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MICROBIAL MERCURY METHYLATION AT CADDO LAKE: A MOLECULAR ECOLOGY APPROACH

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MICROBIAL MERCURY METHYLATION AT CADDO LAKE:

A MOLECULAR ECOLOGY APPROACH

by

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Dedication

I dedicate this Thesis to my children Taytem and Dakoda King—I do everything with you in my heart.

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Abstract

MICROBIAL MERCURY METHYLATION AT CADDO LAKE: A MOLECULAR ECOLOGY APPROACH

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Caddo Lake in northeastern Texas is a cypress-Spanish moss dominated lake ecosystem. Contamination of mercury (Hg), especially methylmercury (MeHg), was reported in this lake a decade ago. MeHg is a neurotoxicant accumulated in major fish species and reptiles. Due to the biomagnification feature of MeHg transfer, Hg contamination in the fishes of Caddo Lake has caused health concerns for the wildlife and local people. However, the source and synthesis of MeHg in this lake, primarily the microbial Hg methylation mechanisms, have not been investigated. We investigated the lake for the past three years (2016-2018), by taking sediment and plant samples in several locations of the lake wetland habitats which showed high MeHg levels in fish from previous studies. We employed a culture-independent molecular approach to identify the Hg-methylating microbial community present in sediment as well as the sporangia of the invasive species Giant salvania (*Salvania molesta*). Total organic carbon, total Hg, MeHg, sulfate, iron(III) and other biogeochemical factors were analyzed in the lake ecosystem. We extracted genomic DNA from all samples and detected functioning genes including the Hg methylation genes (*hgcAB*), methyl-coenzyme M reductase genes (*mcrA*) as well as

16S rRNA genes. The 16S rRNA genes were characterized by high throughput next generation sequencing on Illumina MiSeq. In lake sediment samples, a total of 6402 OTUs were discovered, dominated with *Crenarcheales* (9.7%), *Bacteroidales* (5.2%), *Syntrophobacterales* (3.1%). Our results indicated that the lake sediment samples contained diverse potential mercury methylators, including *Syntrophobacteraceae* (1.4%), *Geobacter* spp. (1.1%), SRB *Desulfovibrio-Desulfobulbus-Desulfobacter* (0.6%), and methanogenic archaea (0.6%). It seems that microbial MeHg production in this wetland habitat could be influenced by a complex syntropy among *Syntrophobacterales*, methanogens, and sulfate reducing bacteria. Results based on the geochemical data and *hgcA* gene detection and quantification suggest that, Johnsons Ranch and Judd Hole are likely the hot spots for MeHg production in this lake ecosystem.

Chapter 1

Introduction and Background Information

Caddo Lake is located in the northeastern part of Texas with the Texas-Louisiana border running through Caddo Lake. It is one of the largest flooded cypress forests in the United States with an area of 25,400 acres. Caddo Lake is a freshwater lake ecosystem which falls under a forested wetland. Caddo Lake has an interesting history: in 1811, the 161-km natural log jam on the Red River (called "The Great Log Jam") ruptured during a severe earthquake (8.9 on the Richter Scale) (Kley & Hine, 1998). This influx of water from the Red River formed Caddo Lake. The lake is dominated by cypress trees, along with several native and invasive species. The lake has a variety of vegetation which makes it naturally organic-rich for carbon and nitrogen. In October 1993, Caddo Lake became one of thirteen areas in the United States protected by the Ramsar Treaty, which protects certain endangered birds and habitats (Kley & Hine, 1998). The lake is fed by the Big Cypress Tributary and drains through an artificial dam into the Red River system. This lake ecosystem contains endangered species that inhabit the area. The recently invading species, Giant salvania (*Salvania molesta*), is an aquatic macrophyte fern that forms mats. These plants can restrict sunlight in the water column and create an anaerobic setting on the lake bottom (Thomas & Room, 1986).

Mercury transfer through trophic levels occurred widely in this aquatic ecosystem under the mechanism of bioaccumulation (Chumchal et al., 2011). Chumchal's team has investigated Hg contamination in various classes of organisms in the lake for years, showing that the apex predators in the lake contained the highest concentrations of MeHg. Contamination of MeHg as a neurotoxicant in the lake fish has caused an increased health concern on wildlife and local residents. The lake is currently under an advisory from the EPA for fish consumption due to Hg contamination in the lake. The potential contamination source for the lake is from the atmospherically deposited Hg emitted from several coal-burned electrical stations in East Texas.

Freshwater lake wetlands such as Caddo Lake act as major sites for receiving airborne Hg and producing MeHg into aquatic ecosystems. In the system with aquatic vegetation, litterfall in the sediment is primarily subjected to anaerobic degradation, dependent on biogeochemical factors, available electron acceptors such as nitrate, manganese (IV), iron (III), and sulfate, and which members of the microbial community are active as well. The MeHg production is primarily considered as a biological process (Compeau & Bartha, 1985). Under organic-rich and mineral-limited conditions such as those for Caddo Lake, syntrophy (or fermentation) and methanogenesis may play a dominant role in the end mineralization processes of carbon cycling. Generally, less toxic Hg (II) which originates from air deposition can readily be methylated into highly toxic MeHg (a neurotoxin) in aquatic environments, primarily by sulfate reducing bacteria (SRB), iron reducing bacteria (IRB), and methanogen (Compeau & Bartha, 1985; Fleming et al., 2006; Yu et al., 2013), through putative Hg methylation genes *hgcA* and *hgcB* via the acetyl CoA pathway (Parks et al., 2013). Caddo Lake is a cypress-Spanish moss

dominated lake ecosystem with the favorable biogeochemical conditions for methanogenesis and syntrophy. Previous investigations showed that the guilds of syntrophs (e.g., *Syntrophobacteriales* and SRB acting as fermentative partners) and methanogens are also crucial in MeHg synthesis (Bae et al., 2014; Yu et al., 2010; Yu et al., 2018). Compared with the SRB monocultures, previous studies revealed that, syntrophic association of SRB *Desulfovibrio desulfuricans* ND132 with a methanogen in a sulfate-deplete medium stimulated potential methylation rates 2-9 fold, while the association of *Syntrophobacter wolinii*, a newly identified Hg methylator, with the methanogen increased the rates two fold (Yu et al., 2018). Previous studies indicated that *Syntrophobacteriales* were likely the major Hg methylating microbes in the *Sphagnum* moss in Adirondacks lakes (Yu et al., 2010), and were the dominant taxonomic group containing the Hg methylation genes *hgcAB* in the Florida Everglades (Bae et al., 2014). However, direct evidence linking the role of syntrophy and methanogens with MeHg has been missing. The identification of Hg methylation genes (Parks et al., 2013) makes it possible to directly relate these functioning genes (Bae et al., 2014; Liu et al., 2014; Schaefer et al., 2014) with in-situ methylation activities (rates). The initial investigation during my undergraduate study also successfully detected both *hgcA* and *mcrA* genes in all sediments collected from the cypress-moss habitats of Caddo Lake (King et al., 2016) . However, the microbial groups involved in Hg methylation in the Caddo Lake environment are unknown, and the interplays of Hg methylation genes with the biogeochemical processes have been little studied.

We hypothesized that the mercury methylation activities in Caddo Lake are mainly dominated through a syntrophic relationship between SRB and MPA. Our goals were:

1. Biogeochemical assessment of sulfate and iron(III) and other parameters (?- Other elements or processes?).
2. Microbial community analysis using next generation sequencing of16S rRNA genes with Illumina MiSeq.
3. Quantification of the Hg methylation genes (*hgcA*) and methane (CH_4) production genes (methyl coenzyme M reductase) *mcrA* though gene abundance with qPCR, and exploration of the relationships between the function genes and the biogeochemical parameters in a cypress–moss-sediment lake ecosystem. Change formatting to 3.

Chapter 2

Literature Review

Mercury

Mercury (Hg) has several speciation forms found in nature and is a naturally occurring element that may originate from the crust or the earth, oceans, and atmosphere. When mercury is found in rocks and mineral deposits, it is a toxic mercury sulfide (HgS) mineral, called cinnabar (Smith et al., 2008). It is the most prominent ore of mercury and has a bright red color. Mercury has unique properties which make it useful in gold and silver mining in amalgamation processes. It has also been used in chlor-alkali manufacturing, as a reaction catalyst and in biocide treatments (Fitzgerald et al., 2005).

Mercury Speciation Forms

Mercury is a transitional metal and has four main chemical species, elemental (Hg^0), mercurous (Hg_2^{2+}), mercuric (Hg^{2+}), and organic mercury (MeHg). Elemental mercury is a silver liquid substance as the main depiction of mercury. This elemental mercury species is liquid at room temperature and can easily vaporize with little to no provocation from outside sources. Most of the Hg in the atmosphere is Hg^0 in a gas form rather than the liquid state with a low Henry's Law constant. Elemental Hg is slightly soluble in water and is normally unreactive. Mercurous Hg has an oxidation of 1 which is extremely rare and is found as mercurous chloride (Hg_2Cl_2) or in calomel, its mineral form. Mercuric Hg compromises most of the ionic forms and has an oxidation number of 2.

Organic mercury is mainly found as Methylmercury (MeHg or CH_3Hg) or Dimethylmercury (Me_2Hg) (Miller & Akagi, 1979) where MeHg is the predominant version found in nature. MeHg is the most toxic form of the metal and is readily accumulated in aquatic environments. First discovered to be an issue in Minamata, Japan (Harada, 1995), MeHg became an immediately noticed problem due to the neurodegenerative symptoms that the local villagers were experiencing. By consumption of shellfish and fish, villagers who were contaminated with MeHg experienced a unique set of symptoms. The disease was coined as Minamata's Disease.

Mercury Cycling

It has been estimated that 36% of all Hg in the environment is released through natural approaches: volcanic eruptions, deep sea vents, hot springs, and evaporation from ocean basins and soils. The other 64% is a result of anthropogenic release of mercury into the environment from other sources (Mason & Sheu, 2002). One of the main anthropogenic inputs is through the stationary fuel combustion during electricity generation, and it accounts for 65% of the atmospheric mercury contamination (Pacyna et al., 2006). Texas contains several big coal-burnt electric power stations with the highest emission output measured at 5,317 kg in 2008 (*Figure 2.1*). The Hg input in the air can travel long distances as Hg^0, and then can go through the mechanism of atmospherics deposition where it can enter the aquatic systems (Wilson et al., 2006). It has been shown that ozone, bromine, and UV can oxidize elemental Hg, where bromine species are the primary oxidants transforming this atmospheric Hg into Hg(II). Once elemental Hg is oxidized and then absorbed by rain or snow particles, the inorganic Hg can contaminate remote and

ubiquitous ecosystems. Ionic Hg is mainly found in the sediments where it is partitioned. Hg (II) is highly reactive and bioavailable, allowing microbes to transform it into MeHg through a metlhylcobalamin cofactor and an acetyl coenzyme pathway (*Figure 2.2*). The factors controlling MeHg production, cycling, and eventually remediation have been sought on the purposes of protection for the environmental health and safety from the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) (Greenstone, 2002). MeHg is a lipophilic form of mercury that is highly toxic, and bioaccumulative. The organic mercury acting as a neurotoxicant can form a complex with amino acid cystine and methionine, which allows for easy access into endothelial cells of the blood-brain barrier (Clarkson & Magos, 2006). The neurotoxic effect of MeHg in the environment is not restricted to humans, therefore making this contaminant a multi-level ecological problem affecting all higher order of organisms (Zillioux et al., 1993). Several environmental drivers have been discovered to affect this global issue. The intertwining of the Hg cycle with other geochemical cycles complicates the study and the understanding of how Hg affects the ecosystem.

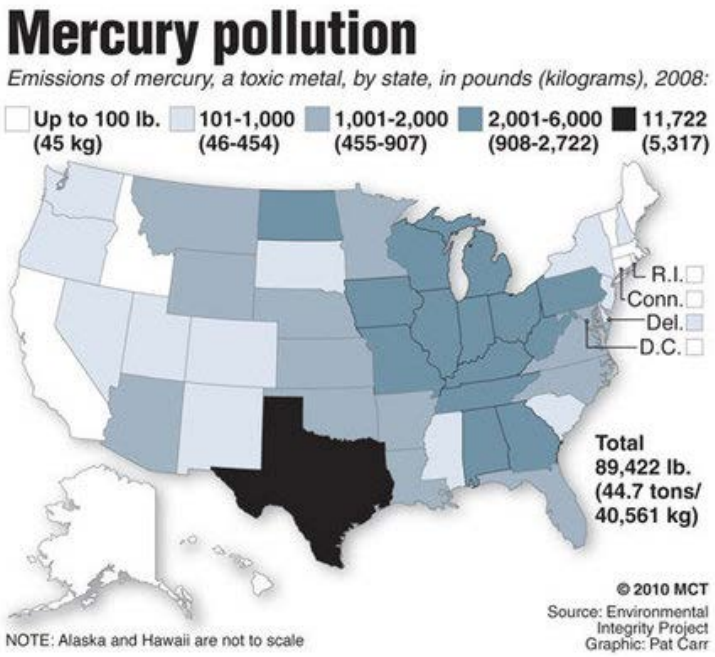

Figure 2. 1 Hg emission in the United States in 2008.

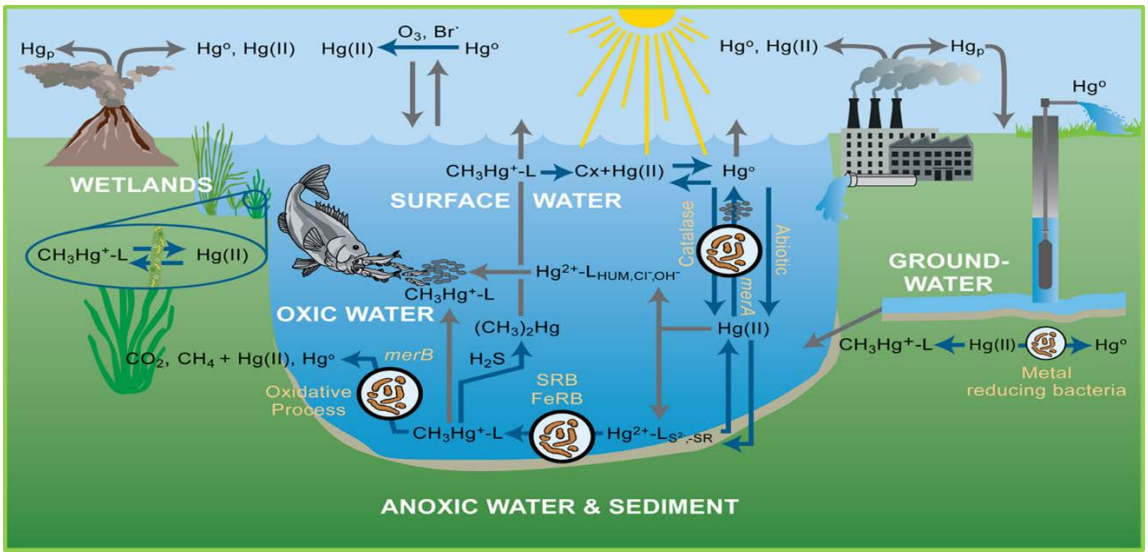

Figure 2. 2 A depiction of the Hg cycle. (Lin et al. 2014).

Mercury Methylation

The production of MeHg is confined to various anaerobic bacteria and archaea. The discovery of the *hgcA* gene coding for a putative corrinoid protein allows for broader investigation approaches, since this is considered as an essential gene to produce MeHg (*Figure 2.3 & 2.4*) (Parks et al., 2013). A previous study on rice paddies near a mercury mine showed that there was a positive correlation of *hgcA* gene abundance with MeHg concentration (Liu et al., 2014). This leads to the idea of using the abundance of the gene to explore the potential hotspots for Hg methylation activities in natural environments. The *hgcA* gene has been located and described across several clades of microbes with different ecological niches. The evolutional change of this gene is probably due to horizontal gene transfer since the clades are so diverse. This phenomenon leads us to determine the dominant potential methylators by investigating electron acceptor metabolism pathways such as sulfate, iron reduction or methanogenesis. Linking these environmental variables is daunting and the interactions can vary from site to site. The different clades consist of sulfur-reducing bacteria (SRB), iron reducing bacteria (IRB) and methanogenic archaea (MPA), representing the majority of the well-focused producers of MeHg (Gilmour et al., 2013; Yu et al., 2013). Few species were found from *Firmicutes* and *Chloroflexi* that contain orthologs of the *hgcA* genes. It has been long believed that the methylation of mercury can only happen under anoxic conditions. However, a recent study has shown that this process may be able to branch into more aerobic conditions as well. Such methylation was found in periphyton, a complex biofilm consisting of algae, bacteria, archaea and fungi (Cleckner et al., 1999). This typical habitat may appear around the roots of aquatic plants. This periphyton methylation seems to be dominated by MPA and SRB

(Correia et al., 2012) assisted by the dynamic creation of an oxygen gradient, allowing for the survivability of a consortium of microbial species with a varying oxygen interaction capacity.

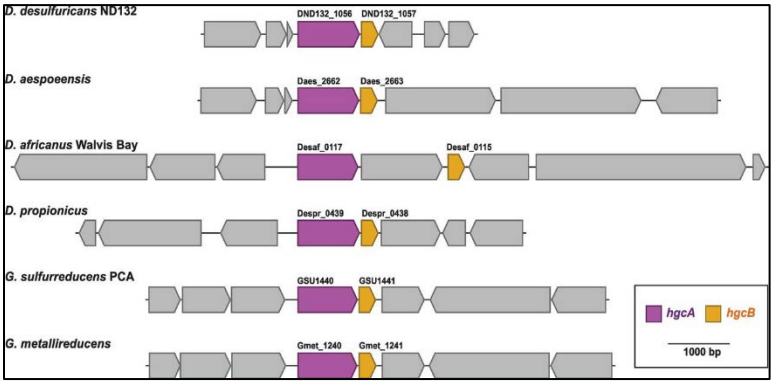

Figure 2. 3 The *hgcAB* genes from several *Deltaproteobacteria* (Parks et al., 2013).

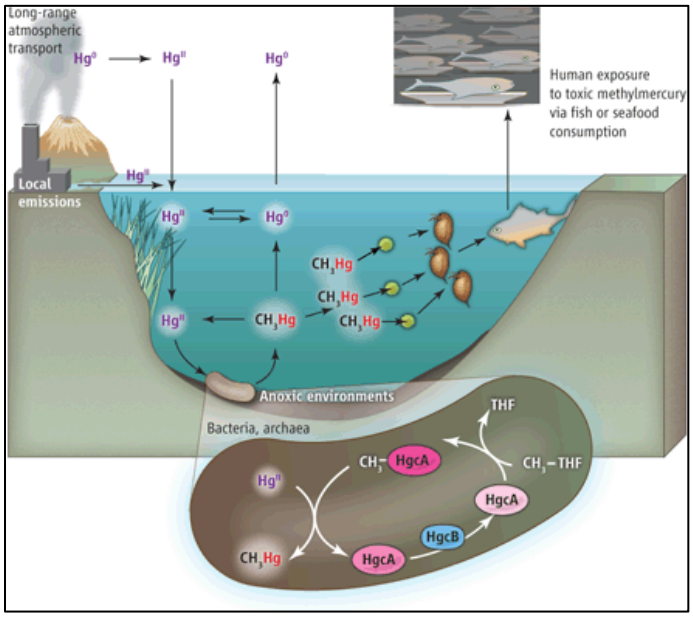

Figure 2. 4 A diagram of the Hg Methylating gene *hgcAB* and its function in Hg methylation.

Methylmercury Demethylation

MeHg is found in many environments even though total Hg concentration is low. Methylmercury in its cationic form (CH_3-Hg^+) is energetically stable in water and is associated with anions such as chlorine and sulfate (Morel et al., 1998). The demethylation aspect is termed as microbial degradation of MeHg, which plays a very important role in mercury cycling. Natural demethylation can occur through microbial demethylation by SRB and methanogens or light photoreduction. There are two types of microbial demethylation reactions through either reductive or oxidative pathways. Reductive demethylation is achieved by the activation of mercury resistance (*mer*) operon which converts CH_3Hg (I) to Hg (0), and this process takes place in more aerobic settings with high Hg level contrary to methylation activities (Schaefer et al., 2004). Oxidative demethylation usually occurs in anaerobic habitats by methanogens and SRB which could convert CH_3Hg (I) into Hg (II) (Barkay & Döbler, 2005). The overall biotransformation processes play the major role in mercury cycling and detoxification.

Methanogens

Methanogens are a class of archaea that are characterized by their ability to produce methane as a metabolic byproduct. Methane Producing Archaea (MPA) all contain the enzyme *methyl coenzyme M reductase*. This enzyme is encoded by a gene known as *mcrA*, which is specific for methanogens (Aschenbach et al., 2013). Methanogens are anaerobic in nature and difficult to culture and identify (Schink & Stams, 2013). Woese and Fox (1977) discovered the entire phylum of archaea using methanogens as a model for their work, which were, at the time, not classified and were not studied intensively, due to their

anaerobic nature. This discovery led to a renditioning of the tree of life and gave way to a three domain classification system (Woese & Fox, 1977).

Archaea are frequently grouped as chemotroph and were considered to be majorly extremophiles. They share many characteristics with *Eubacteria*, containing circular chromosomes, lacking membrane-bound organelles and the ability to reproduce asexually or through conjugation (Schink & Stams, 2013). The cell membrane is made of a pseudopeptidoglycan which is also a similar characteristic with bacteria (Madigan & Martinko, 2006; Schink & Stams, 2013).

Unlike *Eubacteria*, some Archaea utilize a specific mechanistic metabolic pathway known as methanogenesis. Methanogenesis is the process by which methanogens produce methane gas as a byproduct through the consumption of substrates and electron acceptors for energy. This reaction follows a series of steps mediated by different enzymes, and requires an electron acceptor (CO_2), an electron donor, such as H_2 or formate or other organic compounds (Buescher et al., 2015). There are three main classes of methanogens that utilize different substrates including methylotrophic, CO_2 -type, and acetotrophic matters. To date there are eleven known substrates for methanogens (Schink & Stams, 2013). The study of methanogens has great potential for assisting in the production of economically friendly natural gas solution (Strong et al., 2015).

Methanogens are capable of living in a wide range of anaerobic environments including, the digestive tract of ruminant animals, the cecum of cecal animals, monogastric animals, sediments of marshland, rice paddies, swamps, landfills and water treatment plants (Madigan & Martinko, 2006; Schink & Stams, 2013). In order to function properly, methanogens require a source of organic carbon and an absence of oxygen, thus

methanogens are thought to be typically most prolific in wetland sediment (Demirel & Scherer, 2008; Madigan et al., 2010; St-Pierre et al., 2015). We hypothesized that methanogens and their associated partner syntrophs are the main potential groups for microbial Hg methylation in Caddo Lake sediments.

Chapter 3

Materials and Methods

Study Sites, Geochemical Characteristics, and Sampling Methods

Several sites of Caddo Lake served as the experimental points which have had historically high MeHg concentrations in aquatic organisms (Chumchal et al., 2011). Selection of these sampling locations was also based on the amount of human interactions with the invasive species inhabiting the lake. Samples of sediment and several invasive and native aquatic plants in the lake were taken from March 2018 to December 2018. Invasive vegetation and cypress trees covered a majority of the lake surface. The invasive species consisted of a mix of *Salvinia molesta*, *Eichhornia crassipes,* and *Hydrilla verticillate*. Global positioning system (GPS) coordinates (*Figure 3.1; Table 1*) were followed throughout the whole sampling scheme to ensure that the same locations were sampled from. Based on the initial study from 2015 to 2017, the lake was sampled quarterly starting in March 2018. Along with the 2018 collected data, previous sampling data from 2015-2017 were included for multiple analysis. The sampling sites in the southern part of the lake within the Texas border were Johnsons Ranch, Ames Spring Basin, State Park, and Crips Camp. The northern sites included Cross Bayou, Judd Hole and Kane Hole (*see Figure 3.1*). These northern sample sites were likely subjected to a limited management, since it was more secluded to local communities. The northern sampling locations are closer to the main water inlet for the lake, Big Cypress Bayou.

Table 1GPS coordinates for Caddo Lake sampling.

Sampling Location	Longitude	Latitude
State Park	W94 10.544	N32 41.650
Crips Camp	W94 07.353	N32 42.136
Johnsons Ranch	W94 07.096	N32 42.447
Ames Spring Basin	W94 05.719	N32 42.813
Cross Bayou	W94 05.559	N32 44.073
Kane Hole	W94 05.411	N32 44.653
Judd Hole	W94 06.195	N32 44.572

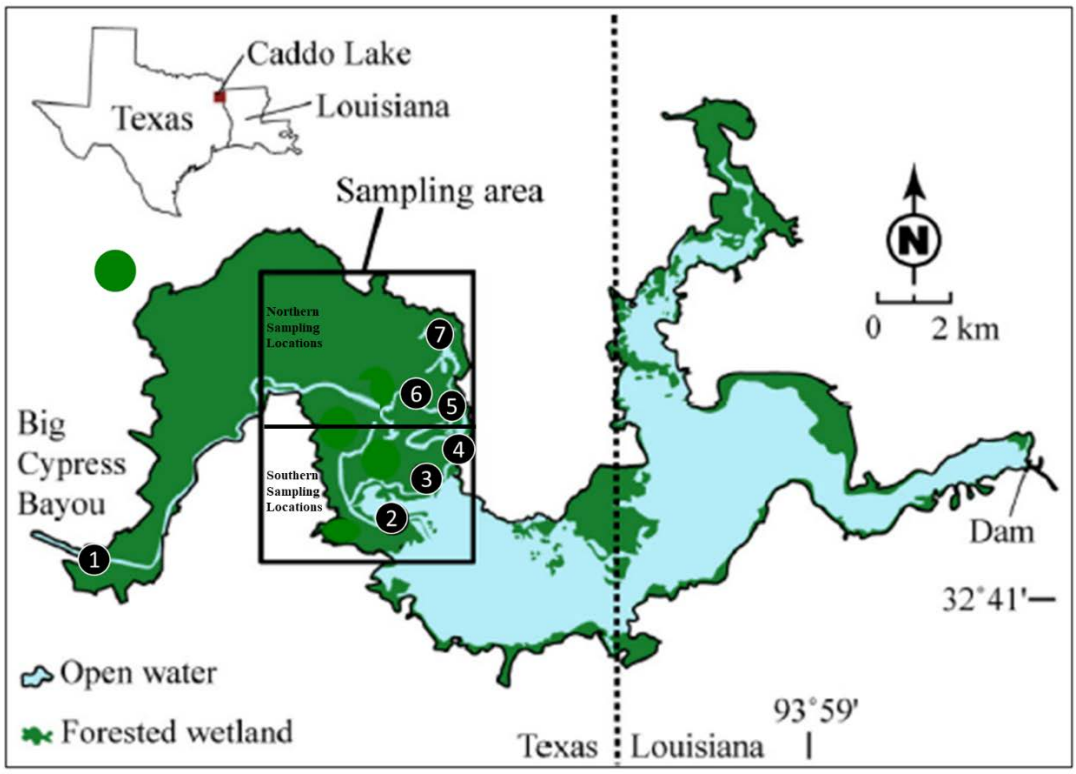

Figure 3. 1 Sample locations at Caddo Lake. The study area was divided into northern and southern regions. Sample location names: 1. State Park; 2. Crips Camp; 3. Johnsons Ranch, 4. Ames Spring Basin; 5. Cross Bayou; 6. Judd Hole; 7. Kane Hole.

Sampling Methods

Sediment samples were taken using an Ekkman grab. In order to obtain an adequate amount of sediment, three or more grabs were taken and the samples were then homogenized on site. Enough sediment was collected to fill two 50 ml Falcon tubes: 50mL for MeHg methylation analysis and another 50mL for DNA extraction. The remaining sediment was stored in double clean plastic bags, transported on ice, and stored at -80°C for further geochemical analysis. Invasive aquatic plant specimens such as: Giant salvaina (*Salvinia molesta*), water hyacinth (*Eichhornia crassipes*) and hydrilla (*Hydrilla verticillate*) were collected in the same manner and separated for later processing. Native Spanish moss (*Tillandsia usneoides*) is an epiphytic flowering plant that grows upon the cypress trees.

Geochemical Analysis of Sediment Samples

A partial sediment sample from the geochemical sampling bag was centrifuged at 4500 g and pore water was extracted and stored frozen at -20 °C prior to analysis. After pore water extraction, dissolved organic carbon and dissolved organic nitrogen content of the samples were analyzed using a Total Organic Carbon Analyzer (Shimadzu TOC-VCSH) with a TNM-1 Total Nitrogen Measuring Unit. Pore water samples were further filtered using a 0.45μm filter syringe, and anion and cation levels in the filtered samples were then analyzed by a Dionex ICS 5000+ ion chromatography (Thermo Scientific) with lab standards as references. A Water Quality Meter (YSI with Multi-Parameter probes) was used *in situ* to measure pH, dissolved oxygen (DO), electrical conductivity (EC), and oxidation reduction potential (ORP). Total nitrogen and total organic carbon of selected

sediment samples were quantified using an Elementar Vario Macrototal Combustion Analyzer on approximately 0.5 g of samples. Approximate 1-2 g of sediment samples were weighed and heated for 24-48 h at 105 °C in aluminum weigh boats. Dry weight was then measured after heating and roughly 5 minutes of cooling within a desiccation chamber. Fe(II) and microbially reducible Fe(III) in the whole sediment and porewater were measured by the previous methods (Lovley & Phillips, 1987; Yu et al., 2012).

Total Hg (THg) concentrations in sediment samples were analyzed by cold-vapor atomic fluorescence spectroscopy (CVAFS) detection following extraction, oxidation and volatilization (Bloom et al., 1988; Flanders et al., 2010). CH_3Hg in wet sediment was separated by a solvent (methylene chloride) extraction procedure, and measured following aqueous ethylation with sodium tetraethylborate, purging and trapping, adsorption and desorption, separation by gas chromatography at 100 °C, reduction by a pyrolytic column, and detection by CVAFS (Bloom et al., 1997).

Culturing of Positive Controls

In order to obtain genomic DNA of positive controls for detecting the gene targets *mcrA* and *hgcA*, *Methanosprillum hungatie* was grown in DSMZ medium 119 and *Desulfovibrio desulfcicans* was grown in DSMZ medium 63. Both of these microbes were cultured under anaerobic conditions for 1-2 weeks. The cultures were centrifuged at 5000 g for 20 min, and the cell pellets were used for DNA extraction.

DNA Extraction, and qPCR Analysis

DNA samples from all sediments were extracted using PowerLyser PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The protocol was modified by starting with an initial sediment aliquant of approximate 0.5 g and by use of a beadbeater for cell lysis. Quality and concentration of extracted DNA were determined spectrophotometrically using a ND-1000 nanodrop (Thermo Scientific) at 260 and 280 nm.

The qPCR analysis was performed using a Corbett Rotor-Gene (model RG-6000) and Rotor-Gene 6000 Series Software 1.7.75. Amplification of bacterial *16S* genes was prepared according to Harter et al. 2014 with minor modifications (Table 2). The following primers were used to target genes including Bacterial *16S*, Archaeal *16S*, *mcrA*, and the methylation gene *hgcA* (Table 2). Several clade-specific primers for *hgcA* gene amplification were used to target Hg methylators, including *Deltaproteobacteria*, methanogenic archaea and *Firmicutes*. Loading of DNA samples for qPCR analyses was performed using a Corbett CAS1200 robot. Based on the full genes found in the genomes of the representative species, the positive controls of *hgcA* genes from *Deltaproteobacteria*, methanogens, and *Firmicutes* were synthesized as gBlocks Gene Fragments of DNA sequence manufactured by ITD DNA Technologies, Inc. (Coralville, Iowa). Standards of the *hgcA* gene controls were prepared via serial dilution. Spikes were composed of equal parts of sample and standard DNA. All standards and no template control (NTC) were run in triplicate while the samples were run as duplicate. Reactions targeting Bacterial *16S* genes were diluted by 10× fold for all samples. All samples were prepared according to the protocol referenced therein.

Table 2 Target genes and primers used in qPCR.

Gene	*Primer Sequences*	*Source*	*Product Size (bp)*
*mcr*A	mcrAlas 5'-GGTGGTGTMGGTTCACMCARTA-3' mcrArev 5'-CGTTCATGGGACTTCTGG-3'	(Steinberg & Regan, 2009)	300-400
Deltaproteobacteria *hgcA*	ORNL-Delta-HgcA -F 5' GCCAACTACAAGMTGASCTWC-3' ORNL-Delta-HgcA -R 5' CCSGCNGCRCACCAGACRTT-3'	(Christensen et al., 2016)	100-200
Archaeal *hgcA*	ORNL-Archaea-HgcA-F 5' AAYTAYWCNCTSAGYTTYGAYGC-3' **ORNL-Archaea-HgcA-R** 5' TCDGTCCCRAABGTSCCYTT-3'	(Christensen et al., 2016)	100-200
Archaeal 16S	Arch 967F 5'-AATTGGCGGGGGAGCAC-3' Arch-1060R 5'-GGCCATGCACCWCCTCTC-3'	(Bengtson, Sterngren, & Rousk, 2012; Bräuer, Cadillo-Quiroz, Yashiro, Yavitt, & Zinder, 2006)	100
Bacterial 16S	Bac16S, F 5'-TGGAGCATGTGGTTTAATTCGA-3' Bac16S, R 5'-TGCGGGACTTAACCCAACA-3'	(Bengtson et al., 2012; Steinberg & Regan, 2008)	160

Table 3 qPCR amplification protocols.

Reaction Protocols		*Archeal 16S*		*Bacterial 16S*		*Deltaprotobacterial hgcA*		*Archeal hgcA*		*mcrA*	
Step	Phase	Temp (ºC)	Time	Temp (ºC)	Time	Temp (ºC)	Time	Temp (ºC)	Time	Temp (ºC)	Time
Step 1	Initiate	94	8 min	98	3 min	95	3 min	95	3 min	98	5 min
Step 2	Denature	98	30 sec	98	30 sec	95	15 sec	95	30 sec	98	30 sec
	Annealing	61	30 sec	61.5	30 sec	65	20 sec	50	10 sec	55	10 sec
40X	Extension	72	30 sec	72	30 sec	65	21 sec	60	60 sec	72	60 sec
Step 3	Melt Curve	55-95	1.0ºC /5 sec	50-99	1.0ºC /5 sec	65-95	0.5ºC /5 sec	55-95	0.5ºC /5 sec	55-98	1.0ºC /5 sec

Next Generation Sequencing

The samples that were sequenced included three replicates from the State Park, Crips Camp, and Johnsons Ranch. Other sample locations that had only one sample for sequencing included Cross Bayou and Kane Hole. Prokaryotic amplicons were generated using primers 519F (5'-CAGCMGCCGCGGTAA-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') that amplify the V4-V5 region of the 16S gene locus (Klindworth et al., 2013; Wang & Qian, 2009). Paired-end sequence data were generated on an Illumina MiSeq instrument using v3 600 cycle kits (Illumina, San Diego, CA) as described in the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2013), except that dual 6 bp instead of 8 bp index sequences were attached to each amplicon during indexing PCR.

The raw sequencing reads were processed with a combination of QIIME (Caporaso et al., 2010) and USEARCH (Edgar, 2010) software packages, as well as custom python scripts. 16S sequences were compared to the Greengenes 13.8 reference database (DeSantis et al., 2006) and AMF sequences were compared to the Silva 128 database (Gurevich et

al., 2013) using UCLUST (Edgar, 2010) in order to pick referenced-based Operational Taxonomic Units (OTUs) at 97% similarity, and to provide taxonomic assignments for each sequence read. The sequencing datasets were normalized with cumulative sum scaling, to an equal sequence count for each sample by randomly subsampling sequences without replacement to provide even measures of microbial alpha- and beta-diversity and to have equal sequencing depth to produce all figures, tables, and statistical analyses. These data outputs were processed through open source console R (RC Team, 2013) using a package called phyloseq (McMurdie & Holmes, 2013) to generate the species diversity, correlation analyses, the abundance heatmap figure (Caporaso et al., 2010). Using the R console, potential methylators were retrieved from the dataset using filter commands to sort the known methylating families based on relative abundance in the sequencing sets.

Statistical Analysis

The geochemical results and qPCR data were analyzed for seasonal and sampling location variations. The analyses of the dataset were conducted by using the statistical software SAS (SAS Institute, 1985). All of the parameters from the experiments were analyzed by Canonical Correspondence Analysis (CCA) using the statistical software PAST (Hammer et al., 2001). These are the list of software. What type of statistical analysis did you use for different sample groups.

Chapter 4

Results

Biogeochemical features of habitats

The basic biogeochemical features of the sampling sites in Caddo Lake are listed in Table 4. Lake sediment from different sites were most weakly acidic to neutral, with pH values ranging from 6.1 to 6.9. With the frequent input of organic litter, most sediment sample sites contained high total organic carbon, especially for the sites covered with cypress-moss including Kane Hole and Judd Hole. However, the highest concentration of water extractable of organic carbon appeared in the State Park, a site which was also fully occupied by the aquatic vegetation. The three highest levels of porewater sulfate appeared in State Park, Crips Camp, and Johnsons Ranch, the three sites where the sediment Fe (III) levels were also higher compared with other locations. The highest concentration of MeHg was found in Johnsons Ranch, followed by Kane Hole and Judd Hole. However, the sites with highest THg were not as same as those of MeHg in the locations. Sediment from Crips Camp showed the highest THg level, following by Judd Hole and State Park (Table 4).

The major biogeochemical parameters including Total Organic Carbon (TOC), sulfate and iron (III) in lake sediment showed seasonal changes (Figure 6.1). TOC measurements were the highest throughout the sampling sites in the summer ($p = 0.0037$, Figure 6.1). There had a significant p-value associated with the Analysis of Variance (ANOVA) when seasonality was considered as the factor ($p = 0.0037$). There was also an obvious spatial distribution trend of TOC among the sample sites for spatial directionality.

Sites that were farther north were higher in TOC than sites toward the south of the lake**.** With increasing TOC from the south to north, the sediment Total Organic Nitrogen (TON) levels were also increased for these sample sites. The differences were also seen in TOC according to seasonality: summer and winter showed increased levels of TOC while spring and autumn showed decreased TOC levels. There were no discernable trends in Water Extractable Organic Carbon (WEOC) or the Total Dissolved Nitrogen (TDN) determined from the pore water analysis (data not shown).

Sulfate concentrations throughout the lake showed no significant trend based on ANOVA, and the seasonal changes were site-specific (Figure 6.1). Sulfate levels in State Park were highest in spring, while Crips Camp sediment in Fall contained the peak levels of sulfate. For spatial distribution, however, it seems that the northern regions of the lake were lower in sulfate concentrations throughout the seasons whereas the southern regions of the lake had a higher overall sulfate concentration. While the ANOVA showed no significant trend, with the p-value close to a slightly higher than the α level of 0.05 ($p = 0.0637$). For Iron (III), no discernable overall trend changes were observed across the sites of Caddo Lake. The large triplicate variations of measurements occurred in the figure were likely due to the elusive oxidative nature of Fe(II) in sediment during sample handling and transport. The two high Fe (II) levels were found in Crips Camp and Johnsons Ranch in the Fall (Figure 6.1).

Microbial communities in lake sediments characterized by high throughput sequencing

The microbial 16S rRNA gene analysis by Illumina MiSeq sequencing in lake sediment samples yielded a total of 6402 OTUs. According to these OTU data, the

dominant clades in the lake sediment were the orders *Cenarchaeales* (9.7%), *Bacteroidales* (5.2%), and *Syntrophobacterales* (3.1%) (Figure 6.2). The alpha diversity Chao1 index is an estimate of diversity using the analyses of abundance and species richness. All the sample sites had a high Chao1 index, with Johnson's Ranch being the highest (Figure 6.3). The alpha diversity index for the Shannon's is interpreted as abundance and species evenness (Figure 6.3). Shannon's diversity index represents the similar ecological significance as the Chao1 index (Figure 6.3).

A maximum-likelihood (ML) phylogenetic tree showed the overall community genetic diversity, phylogenetic relationships, and abundance of microbes in different sampling sites throughout the lake (Figure 6.4). Paired with the abundance table, the phylogenetic tree showed that the dominating microbial phyla across the sites in Caddo Lake were *Proteobacteria* and *Crenarcheaota* (Figure 6.2 and 6.4). The lake sediment samples also contained potential mercury methylators such as *Syntrophobacteraceae* (1.4%), *Geobacter* spp. (1.1%), SRB *Desulfovibrio-Desulfobulbus-Desulfobacter* (0.6%), and methanogenic archaea (0.6%) (Figure 6.5). Comparison analyses of families with methylation potential demonstrated that the dominated families were related to syntrophic bacteria. The genetic diversity and evolution of the potential Hg methylators are shown through a phylogenetic tree (Figure 6.6). A heatmap was created to look at the most prevalent families throughout the sediment samples. The heatmap indicates that several methylating families were in high prevalence in the samples, including *Syntrophaceae*, *Geobacteraceae*, and *Syntrophobacteraceae* (Figure 6.7)**.**

Gene abundance in lake sediment

Bacterial *16S* gene abundance in sediment quantified by qPCR Qualitative Analysis was generally higher than that of Archaeal *16S* (Figure 6.8). The gene abundance reached well over 1,000,000 copies on the dry weight basis. No significant variations in seasonal changes or spatial distribution were observed (Figure 6.8). The seasonal changes of bacterial *16S* genes in lake sediment samples were highly site-specific.

Amount of Hg methylation gene *hgcA* in sediment represents the potential of microbial synthesis of MeHg or microbial biotransformation of Hg (II) into MeHg. The *hgcA* genes in Caddo Lake were assessed for two of the three methylating clades: *Deltaproteobacteria*, and methane producing archaea (methanogens). *Deltaproteobacterial hgcA* represents the methylating deltaproteobacteria which include both SRB and IRB in the lake ecosystem. The abundance of the functioning genes ranged from 10,000 to 450,000 gene copies per gram of sediment among the sample sites, with the high peak appeared in Judd Hole in the spring (Figure 6.9). Archaeal *hgcA* genes were in high abundance throughout the lake for all seasons (Figure 6.9). The northern part of the lake had a higher gene abundance than the southern locations. The gene abundance reached over 500,000 copies per gram of sediment (Figure 6.9). Archaeal *hgcA* genes represent the methylating methanogenic archaea (Figure 6.9). The gene abundance ranged from 5,000 to 35,000 copies per gram of soil (Figure 6.9). Seasonal changes of Archaeal *hgcA* genes were observed in the lake ($p = 0.0059$). The gene abundance was quite high in all the sample locations (Figure 6.9). Spatial distribution of the genes among the sample sites was significantly variated ($p = 0.0356$) (Figure 6.9). The southern part of the lake seemed to have higher overall number of methanogens in terms of *mcrA* gene abundance (Figure 6.9).

The location of a potential hotspot for Hg methylation from this analysis would be Johnsons Ranch which had the highest average abundance of *hgcA* genes for all the seasons from *Deltaproteobacteria* (Figure 6.9). The highest seasonal average occurred in spring, which seemed to be the time where methylating *Deltaproteobacteria* were found in the highest abundance.

Correlation of Hg methylation genes with biogeochemical changes

The potential relationships between *hgcA* genes of bacteria and archaea and sediment electron acceptors including sulfate and Fe(III) were analyzed (Figure 6.10). The correlation analyses showed that no significant relationships were found between *hgcA* abundance and concentrations of sulfate or Fe(III), either for bacteria or archaea. The canonical correspondence analysis (CCA) between the geochemical parameters and the functional gene *hgcA* revealed that, the Hg methylation gene *hgcA* from *Deltaproteobacteria* in the hotspot of Johnsons Ranch had a moderate cluster relationship with MeHg and sulfate, while the genes in the other sites had weak relationships with biogeochemical factors (Figure 6.11)

Chapter 5

Discussion

Biogeochemical cycling of carbon, sulfate and iron

Total organic carbon (TOC) levels are related to the carbon flow throughout Caddo Lake. Some of the key factors of carbon flow include carbon input through vegetation and turnover of sediment organic matter based on water current (Cui et al., 2005). The TOC trends observed in Caddo Lake were supported by the spatial distribution pattern of vegetation growing in the lake sample sites. The aquatic plant beds tend to form a cypress-Spanish moss vegetation with floating invasive plants on lake water surface in the northern-most sites of the lake. Aquatic plant beds directly affected sediment organic carbon content through litterfall during the fall and winter months. Previous literature has shown that the relationship between nitrogen and carbon are directly correlated (Sambrotto et al., 1993). At sites with increased TOC levels, TON levels were also increased; this trend was consistent in terms of site identity and seasonality.

Sulfate and iron(III) concentrations in the lake sediment can be dependent upon the process of carbon metabolism. In unvegetated areas that were iron-rich, carbon oxidation was dominated by iron-reducing bacterium while the methanogenesis was suppressed (Rejmankova & Post, 1996). Vegetated areas that were sulfate- and iron-limited were dominated by methanogenic activity (Rejmankova & Post, 1996). Caddo Lake is comprised of both sulfate- and iron-limited areas. These biogeochemical characteristics

structure the site-dependent community assemblages in the lake ecosystem. The sulfate levels in Caddo Lake varied seasonally, leading to a seasonal shift of the dominating communities that depended on electron acceptors and oxidation-reducing conditions across the lake. Generally, sulfate levels ranged from higher concentrations in more southern regions to lower concentrations in more northern regions.

Potential Hg methylators in benthic microbial communities

The functional roles of the microbial communities identified in Caddo Lake likely represent or stem from the ecological adaptation to the geochemical nutrient cycling or metabolisms of carbon and nitrogen. Caddo Lake is rich in organic matter and there are diverse and abundant microbiota living in the sediment. The microbial members in the lake can actively transform or metabolize litterfall from the surrounding vegetation. The sequences of microbial members in this system were similar to other nutrient-rich forested wetlands, and these nutrient-rich ecosystems were characterized by their high number of *Proteobacteria* (Lv et al., 2014). We also revealed a large amount of sequences from archaea from the samples, which might also involve in the cycling of organic carbon, nitrogen, or toxic metals.

The 16S sequencing was used to examine potential mercury methylating species. The 16S sequencing data grant us a first look into the community structure of these Hg methylating groups in Caddo Lake. The functional capability to methylate mercury varies across multiple clades and is likely evolved through horizontal gene transfer (Bravo et al., 2018; Parks et al., 2013; Yu et al., 2013). Although the sequencing of the function gene *hgcA* would reveal more explicit details regarding the abundance of each species, the sequencing data of *16S* genes presented here indicated several families known to contain

Hg methylators, including *Syntrophobacteraceae* (1.4%), *Geobacter* spp. (1.1%), SRB *Desulfovibrio-Desulfobulbus-Desulfobacter* (0.6%), and methanogenic archaea (0.6%). It seems that, the dominant species of potential Hg methylators in the lake were mainly from *Deltaproteobacteria*, representing 3.1% of total sediment community diversity. Recent study by Yu et al. (2018) revealed that, *Syntrophobacter wolinii* from *Syntrophobacteraceae* is a weak Hg methylator by itself. More importantly, once *S. wolinii* was associated with methanogen *Methanospirillum hungatei* or with SRB *Desulfovibrio desulfuricans* ND 132 by syntrophy, the syntrophic associations could significantly stimulate Hg methylation under no sulfate conditions. The dominant distribution of the three groups of microbes in the sediment suggests that, except for the roles of Hg methylation contributed by SRB, IRB and methanogens, syntrophy of *Syntrophobacter* spp., SRB *Desulfovibrio* spp., and methanogens might play critical role in microbial MeHg production in Caddo Lake, especially under sulfate- and iron-limited conditions. In natural habitat such as in Florida Everglades wetland, the sulfate-limiting conditions could lead to form prevailingly syntrophic relationships of methanogens with sulfate reducing bacteria or *Syntrophobacterales*, likely dictating the Hg methylation activities (Bae et al., 2014; Yu et al., 2018).

The comparison of qPCR data of *16S* gene copies in sediment samples showed that bacteria were significantly more abundant than archaea in Caddo benthos microbes. The same scenario was also observed when we compared the *hgcA* gene copies between the *Deltaproteobacteria* and archaea. For instance, in the spring sediment samples, *hgcA* gene copies from *Deltaproteobacteria* were roughly 3-15 times higher than those from archaea in the samples (Figure 6.9). However, copies of *mcrA* genes which uniquely represent

methanogens were even higher than the *hgcA* copies from *Deltaproteobacteria*. That implicates that only partial of methanogens might contain the detectable *hgcA* genes or involve in Hg methylation (Podar et al., 2015). In the general perspective, the dominance of functional gene copies usually means a higher capacity of the guild in microbial metabolism activities. However, it might be not the case when microbial Hg methylation is occurring at *in situ* conditions, considering the huge variations of Hg methylation capacity among the microbes and their interactions.

Based on the previous MeHg analyses and *hgcA* copies detected in the sediment samples, we proposed that Johnsons Ranch and Judd Hole were probably the key sites in Caddo Lake, acting likely as the hotspots for MeHg production. Johnsons Ranch seemed to be dominated by sulfate-reducing bacteria which was most dependent on sulfate concentrations.

Correlation of Hg methylation genes with biogeochemical changes

After Parks et al. (2013) initially identified the functioning gene *hgcAB* for Hg methylation from the two classic species *D. desulfuricans* ND 132 and *Geobacter sulfurreducens* PCA, direct *hgcA* gene detection to explore environmental Hg contamination has been broadly employed in many current studies. However, whether or how the gene abundance of *hgcA* is related with the typical electron acceptors such as sulfate and Fe(III) in environments is unknown. It is quite unexpected that the correlation analysis in this study showed no positive relationships between concentrations of sulfate and Fe(III) and *hgcA* gene abundance. The principal component analysis showed a high correlation of *Deltaproteobacterial hgcA* gene abundance with MeHg and sulfate concentrations in Johnsons Ranch (Figure 6.11). The average sulfate levels cross the lake

sampling sites ranged from 4.6 to 73.3 μM, a lower end of typical sulfate range in freshwater lakes (0-200 μM) (Yu et al., 2018). Slightly seasonal changes of sulfate were observed in this site, ranging from 15 to 60 μM (Table 4 and Figure 6.1). The relatively stable sulfate levels in Johnsons Ranch might support the growth of SRB and their persistent Hg methylation activities. However, the relationships between the *hgcA* genes and Hg methylation rates and other mechanisms are still remained unknown in Caddo Lake ecosystem and are warranted to be further investigated.

Chapter 6

Summary

This study explored the biogeochemical features, microbial communities, Hg methylation genes in sediment samples collected seasonally from several sites for two years. East Texas holds several coal burning power stations which have historically emitted a large quantity of Hg and eventually caused fish contamination in nearby lakes. The study further analyzed their relationships with environmental factors in Caddo Lake, an organic forest lake with wetlands. The results indicated that several biogeochemical parameters changed during seasons. Diverse microbial communities, and higher abundance of bacterial functioning gene *hgcA* were observed in some habitats. The potential Hg methylating species were dominated by SRB, IRB, and MPA, with the largest group of *Syntrophobacteraceae* involving in the syntrophic relations. In order to protect habitats like Caddo Lake with endangered species, environmental variables especially MeHg and sequences of the Hg methylating genes in microbial communities should be further evaluated in the search of remediation strategies to mitigate this toxic metal contamination in East Texas. The identification of the hotspots for Hg methylation will provide scientific evidence for establishing environmental regulation policies in order to reduce future Hg emissions and to protect human health and natural wildlife in East Texas and beyon

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Table 4 Sediment biogeochemical characteristics in Caddo Lake, Texas.

Sample	*pH*	*Total Organic Carbon (g/kg)*	*Pore Water Organic Carbon (mg/kg)*	*Total Nitrogen (g/kg)*	*Sulfate (µM)*	*Iron (III) dwt (mg/g)*	*THg dwt (µg/kg)*	*MeHg dwt (µg/kg)*
State Park	6.9	60.4 ± 21.9	94.5 ± 102.3	3.8 ± 1.2	73.3 ± 33.2	0.9 ± 0.5	164.8	0.144
Crips Camp	6.1	61.7 ± 32.6	46.7 ± 26.2	4.0 ± 1.7	53.7 ± 49.0	1.0 ± 0.8	333.6	0.732
Johnsons Ranch	6.5	98.8 ± 52.3	45.3 ± 30.3	7.4 ± 4.9	45.4 ± 20.4	0.9 ± 0.5	113	4.255
Ames Spring Basin	6.4	99.0 ± 17.5	27.7 ± 11.4	6.4 ± 2.1	17.0 ± 5.4	0.3 ± 0.4	122.3	0.881
Cross Bayou	6.4	105.7 ± 70.4	28.7 ± 12.2	6.5 ± 4.4	17.9 ± 8.7	0.5 ± 0.5	165	0.284
Judd Hole	6.5	154.1 ± 19.3	27.8 ± 12.6	9.4 ± 1.4	19.3 ± 3.9	0.6 ± 0.5	223.8	1.254
Kane Hole	6.5	179.9 ± 104.4	38.0 ± 15.2	11.9 ± 6.5	4.6 ± 5.0	0.1 ± 0.1	147.6	1.767

* The data were the average of measurements from four seasons from 2018, with the standard deviations. THg: Total mercury. MeHg: Methylmercury.

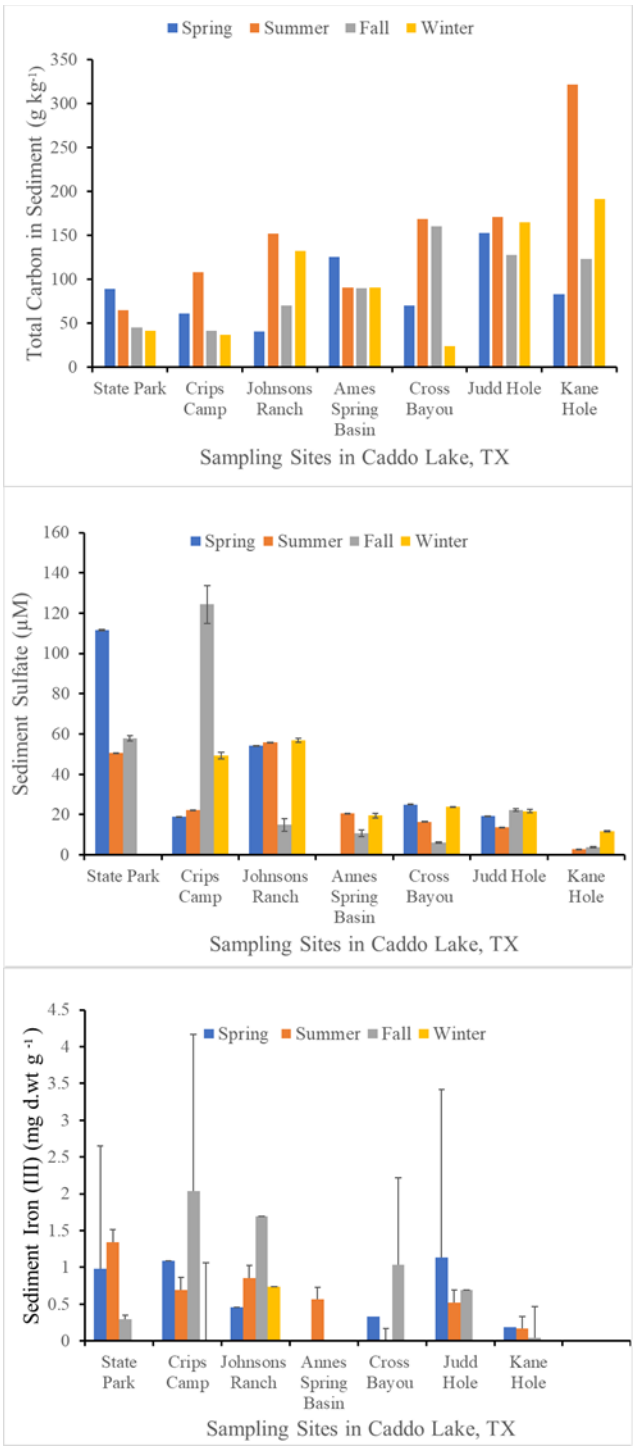
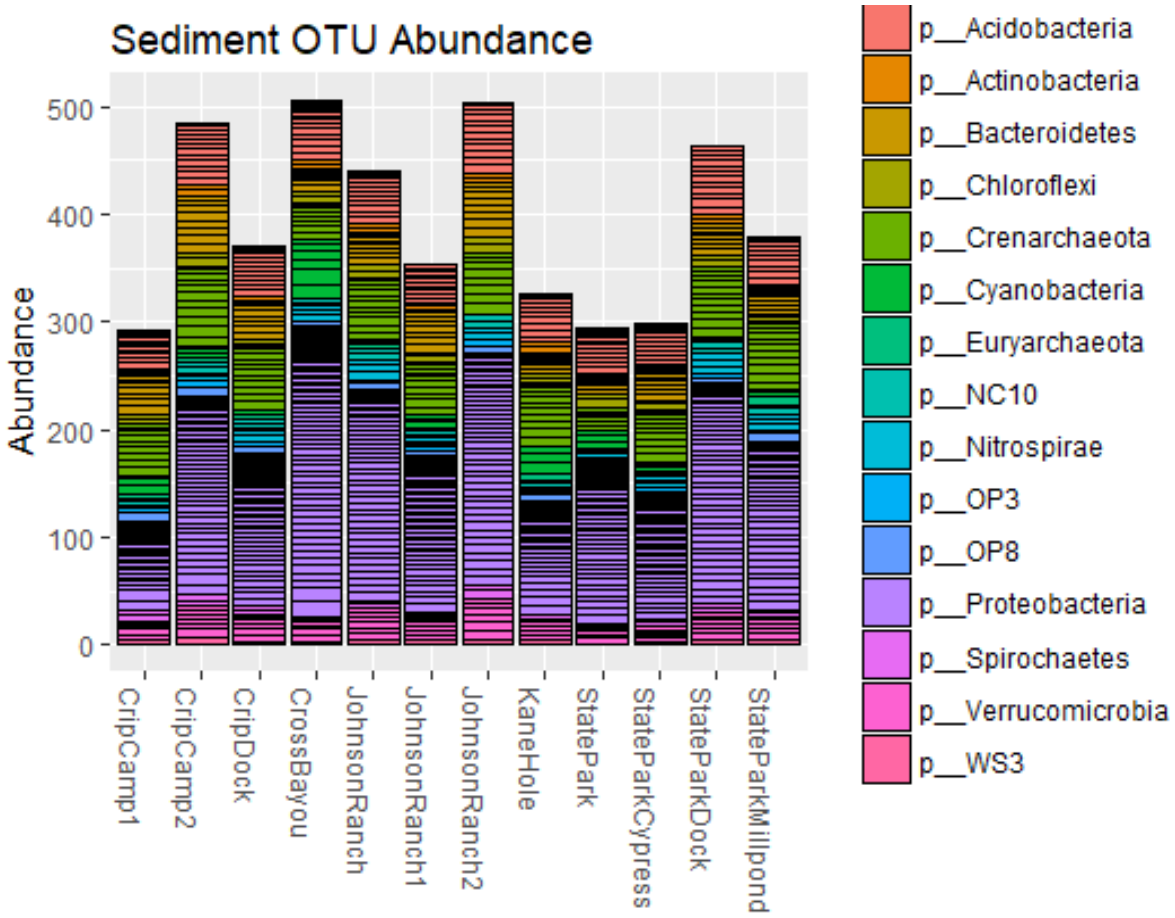
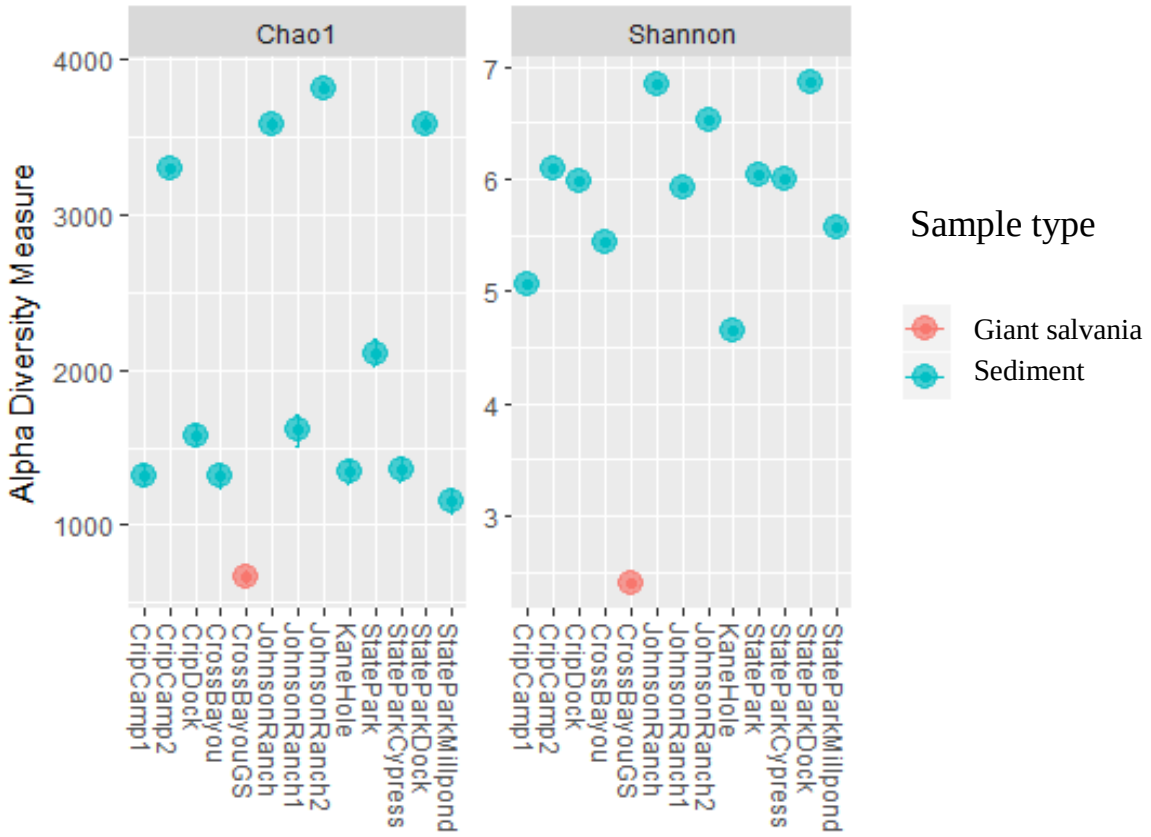


Figure 6. 1 Seasonal changes of sediment total carbon, sulfate, and iron (III) in Caddo Lake, Texas from March to December 2018.



Sampling sites in Caddo Lake, Texas

Figure 6. 2 Microbial community abundance in the sediment samples of Caddo Lake, Texas.



Sample sites in sediment Caddo Lake, Texas

Figure 6. 3 Microbial community alpha diversity in the sediment samples of Caddo Lake, Texas.

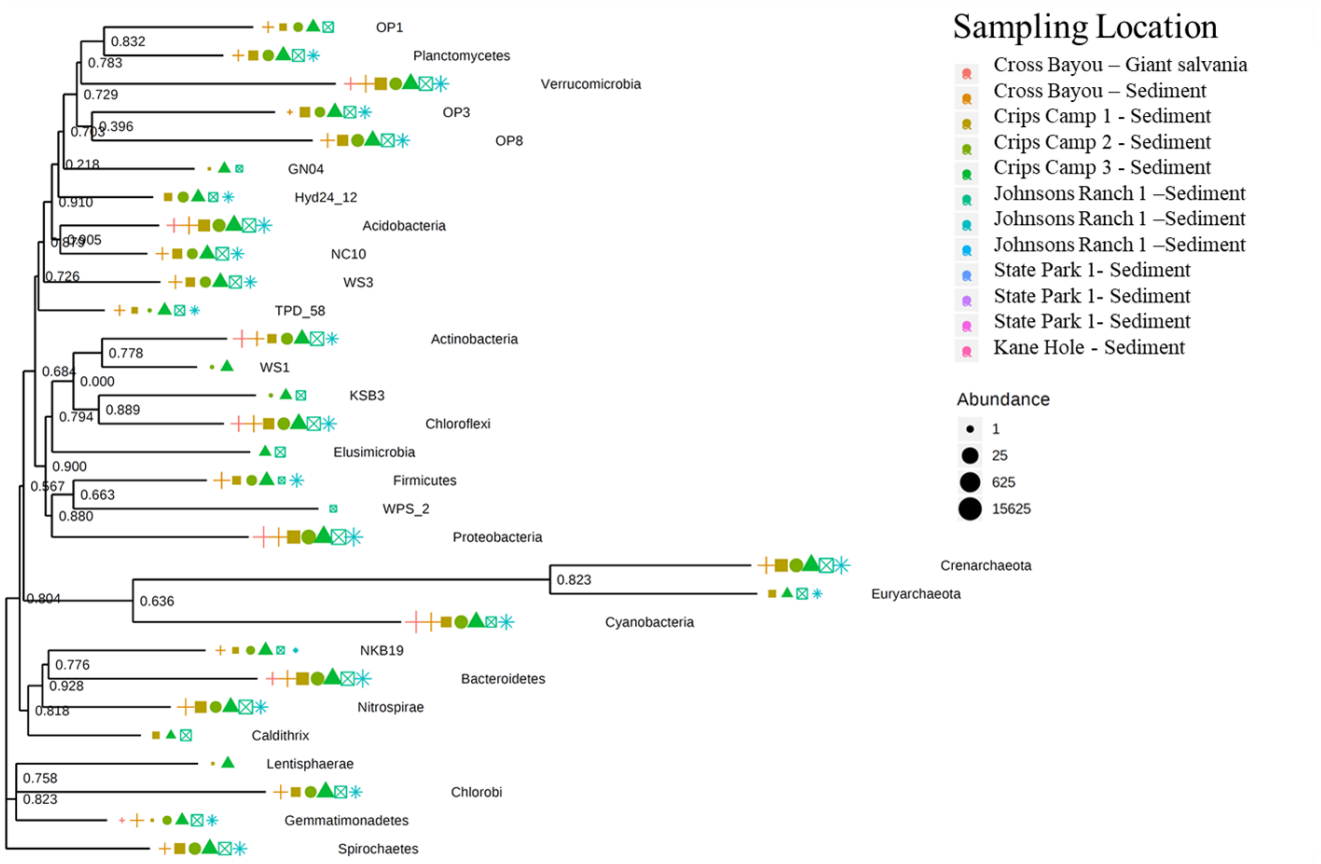


Figure 6. 4 Phylogenetic relationships of microbial communities in the sediment samples of Caddo Lake, Texas.

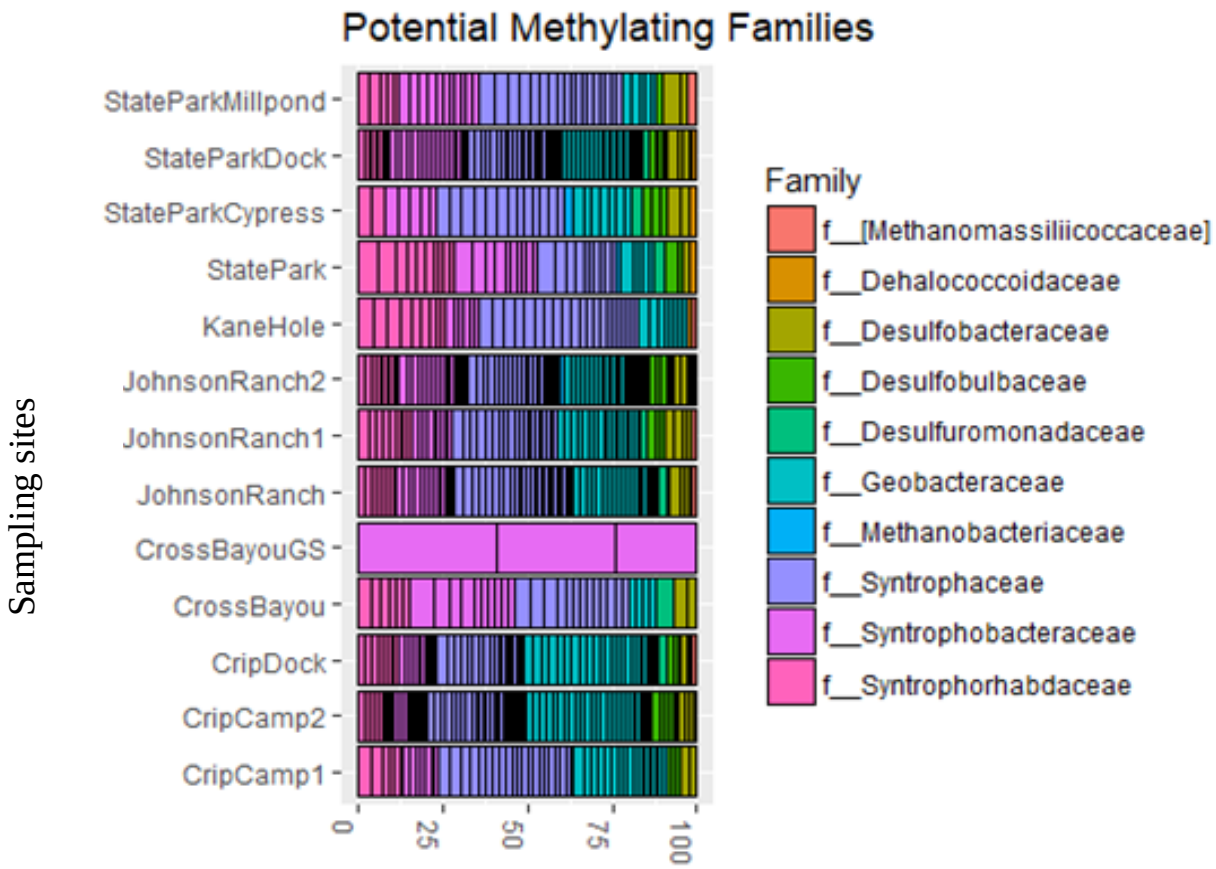


Figure 6. 5 Potential mercury methylating microbes with relative abundance in the sediment samples of Caddo Lake, Texas.

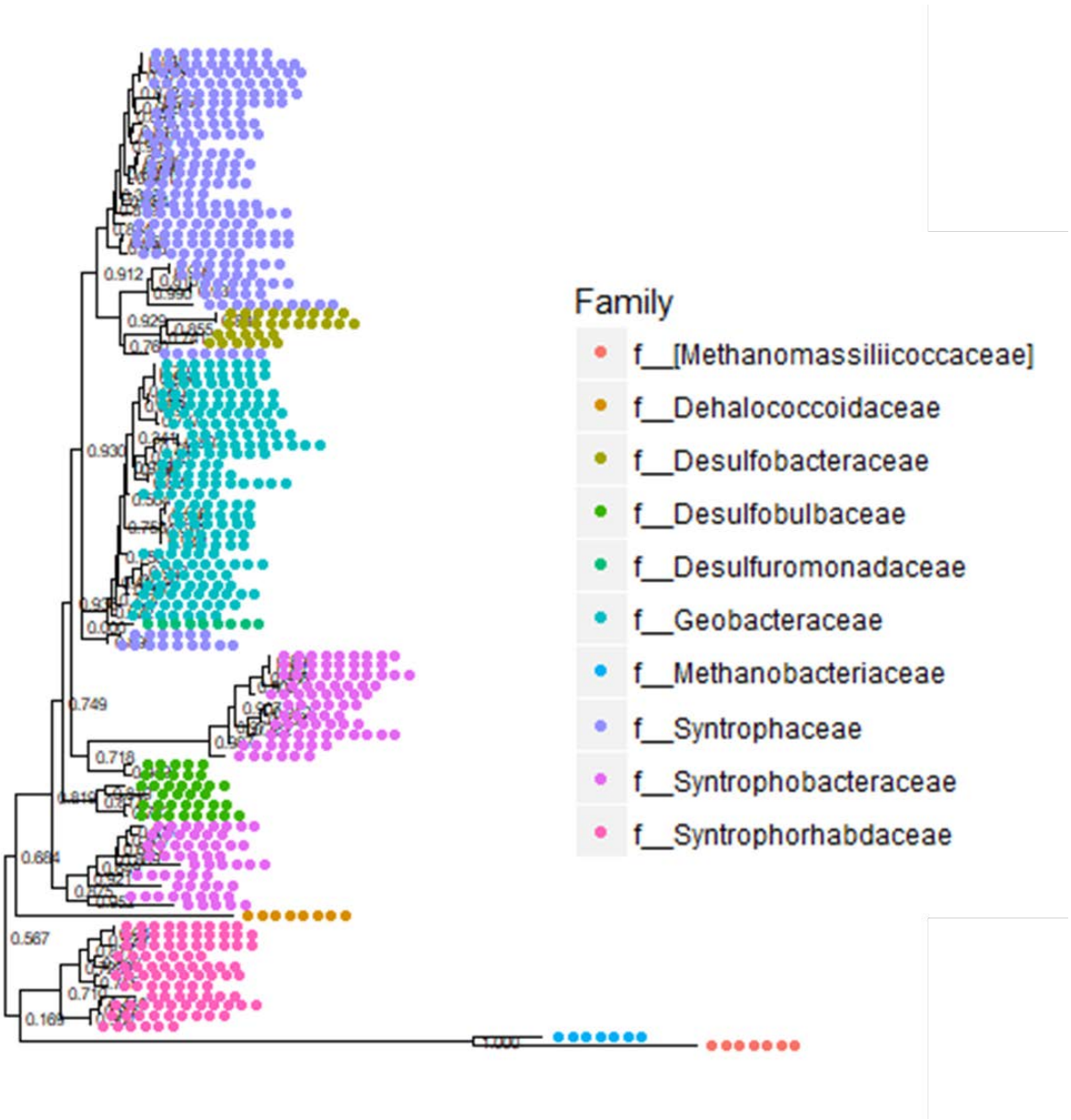


Figure 6. 6 Phylogenetic relationships of potential mercury methylating microbes in the sediment samples of Caddo Lake, Texas.

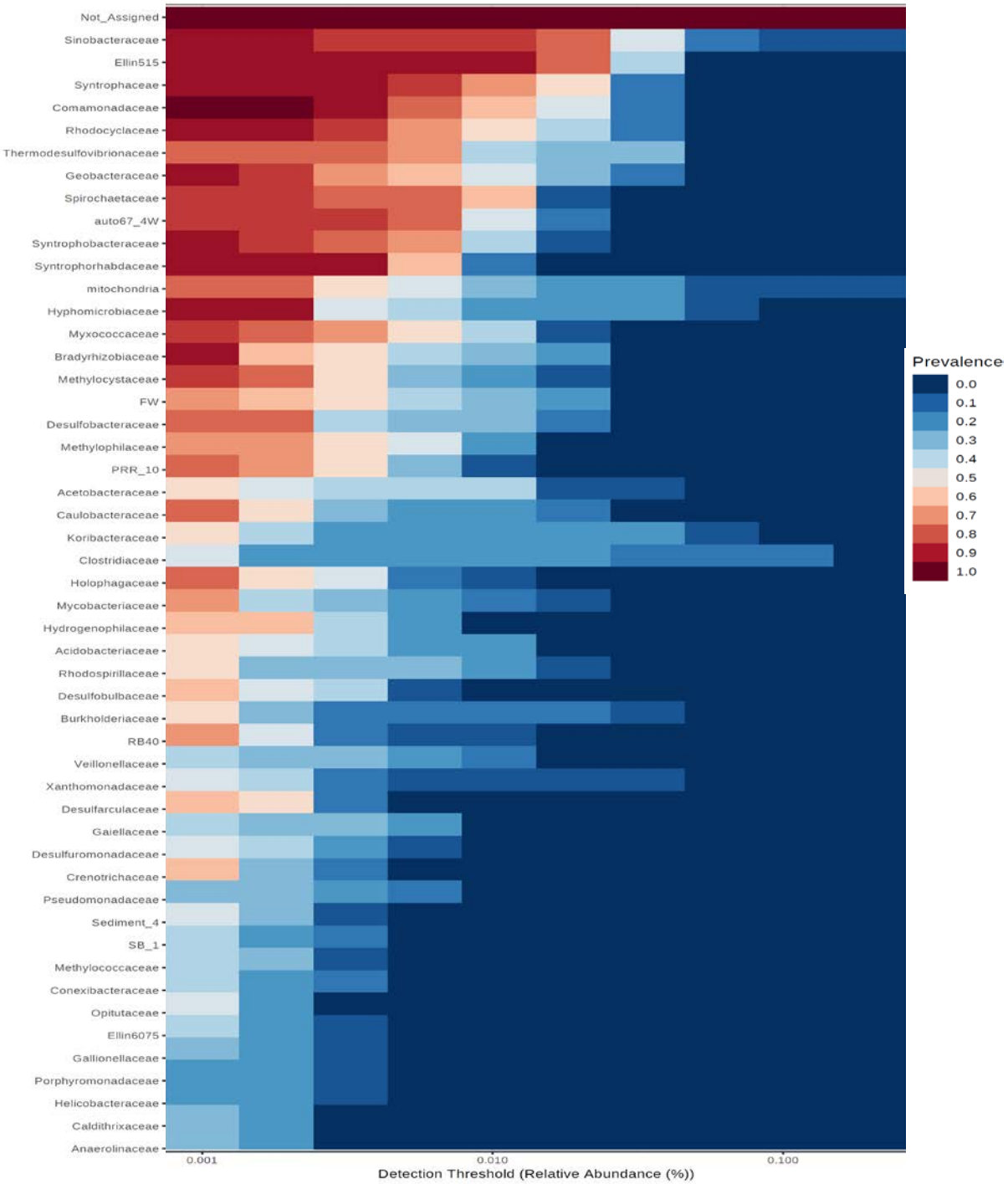


Figure 6. 6 Relative abundance prevalence of potential mercury methylating families in the sediment samples of Caddo Lake, Texas.

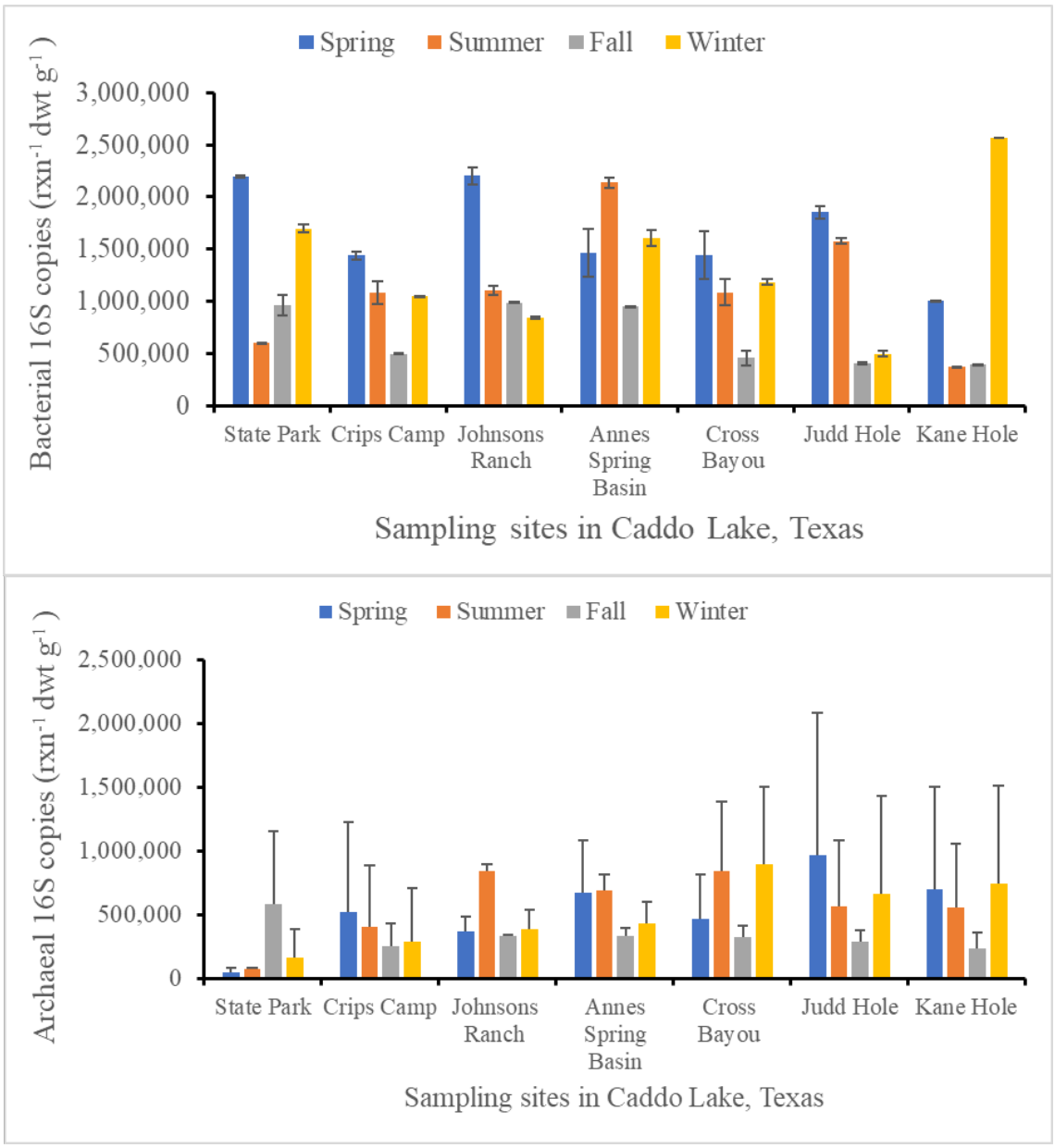


Figure 6. 7 Seasonal gene abundance of the microbial 16S rRNA in the sediment samples of Caddo Lake, Texas from March to December 2018.

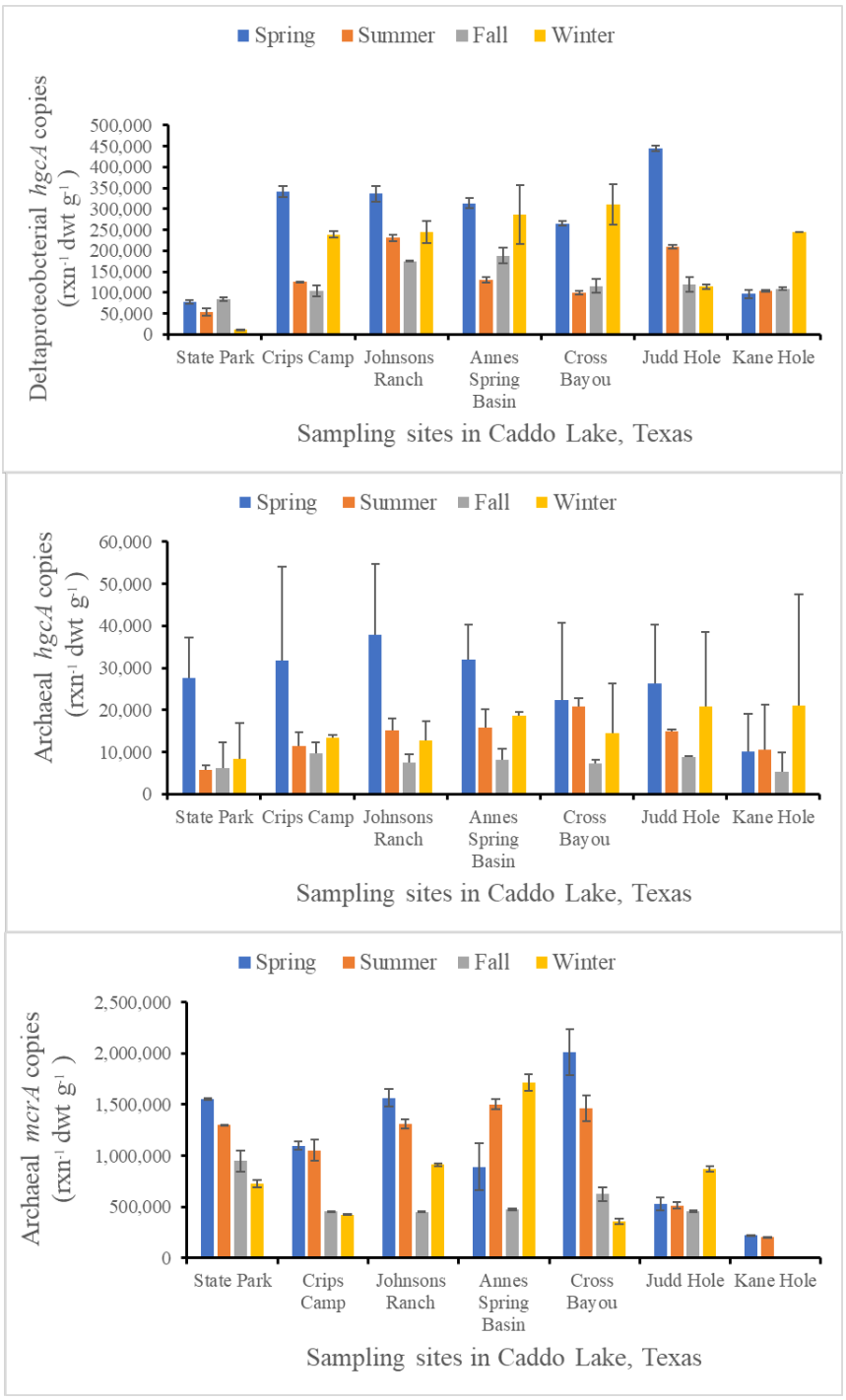


Figure 6. 8 Abundance of the function gene *hgcA* in *Deltaproteobacteria* and Archaea, and *mcrA* in methanogens in the sediment samples of Caddo Lake, Texas from March to December 2018.

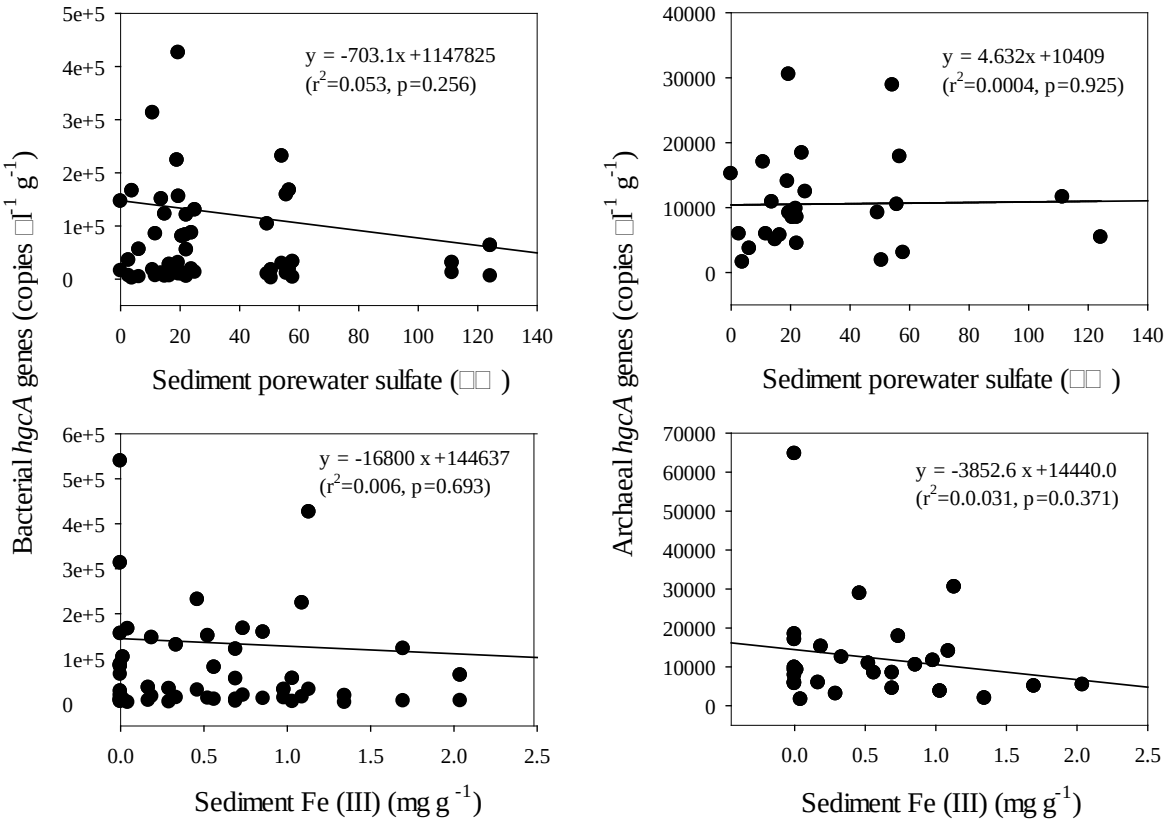


Figure 6. 9 Correlation analyses of the abundance of function gene *hgcA* in *Deltaproteobacteria* and Archaea and pore water sulfate and iron (III) levels in the sediment samples of Caddo Lake, Texas.

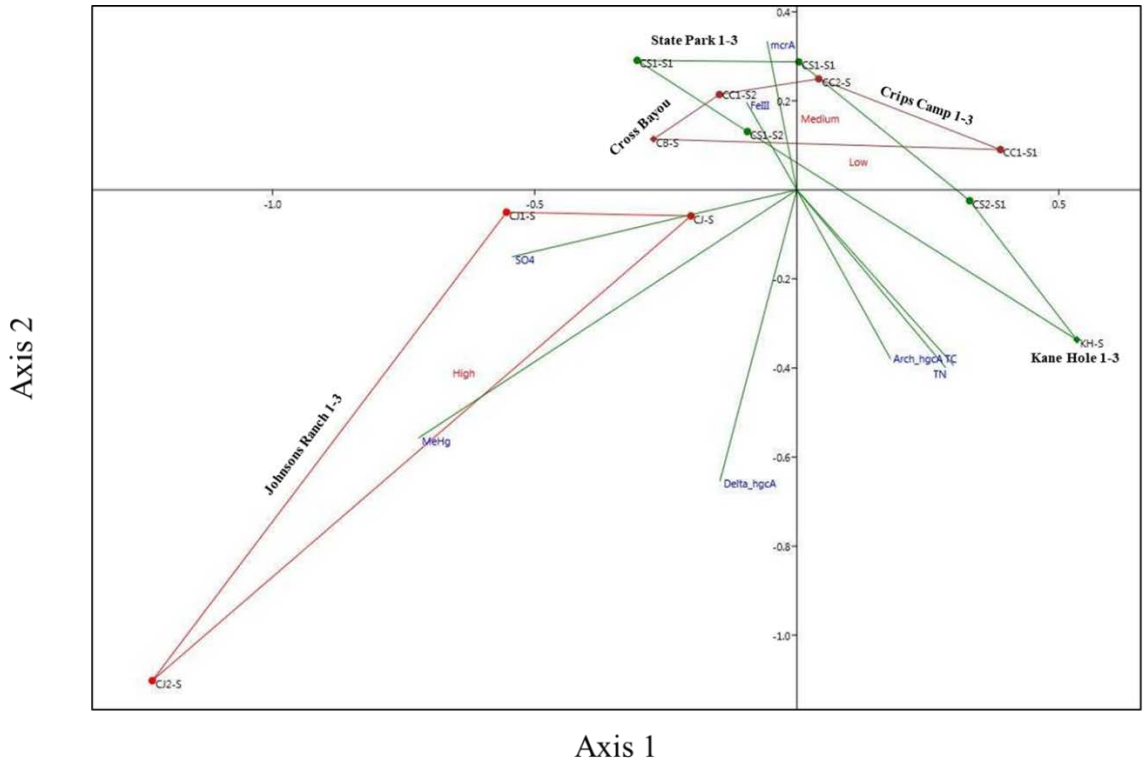


Figure 6. 10 Canonical correspondence analysis on the sediment samples of Caddo Lake, Texas. The hot spot Johnsons Ranch is labelled as a red triangle.

Appendix: Microbial Mercury Methylation at Caddo Lake: A Molecular Ecology Approach

Figure A.1 Sampling area near Kane Hole covered by Giant salvania, Cypress and Spanish moss.

Figure A.2 Sampling location in Ames Spring Basin covered by Giant salvania.

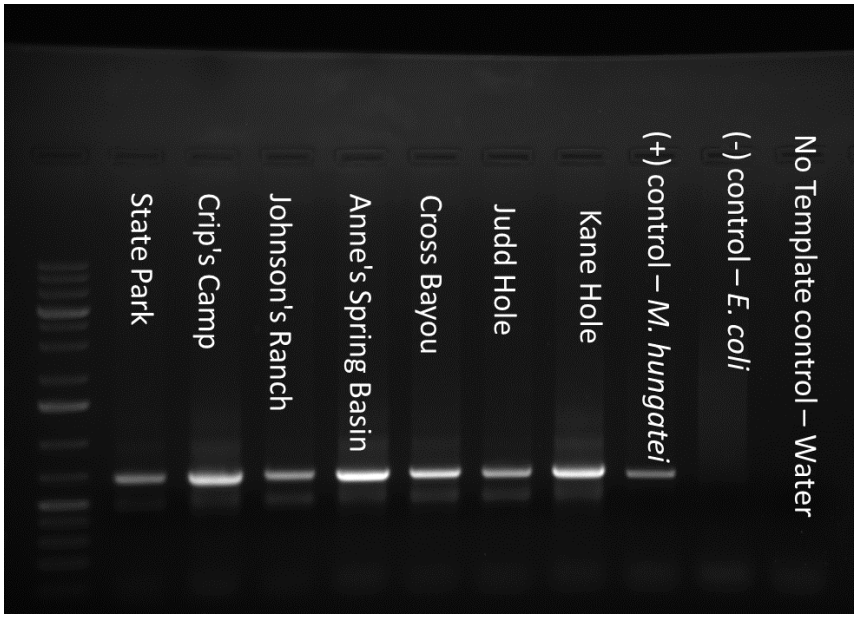


Figure A.3 Gene *hgcA* amplified by Liu et al. (2014) primer set of sediment samples taken in March 2018 with a target product size of 680 bp.

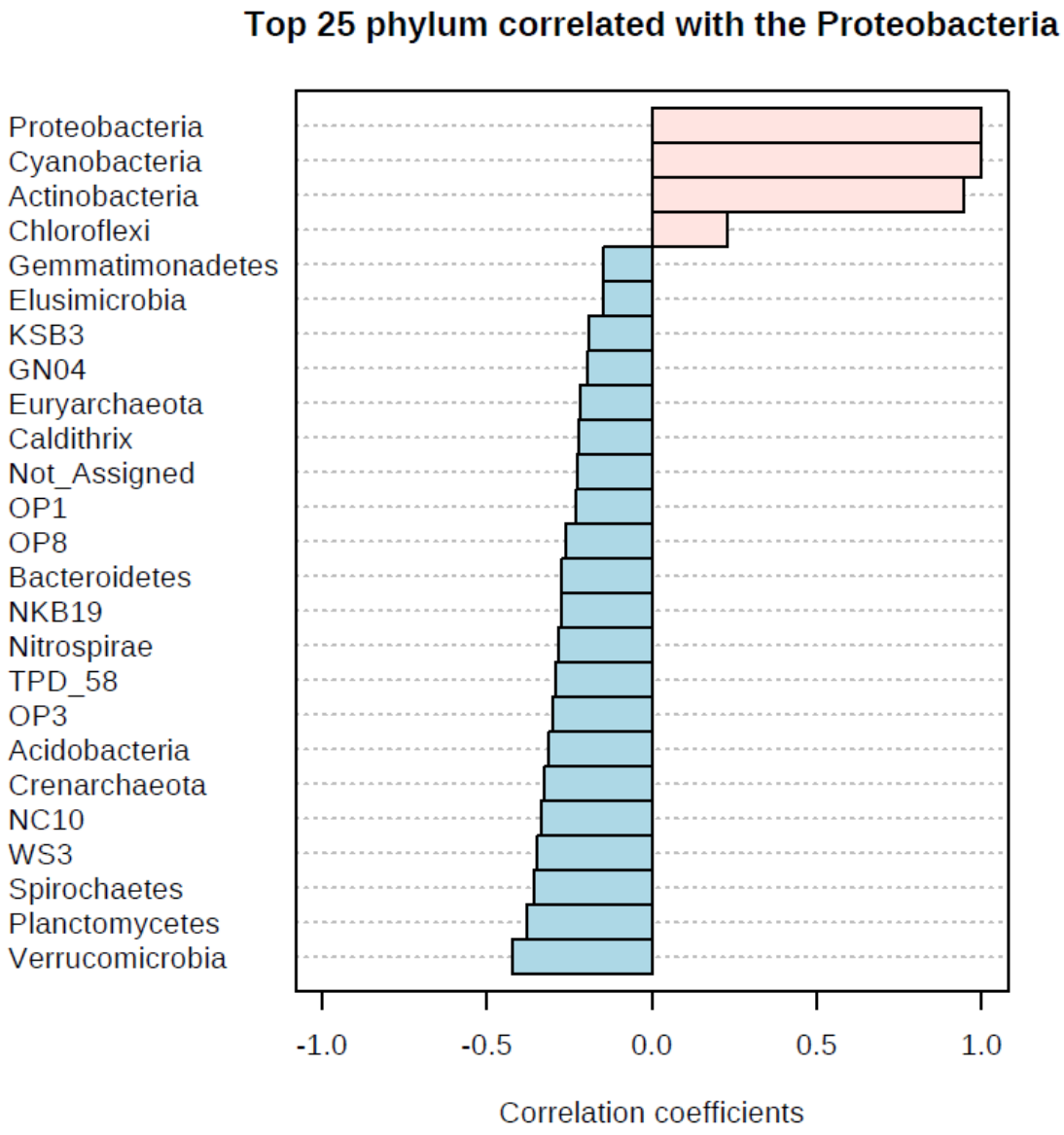


Figure A.4 Phyla that correlate with proteobacteria in the Caddo Lake sediment samples.

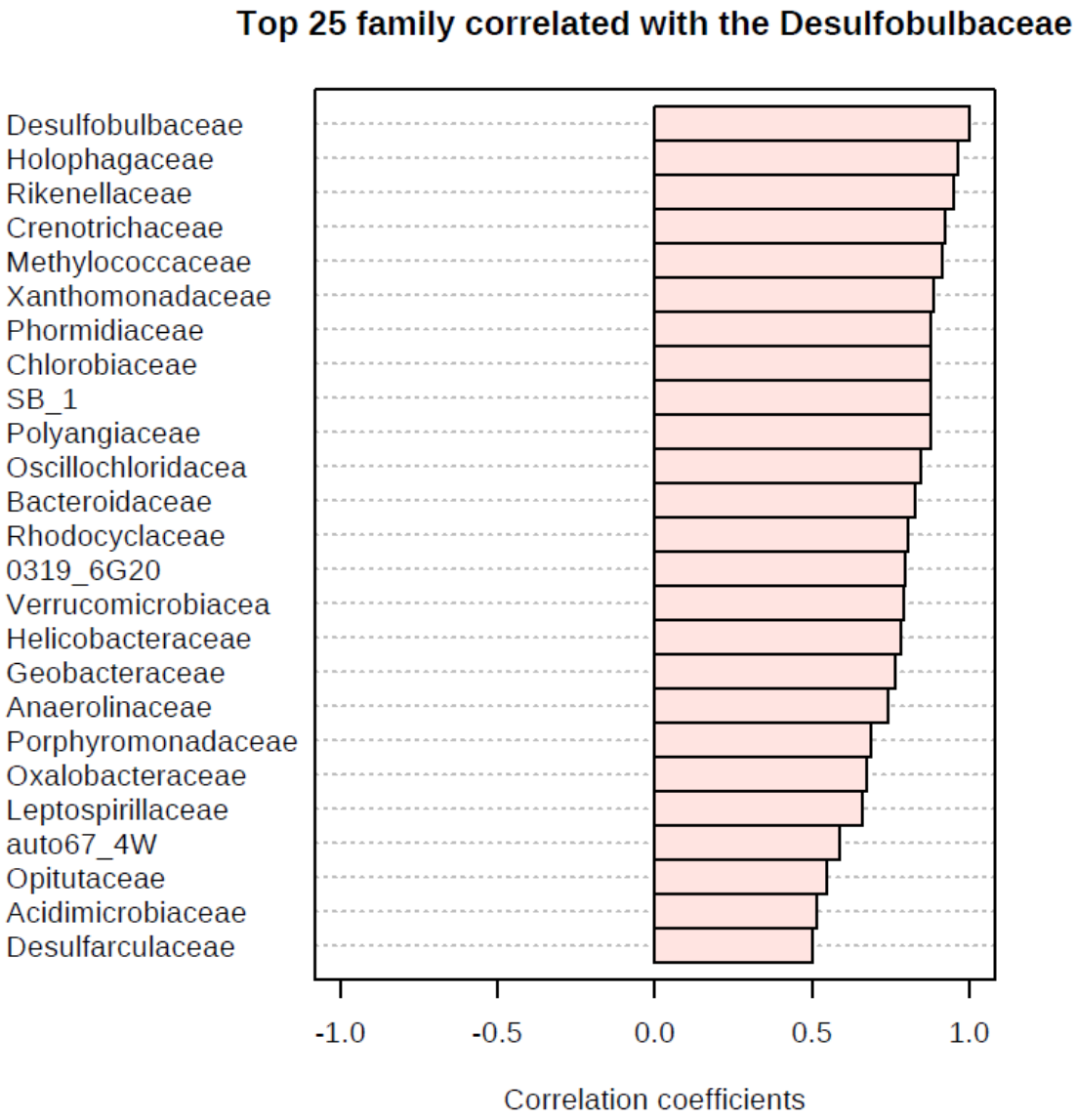


Figure A.5 Families that correlate with *Desulfobulbaceae* as a known Hg Methylating family.