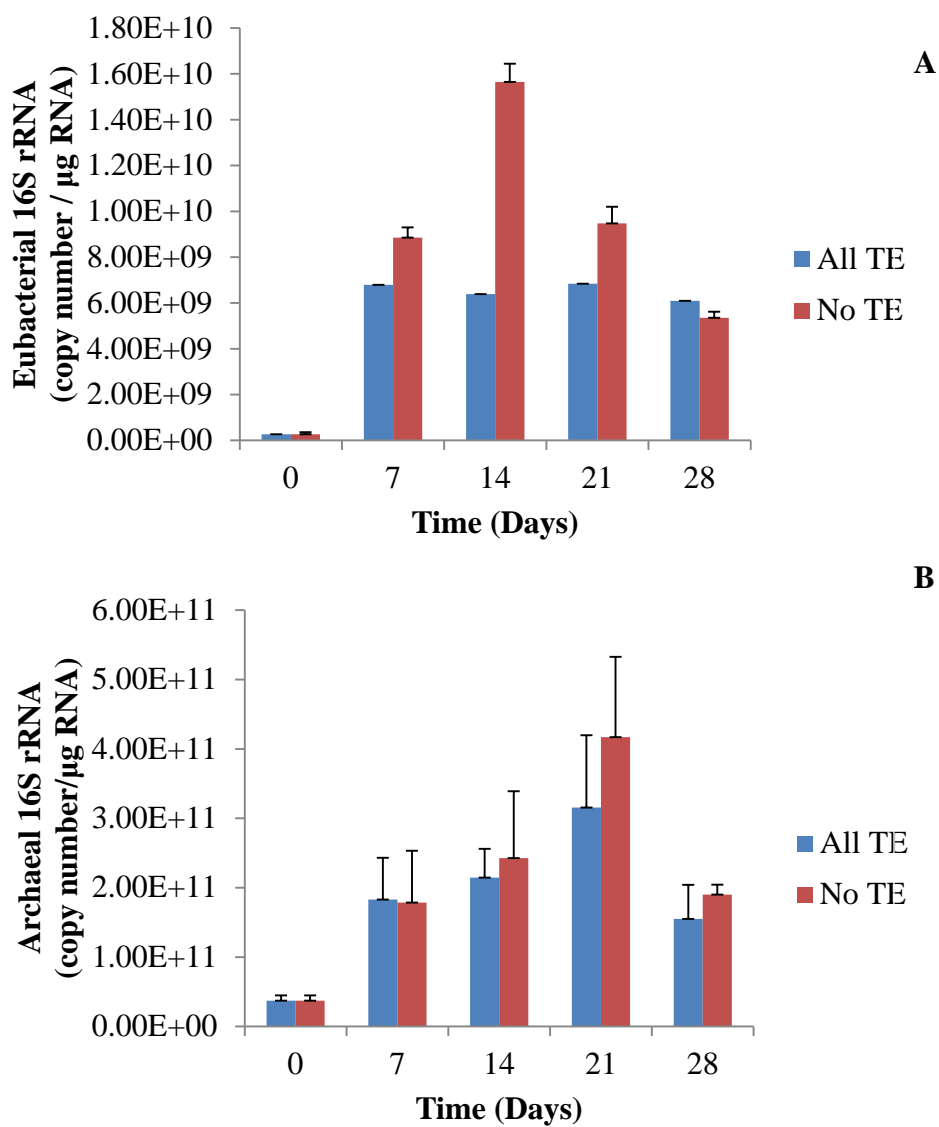


Figure 5.3. Expression levels of archaeal (A) and eubacterial (B) 16S rRNA in coal bed production water enrichment cultures with all trace element amendment (All TE) and without any amendment (No TE)

5.3 Discussion

Quantification of gene biomarker transcripts has been used as a means to determine metabolic activity of microbial communities. In this study, *mcrA* expression correlates with methane production rates in trace element amended enrichment cultures. This finding corroborates the findings made by previous studies that demonstrate that functional gene expression assessment accurately estimates activity rates (Chin *et al.*, 2004; Chin *et al.*, 2008; Akob *et al.*, 2012). While *mcrA* expression and methane production rates establish a trend in metabolic activity of the methanogenic enrichment cultures over time, overall biomass assessed by qPCR of 16S rRNA does not match with functional gene expression. While *mcrA* expression and methane production peak at 7 days, archaeal biomass continues to steadily increase in both All TE and No TE enrichments until 21 days of incubation. Eubacterial biomass peaked at 14 days in No TE enrichments while All TE enrichments remained stable after 7 days. Interestingly, overall eubacterial and archaeal biomass was higher in No TE than All TE enrichments. While 16S rRNA has traditionally been valued as the most direct means to evaluate abundance and activity of microbial communities, we clearly see here that functional gene expression more accurately correlates with biogeochemical process rates (Ünal *et al.*, 2012). In terms of the effects of trace elements on methanogenic activities of coal bed enrichments cultures, this study provides evidence that trace element availability may have a significant influence on methane production *in situ* and should be considered when exploring coal bed methane as a viable source of natural gas (Mesle *et al.*, 2013).

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 Significance and impact of anaerobic microbial activity study

Anaerobic microorganisms and the biogeochemical processes which they mediate are important for exploiting nature's bioremediation and sustainable energy production potentials. The dissimilatory sulfate- and iron-reducing, and methanogenic prokaryotes are known to couple anaerobic respiration to direct or indirect petroleum hydrocarbon degradation. Individual strains and, more accurately, the communities which they form are not only involved in the biodegradation of a significant portion of organic material on Earth, but they are exclusively responsible for bioremediation activities in the anaerobic environments which dominate the Earth's subsurface (Lovely and Coates, 2000; Coates and Achenbach, 2001; Jones *et al.*, 2008).

Studies aimed at monitoring the diversity and activities of anaerobic microbial communities in diverse ecosystems have been conducted with increased frequency in aquifers and marine sediments (Coates *et al.*, 1997; Chin *et al.*, 2008; Yagi *et al.*, 2010; Akob *et al.*, 2012). The *Deepwater Horizon* oil spill in 2010 provided an opportunity and an impetus for studying the bioremediation potential of anaerobic microbial communities in the heavily impacted salt marshes of southern Louisiana.

The role of high surface-charge, montmorillonite clay in acceleration of anaerobic crude oil biodegradation was previously investigated (Carmichael and Pfaender, 1997; Chaerun *et al.*,

2005; Warr *et al.*, 2009), but there have been no studies exploring *in situ* metabolic activity and diversity with application of clay mineral *in situ*. To the best of our knowledge, this is the first *in situ* study which employs key functional gene markers molecular proxies for metabolic activity and diversity assessments in anaerobic oil-contaminated salt marsh sediment and also elucidates clay-enhanced petroleum *in situ* bioremediation.

In hopes of increasing our reliance on sustainable energy alternatives, an interest in coal bed methane has been fueled by advances in methods for studying *in situ* biological methane production (Gale and Freund, 2001; Thielemann *et al.*, 2004). This study aimed to assist in efforts to better characterize methanogenic communities and assess their methane production in response to trace element amendment (Unal *et al.*, 2012).

6.2 Functional gene transcripts as molecular proxies for *in situ* microbial activity

In line with other studies of its nature, this study employed molecular methods relying on the isolation of high quality RNA and mRNA for phylogenetic and metabolic activity assessments of anaerobic communities of biogeochemical significance (Akob *et al.*, 2012; Chin *et al.*, 2004; Chin *et al.*, 2008). The advantage of this approach over studies using quantitative methods relying on environmental DNA is that literal activities are represented rather than just the genetic *potential* for activity that is elucidated from gene sequencing and quantification. Activity assessments based on mRNA quantification for several key functional genes of interest have provided tremendous insight into the respective activities of the dominant anaerobic respiratory guilds responsible for oxidation of petroleum hydrocarbon in *Deepwater*

Horizon crude oil-impacted salt marsh sediments. Previous findings on the dynamics between dissimilatory sulfate- and iron reducing and methanogenic prokaryotes were revalidated by molecular activity assays in this study, supporting running hypotheses on seasonal variations in sulfate- and iron-reducing activities in actively growing *Spartina* sp. marsh (Koretsky *et al.*, 2002; Kostka *et al.*, 2002; Koretsky *et al.*, 2003; Koretsky *et al.*, 2005). In fact it has been suggested that increased rates of *in situ* petroleum hydrocarbon degradation in vegetation regions of the marsh may be promoted by rhizospheric microbial communities living in association with the root systems of living *Spartina* sp. (Beazley *et al.*, 2012).

Special attention needs to be given to the possibility of accelerating *in situ* crude oil biodegradation rates by the application of montmorillonite clay. Molecular activity assessments *in situ* and in sediment microcosm incubations demonstrate that microbial respiratory and oxidative activities increase in oil impacted sediments in the presence of crude oil. These findings are supported by *in situ* measurements of crude oil hydrocarbons in control and clay-amended sites in Bay Jimmy by Ghelerter *et al.* (unpublished) which demonstrated that alkane degradation is significantly accelerated in clay-amended sites compared to control sites. Prior studies have suggested the usefulness of clay at adsorption of *in situ* organic contaminants (Sharmasarkar *et al.*, 2000; Witthuhn *et al.*, 2006), and the effects of clay addition on promoting microbial growth and petroleum hydrocarbon degrading-activities have recently gained attention (Carmichael and Pfaender, 1997; Chaerun *et al.*, 2005; Warr *et al.*, 2009). The results from the present study in conjunction with the findings of Ghelerter *et al.*, (unpublished), prove that clay addition in the presence of crude oil leads to accelerated biodegradation of particular classes of petroleum hydrocarbons as a results of direct

stimulation of microbial activities and also montmorillonite-enhanced bioremediation may be a viable method for accelerated petroleum hydrocarbon biodegradation in contaminated environments. A detailed investigation on the biophysicochemical interactions between microbe, clay and petroleum hydrocarbon are of great importance and necessary in order to extend our knowledge and further to design a long-term effective oil spill bioremediation strategy using clay minerals.

In a similar study, which was extended to biogas production, gene expression analysis on methane production in coal bed production water enrichment cultures also demonstrated that transcript levels of *mcrA*, a molecular proxy gene for methanogenesis, correlated with methane production rates (Unal *et al.*, 2012). Interesting microbial community dynamics were observed between eubacterial and archaeal communities as revealed by quantification of 16S rRNA RT-PCR products.

These findings confirm that significance of use of gene markers as molecular proxies for estimating *in situ* microbial activities for bioremediation of contaminated environments and sustainable bioenergy production in environment as well.

6.3 Estimate of Microbial diversity using sequencing technologies in environmental samples

Pyrosequencing presents a powerful method to rapidly obtain large volumes of community diversity and phylogenetic data from relatively simple nucleic acid and purification procedures coupled to next generation sequencing and analysis (Quail *et al.*, 2012). Ion Torrent™ sequencing using wide-coverage, degenerate 16S rRNA gene primers on environment DNA templates reveal a diverse and complex community in vegetated and non-vegetated, crude oil impacted salt marsh sediment as well as within oil-spiked and clay-amended microcosms. Results reveal that community diversity is relatively stable regardless of seasonal changes and clay amendment *in situ*. Similar findings were also indicative of stable community diversity in microcosm incubations. Rather, sample site location and, quite probably, vegetation dynamics, seem to have a greater influence on diversity and community structure whereas activity levels of resident communities are tied to season fluctuations in environmental conditions (Howes and Teal, 1995; Koretsky *et al.*, 2002, Koretsky *et al.*, 2004; Koretsky *et al.*, 2005). A more even distribution of OTU rankings were observed in the non-vegetated and more highly impacted BJ3 site, which was quite interesting considering the ecological disturbance arising from wrack accumulation. 454 sequencing of environmental RT-PCR 16S rRNA amplicons reveal trend in regards to diversity, showing that the dominant metabolically active OTUs are more or less represented by pyrosequencing of environmental DNA, though some reportedly minor phyla in terms of OTU abundance are better represented by RNA in regards to activity.

6.4 Challenges in molecular microbial ecology study

Microbial activity and phylogenetic analyses based on quantification of gene expression and next generation pyrosequencing are promising tools for determining structure and function of metabolically-active microbial communities. Nevertheless there are limitations to its application. The nature of qPCR requires a smaller size RT-PCR amplicon (100 – 200 bp) which limits the degree of phylogenetic coverage. Also, shorter transcripts are less suitable for assigning phylogenetic identity through bioinformatics databases such as BLAST. This is compensated for, in part, by the use of a larger base pair amplicon of the 16S rRNA gene used for pyrosequencing, which is standard for deep sequencing and community analysis and has been the default gene used for phylogenetic assessments in the field of microbial ecology (Caporaso *et al.*, 2012; Edwards *et al.*, 1989; Ludwig, 2007). This study is also limited by the current knowledge base of anaerobic microbial respiratory and oxidative pathways which provide potential biomarkers for activity assessment. As pathways and the genes that encode them are better understood, more biomarkers will become available for *in situ* studies leading to advances in engineered and intrinsic bioremediation of contaminated environments and bioenergy production (Lovley, 2003; Akob *et al.*, 2009; Andreoni and Gianfreda, 2007; Beller *et al.*, 2002; Carmona *et al.*, 2009; Chin *et al.*, 2008; DiDonato *et al.*, 2010; Selesi *et al.*, 2010; Unal *et al.*, 2012).

6.5 Future studies

Like other studies of this nature, the advantages of gene expression assays for the assessment of microbial activity rates promoting biogeochemical cycles are clearly defined and supported by this study's findings. More comprehensive insight into *in situ* biodegradation activities by diverse groups of anaerobic microorganisms will be possible as specific catabolic and respiratory pathways are characterized. For this reason, cultivation-based studies on pure culture isolates from petroleum hydrocarbon contaminated environments are crucial for the development of genetic systems integral to metabolic pathway elucidation. Anaerobic isolation and cultivation are wrought with challenges in their own right, so findings such as ours that highlight the implications of anaerobic microbiology on the broader fields of environmental remediation and bioenergy production lend support to the investment of time and resources in classical methodologies within the discipline. Enrichment culture development and strain isolation attempts are currently underway to enrich our culture collection of petroleum hydrocarbon degrading dissimilatory iron- and sulfate-reducing prokaryotes.

The relationship between rhizospheric communities, montmorillonite amendment, and microbial diversity and activity warrant further elaboration, particularly in regards to clay-hydrocarbon-microbe interactions. Gene expression activity assessments indicate a stabilization of microbial metabolism in the presence of montmorillonite and high amounts of crude oil. Specific mechanisms for this phenomenon are as yet undetermined and warrant further investigation. Close collaboration between geoscientists, mineralogists, and microbiologists are necessary for such interdisciplinary work which would contribute

tremendously to the fields of geomicrobiology and microbial biogeochemistry while having direct implications for environmental remediation

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