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DETERMINING BIOINDICATORS FOR COASTAL TIDAL MARSH HEALTH USING THE FOOD WEB OF LARVAE OF THE GREENHEAD HORSE FLY (*Tabanus nigrovittatus*)

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

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by
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Abstract

The greenhead horse fly *Tabanus nigrovittatus* Macquart is native to coastal marshlands from Texas to Nova Scotia. The larvae are apex invertebrate predators and their development is dependent on the food web in the soil. Surveillance of *T. nigrovittatus* after the 2010 Deepwater Horizon oil spill in the Gulf of Mexico showed population crashes of adults in the coastal marshes of East Louisiana near places where oil made landfall, but not in West Louisiana where the oil did not reach. Sediment collection in 2011 from West and East Louisiana revealed larval population crashes in the Eastern coastal region. We hypothesized that due to the oil contamination a critical component of the food web of the larvae was destroyed and/or direct toxicity of the oil led to tabanid larval population crashes. With the aim of deciphering the tabanid larval food web in the Louisiana marshes, we used 18SrRNA metagenomics to identify the components of the food web in larval guts (n=16) and the surrounding sediment (n=25) from East and West Louisiana. An approximately 400 bp region at the 5' end of the 18SrRNAgene was sequenced using the Illumina MiSeq sequencing platform. Downstream analysis was conducted in QIIME (v1.9). Effect of oil, sediment chemistry and sediment toxicity on larvae and their food web was determined to explain larval decline in East Louisiana. We found insects and fungi to dominate the tabanid larval guts. Insects identified belonged to the families Drosophilidae, Culicidae and Tabanidae. Bioindicators of minimally oiled sediments are Hymenostomatia (*Tetrahymena*), Bivalvia (mollusks), Maxillopoda (Crustacea) and Peronosporomycetes (fungus like eukaryotes). "Phylum" Opisthokonta dominance in sediments is an indicator of larval presence in West Louisiana but not the East. Oil contamination, sediment chemistry and toxicity could not explain larval population crashes in the East. Decline in the adult population led to fewer breeders and subsequently fewer larvae.

Chapter 1. Literature review

1.1 Wetlands, Coastal Wetlands and Salt Marshes

Wetlands are land areas saturated with water permanently or seasonally. Wetlands including marshes, mires, ponds, swamps, bogs lagoons, mudflats, floodplains and shallow seas are found in almost every country in this world (Whigham, 2009). Coastal wetlands are located in coastal watersheds and include salt and freshwater wetlands, which can be further classified as tidal and non-tidal (Whigham, 2009). Total wetland acreage in the contiguous United States is 38 percent out of which 81 percent is located in the southeast United States (Dahl and Stedman, 2013). In the Gulf of Mexico, salt marshes are the tidally flooded areas of the marsh closest to the beach rim dominated by *Spartina alterniflora* (smooth cordgrass) and vary from 1 to 15 miles in width (Boesch et al., 1994)**.**

1.2 Importance of Wetlands, Coastal Wetlands and the Salt Marshes of Louisiana

Wetlands provide for two thirds of the fish harvest on this planet (Maltby, 2013). Coastal marsh ecosystems offer flood protection, erosion control and carbon sequestration (Beaumont et al., 2007, US-EPA). In addition, they provide habitats and food for wildlife, recreational services and support for commercial fisheries with over 50% of the fishery industry in the Southeastern United States relying on coastal wetlands (US-EPA, Beaumont et al., 2007). Over 2 million people in Louisiana would have to relocate and the lifestyle that has been preserved for centuries would be diminished without the coastal marshes (Reed and Wilson, 2004). In the salt marshes of Louisiana, the osmotolerant and dominating plant *Spartina alterniflora* houses a great diversity of herbivorous insects with over 100 species (Wimp et al., 2010, Pfeiffer and Wiegert, 1981). In addition, *Spartina* marshes are highly productive and act as a breeding ground for the

larval stages of crabs, shrimp, sea trout and other economically important fishes (White et al., 1978). Salt marshes also act as seasonal nitrogen and phosphorus sinks increasing water quality and act as water reservoirs (Boorman, 1999).

1.3 Vulnerability of Coastal Wetlands globally and within the United States with special emphasis on the Salt Marshes of Louisiana

Globally, coastal ecosystems provide extensive ecosystem services but face severe threats from natural processes (Barbier et al., 2011, Orth et al., 2006) and human activities (Kirwan and Megonigal, 2013, Gundlach and Hayes, 1978, US-EPA). The vulnerability of coastal marshlands to climate change, sea level rise, land subsidence, hydrologic modifications, development (residential and commercial), invasive species and pollution, i.e. by agricultural fertilizer runoff, chemical and oil spills, has been realized. A combination of such natural and anthropogenic stressors contribute to the degradation of the coastal marshes at a rapid rate (O'Riordan et al., 2000). Anthropogenic stressors such as agriculture and pollution have led to destruction of mangroves (Valiela et al., 2001). Salt marshes of Louisiana are primarily affected by salt water intrusion and inundation due to sea level rise and land subsidence exacerbated by hurricanes (Chesney et al., 2000). Growth of *Spartina* marshes is dependent on adequate rainfall, nutrients in the soil and nutrients transported by the tides making them vulnerable to both natural and anthropogenic stressors (Weinstein, 1996, Anthony et al., 2009).

1.4 Oil spills – a major anthropogenic stressor of Coastal Wetlands

A common anthropogenic stressor in coastal areas is oil spillage (Crain et al., 2009,Silliman et al., 2012,Pezeshki et al., 2000, Reddy et al., 2002). Oil and gas development in the Gulf of Mexico has increased the Gulf's vulnerability to oil spills. Louisiana coastal wetlands supports processing and transportation of US oil production and 24% of US natural gas production

(Tunnell et al., 2009). Thus, elevated background levels for oil in Gulf of Mexico are always present. Oil spills are a lurking danger in offshore waters (Tunnell et al., 2009). In the period from 2001 to 2015 the total quantity of oil spilled offshore in US was 4,937,607 barrels. Primary causes of oil spills are equipment failure, vessel damage, corrosion, external force or effects of weather including hurricanes on platforms, pipelines and tankers (Tunnell et al., 2009). Major oil spills that have affected US waters according to the National Oceanic and Atmospheric Administration (https://response.restoration.noaa.gov/oil-and-chemical-spills/oil-spills/largest-oil-spills-affecting-us-waters-1969.html) are Deepwater Horizon Oil Spill (2010), Exxon Valdez (1989), Ixtoc-1 (1979), Burmah Agate (1979), Hawaiian Patriot (1977), Epic Colocotronis (1975). Every year the quantity of oil transported off the US coast by ships, tankers and barges is increasing (BOEM, 2016).

1.5 Impacts and restoration efforts of damaged Coastal Wetlands within the United States with special emphasis on the Salt Marshes of Louisiana

Coastal wetlands are diminishing rapidly in comparison to other types of wetlands in the United States (Dahl and Stedman, 2013). Within the United States, wetland loss was at an average rate of 80,000 acres per year (2004-2009) across the Atlantic, Pacific, the Great Lakes and the Gulf of Mexico, thereby threatening the habitat of 75% of the nation's waterfowl and migratory birds and 45% of the nation's endangered species (Dahl and Stedman, 2013). The United States Environmental Protection Agency has started programs that create awareness among people to help restore and maintain coastal wetlands (US-EPA). The Gulf of Mexico coast suffers 71% of the estimated wetland losses and the primary reason is human activities (Dahl and Stedman, 2013). In the salt marshes of Louisiana, human activities such as construction of roads, pipelines and destroy, disturb or convert habitats resulting in habitat fragmentation (DeLaune et al., 1983, Reed and Wilson, 2004). Construction of waterways and levees not only causes habitat

destruction and disturbance but also shoreline erosion (DeLaune et al., 1983, Reed and Wilson, 2004). Salt marshes also have been lost due to hurricanes, such as Katrina, Rita and Ike, due to increased rainfall, abnormally high tides, storm surges, runoff and debris deposition (Dahl and Stedman, 2013). With all of these factors causing impact on coastal estuaries, Louisiana was the first state bordering the Gulf of Mexico to develop a comprehensive program to protect and restore coastal wetlands (Dahl and Stedman, 2013).

1.6 Impacts and restoration effects after oil spills on marine and coastal ecosystems

Oil spills are known to have caused ecosystem devastation in the past and pose as a deadly threat due to their unpredictable nature (Etkin, 2000, Dickins, 2011) and because resources to contain oil spills are not readably available (Silliman et al., 2012). Spilled oil can degrade ecosystems either directly or indirectly (Geraci and Smith, 1976). Direct toxicity includes physical smothering or chemical toxicity of the oil resulting in lethality. For example, when oil makes landfall, it smothers vegetation (Pezeshki et al., 2000) and contaminates sediments with toxic residues and creates anoxic conditions, thereby destroying communities living in or dependent on sediments (Teal and Howarth, 1984). Oil spills decrease the abundance and diversity of marine pelagic and benthic communities, micro zooplankton, benthic invertebrates, fish (Teal and Howarth, 1984), and marine vertebrates such as birds (migratory, Seaside Sparrows), turtles, mammals and dolphins (Dahl and Stedman, 2013). In addition to these immediate effects, stranded oil in shorelines and salt marshes can cause long term impacts on the coastal ecosystem because vegetation loss can lead to erosion (Baker et al., 1993) and habitat loss, which endangers endemic species. Indirect toxicity involves loss of habitat/ shelter and partial or complete elimination of key taxa involved in multiple trophic levels (Fleeger et al., 2003).

Ecological interactions between species can affect inter-related ecosystems in the Gulf ranging from the estuaries, coastal wetlands to the open ocean (Silliman et al., 2012).

1.7 Deepwater Horizon Oil Spill – A wake up call for urgent assessment methods

1.7.1 Distribution of oil

The Deepwater Horizon Oil Spill, the largest man-made marine oil spill in history, was caused by the 20 April 2010 due to explosion of a drilling rig. (Crone and Tolstoy, 2010b, McNutt et al., 2012). Eleven people died and 17 were injured during his explosion. Macondo Mississippi Canyon block 252 oil gushed from the exploded rig for four months into the surrounding Gulf waters with a total quantity of 210 million gallons being released (Rabalais and Turner, 2016). The Shoreline Cleanup Assessment Technique (SCAT) showed that 2113 km out of 9545 km of the Gulf of Mexico were oiled (Nixon et al., 2016). East Louisiana coastal marshes took the brunt of oiling after 75 km of shoreline in the Barataria Bay had moderate to heavy oiling (Silliman et al., 2012). However, the distribution of oil in the coastal wetlands was patchy where some areas were affected more than others (Rabalais and Turner, 2016). High spatial heterogeneity within as little as 5m in the marsh was observed (Turner et al., 2014) suggested that site-specific measurements of oil should be taken for each study rather than rely on surveys measuring oil along the marsh shoreline based on data acquire from 2010 to 2011.

1.7.2 PAH assessment

Distribution of petroleum hydrocarbons in an environment associated with a spill can be assessed by determining the Polycyclic Aromatic Hydrocarbons levels. Total Polycyclic Hydrocarbons (TPH) and Polycyclic Aromatic Hydrocarbons (PAH) were measured from plant

and animal tissues, sediments and sea water in many studies after the DHS to determine the extent of oiling.

Detection of oil after the Deepwater Horizon Oil Spill was complicated by the fact that the physical and chemical properties of the oil kept changing with time (White et al., 2016). Oil was detected *in situ* and in the laboratory using different instruments and evaluating components such as total PAH, oil droplets, bulk oil and polarized oil (White et al., 2016). Inlet mass spectrometer and fluorometers were used in situ to measure aromatic hydrocarbons. In laboratory studies, GC-FID, GC- GC-MS in SIM mode, GC×GC-FID, GC×GC-TOF-MS were techniques used to measure the Total Polycyclic Hydrocarbons (TPH's) (White et al., 2016). The total PAH content in the Macondo Mississippi Canyon block 252 oil was low in comparison to other crude oils and decreased as the oil made its way to the marshes (Olson et al., 2017). Weathering of oil leading to a loss of PAHs occurred as the oil travelled from the Gulf waters into the marshes (Olson et al., 2017) potentially decreasing the toxicity of the oil.

1.7.3 Impact of the spill

Multiple field studies assessed the oil contamination which had an adverse impact on marine as well as terrestrial and intertidal ecosystems including marshes, beaches, and barrier islands in the Gulf of Mexico (Reddy et al., 2012, Walker, 2010). The marine ecosystem was impacted by the oil on the surface, suspended in the water column and buried in the deep sediments. Stranded oil in the coastal areas was transported in through tides and wind from the marine ecosystem into the salt marshes endangering the coastal salt marsh ecosystem (Crain et al., 2009, Walker, 2010). Adverse effects of the oil were seen on populations of the marine ecosystem such as corals (White et al., 2012), microbial communities (Redmond and Valentine, 2012), benthic

eukaryotes (Bik et al., 2012), fish assemblages (Able et al., 2015), marine mammals, sea turtles, among others (Barron, 2012).

Hydrocarbon degrading microbial communities in the salt marshes, such as *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, increased in oiled areas temporarily, but microbial abundance decreased subsequently after oil levels went down. Upregulation of hydrocarbon degrading genes of microbes also was observed in oiled environments (Beazley et al., 2012). Impact on terrestrial ecosystem included terrestrial vertebrates and birds such as shore birds, migratory birds, sea side sparrows (Bergeon Burns et al., 2014). Seaside Sparrows that perched on *Spartina* stems or fed on organisms in the oil contaminated sites suffered population crashes (Rabalais and Turner, 2016).

The oil also impacted the meiofauna (organisms that are retained on a 500 um sieve but pass through 50 um populations); small benthic invertebrates (Fleeger et al., 2015), marsh edge fishes (Roth and Baltz, 2009, Rozas and Minello, 1997), decapod crustaceans (Roth and Baltz, 2009, Rozas and Minello, 1997) especially blue crabs and shrimp (Armstrong et al., 1998), arthropods (Day et al., Husseneder et al., 2016, Pennings et al., 2014, McCall and Pennings, 2012, Bam, 2015), and marsh rice rat (Rabalais and Turner, 2016).

Vast devastation of *Spartina alterniflora* was widely reported as well (Silliman et al., 2012, DeLaune et al., 1979, Lin and Mendelssohn, 2012). Herbivores and other associated insects declined due to *Spartina* loss (McCall and Pennings, 2012). Periwinkles that survive on epiphytes attached to live or decaying *Spartina* leaves were indirectly affected and displayed reduction in mean shell length (Rabalais and Turner, 2016). Meiofauna feed on the benthic microalgae that live in the sediment in the *Spartina* marshes. Following *Spartina* recovery, both microalgae and most meiofauna increased in abundance and diversity (Rabalais and Turner,

2016). However, other meiofauna such as less mobile species of polychaetes, ostracods, and kinorhynchs had not recovered even 4 years post spill (Rabalais and Turner, 2016). Insects associated with *Spartina* that are predators, sucking herbivores, stem-boring herbivores, parasitoids, and detritivores recovered a year after the spill (McCall and Pennings, 2012) . Laboratory toxicity assays included a comprehensive toxicity testing program called the Trustees toxicity program to conduct toxicity assays to gauge the impact of the Deepwater Horizon Oil Spill (Morris et al., 2015). Toxicity of oil can act via direct contact with oil, ingestion, contaminated seawater and sediment routes (Morris et al., 2015). Toxicity on different life stages of over 40 species of fishes, reptiles, birds, aquatic invertebrates and phytoplankton was tested as a part of this program (Morris et al., 2015). Fish populations suffered multiple effects. For example, oil disrupted the heart function of pelagic fishes, such as mackerel and tuna, because oil can be absorbed by fish eggs accumulating to lethal levels attributed to blocking of ion channels (Brette et al., 2017, Incardona et al., 2014). Physiological and reproductive impairment of killifish also was observed in *in vitro* studies (Whitehead et al., 2012). In addition, genotoxic effects and immuntoxic effects were observed using a large battery of fish cell lines (Barron, 2012). Food chain disturbances also were detected using laboratory toxicity studies. For example, Laughing Gulls were fed fish injected with oil at a concentration of 5 or 10mL/kg and showed muscle wastage and diminished structural integrity of the cardiac tissue leading to death indicating that oil affects food chains (Horak et al., 2017).

Toxicity assays on invertebrates were conducted using classical and commercial methods. An example of the *in vitro* classical method includes toxicity assays on natural assemblages of zooplankton with sea water spiked with Louisiana Sweet Crude Oil showed 96% mortality at 100ug/L due to accumulation of toxins on eggs and tissues (Almeda et al., 2013).

Commercial assays such as Microtox microbial toxicity assay (SDI, Inc.) using *Vibrio fischeri* and QwikLite toxicity assay (Assure Controls, Inc.) using the dinoflagellate *Pyrocystis lunula* were used to determine the toxicity of filtered seawater spiked with Louisiana Crude Oil. Mutagenic effects were observed starting from 0.1ppm on dinoflagellates in the QwikLite assay and from10 ppm on *Vibrio fischeri* using the Microtox assay (Paul et al., 2013).

Amphipods and copepods were used in multiple toxicity assays involving spiked sea water and sediment bioassays because oil enters amphipods and copepods via ingestion and dermal uptake (Cohen et al., 2014, Almeda et al., 2015, Landrum et al., 1991, Lotufo et al., 2016). An array of commercial sediment assays exists to study sediment toxicity. However, sediment toxicity tests with amphipods have been found to be more sensitive to overall sediment contamination in comparison to commercial tests that are used to measure sediment toxicity such as MicrotoxTM, Thamnotox Kit, and Rototox Kit (Burton Jr et al., 2001).

1.8 Need for assessment tools for marsh health and role of bioindicators

Degradation of coastal ecosystems has led to declining water quality, biological invasions and decreased coastal protection from hurricanes and storm surges (Crain et al., 2009). Coastal marsh health assessment is required after pollution (through chemical and oil spills, etc.), land loss due to sea level rise, erosion and subsidence, as well as during coastal restoration land building efforts (Morris et al., 2015, O'Riordan et al., 2000). (Li et al., 2016). Ecosystem damage assessment, monitoring and control are extremely important, underlining the need for quick and affordable methods to assess ecosystem health as degradation of ecosystems has multiple effects (Schindler, 1987, Kokaly et al., 2013).

Bioindicators of coastal marsh health are a promising way forward to determine the success of remediation efforts in marshes (Burger and Gochfeld, 2001). Various bioindicators have been

used to detect ecosystem health after the Deepwater Horizon oil spill (Snyder et al., 2014, Fleeger et al., 2015, Deis et al., 2017, Beazley et al., 2012). Salt marsh periwinkle mean shell length and length-frequency distributions have been used as indicators to monitor health and recovery of Louisiana marshes (Deis et al., 2017). Reduction in size, abundance and species composition of fiddler crabs were observed in the oiled areas making them promising models of ecosystem health (Deis et al., 2017). Nematode bioindicators include *Sabatieria*, *Dorylaimopsis* and *Cheironchus*, three nematode genera that became dominant in the oiled areas due to their ability to tolerate high PAH levels and due to the decline of PAH intolerant nematode species (Soto et al., 2017).

1.9 Environmental monitoring using invertebrate bioindicators with special emphasis on insects and food webs

Matthew K. Lau et al. in 2016 reviewed 1914 papers (over a span of 14 years) from 53 countries across 6 continents and revealed that 50% of the taxa used in environmental monitoring were animals from which 70% were invertebrates (Siddig et al., 2016). Arthropoda is a major Phylum in the invertebrate community. Arthropod assemblages are better indicators of ecological changes in comparison to vertebrates because they provide signs of ecological changes such as habitat fragmentation in areas which no longer support vertebrate indicator species (Kremen et al., 1993). In addition, monitoring arthropods which are more sensitive to environmental stress than vertebrates can lead to proactive management (Kremen et al., 1993).

Insects are widespread and abundant, have a broad spectrum, acute sensory perception and occupy varied functional niches and microhabitats making them reliable bioindicators in ecosystems (McGeoch, 1998). Insects, especially soil dwelling dipterans have been suggested to be reliable bioindicators for health in various ecosystems including marshes (Frouz, 1999, Rochlin et al., 2011).Woodlice have been used to assess heavy contaminant pollution in

grasslands because they are widespread, easy to sample and identify besides being an important detrivore in the grasslands (Paoletti and Hassall, 1999). Predatory insects namely, Belostomatidae (Hemiptera) and larvae of Libellulidae (Odonata) have been used as bioindicators of heavy pollutants in sugarcane fields (Corbi et al., 2011).

Soil dwelling dipteran larvae and their food webs are considered dependable bioindicators of ecosystems due to their abundance, distribution and participation in the biological processes in the soil and long periods of sediment exposure (Frouz, 1999, Rochlin et al., 2011, Paoletti, 2012). Soil dwelling Diptera in the families Sciaridae and Ceciomyiidae are abundant, and both Sciaridae and Ceciomyiidae larvae react differently to different toxins and were, thus, suggested as important candidates for bioindication (Paoletti, 2012). A disadvantage of using Sciaridae and Ceciomyiidae larvae has been poor taxonomic identification of the larvae (Paoletti, 2012). Previous evidence suggests that soil dwelling larvae from sarcophagus fly families (Sphaeroceridae and Lonchopteridae) associated with vegetation are not influenced by the factors affecting vegetation but are responsive to the soil parameters making them promising indicators of environmental health in a heathland ecosystem (De Bruyn et al., 2001). Salt marsh arthropod communities have shown sensitivity to oil spills (Pennings et al., 2014). Adult and larval tabanid populations (*T. nigrovittatus*) declined in areas where the oil spill occurred (Husseneder et al., 2016). Besides population crashes, genetic bottlenecks, reduction in number of breeders, and reduced gene flow also was detected in the adult tabanid population in oiled areas (Husseneder et al., 2016).

Importance of food webs for environmental monitoring also is recognized. Burger et al. in 2001 recommended the use of deterministic based, population based food web models for ecological risk assessment (Burger et al., 2001). Nematode food webs have been used as bioindicators of a

disturbed soil environment by evaluating ratios of the weighted abundance of functional guilds (Ferris et al., 2001). Abundance ratios of different types of phytoplankton have been used to study the disturbances in the aquatic environment of the Baltic sea as phytoplankton are the primary producers and its abundance affects all trophic levels (Gorokhova et al., 2016).

1.10 Food webs for understanding ecosystem dynamics

Food webs in ecosystems are comprised of taxa which show varied response to environmental stressors (McCann et al., 2017, Barbier et al., 2011)**.** Food web investigations are important for understanding the health (Berry et al., 2017), functioning and dynamics of ecosystems including varied responses of taxa within food webs to environmental stressors (Blankenship and Yayanos, 2005, McCann et al., 2017, Berry et al., 2017). Diets of invertebrates are not widely studied even if they are central to studies of ecological processes, energetics, natural history, and food webs (Blankenship and Yayanos, 2005).

Previous studies of apex predators have shown to deliver valuable insights into ecosystem health (Berry et al., 2017) . For example, the diet of the endangered Australian sea lion (*Neophoca cinerea)* was deciphered using DNA metabarcoding to confirm that it is a wide ranging opportunistic predator that feeds on demersal fauna (Davenport and Bax, 2002). Primary taxa associated with the sea lion diet and variation in diets due to location also was observed (Davenport and Bax, 2002). Identification of the components of the greenhead horse fly larval food web is the first step to decipher the functioning and dynamics of this apex invertebrate predator's food web in the marshes.

Food web components or taxa can be divided into critically resilient, critically sensitive or sensitive with few effects on the food web depending on their role in the food web (McCann et

al., 2017). Taxa in ecosystems that are sensitive to environmental insults are of paramount importance as they can destabilize or alter food webs (Barbier et al., 2011). As the Deepwater Horizon oil spill was a major environmental stressor, comparing food web components at oiled versus non-oiled locations might possibly lead to identification *of* taxa that are critically resilient, critically sensitive or sensitive but with no effect on the larval food web (McCann et al., 2017). This could shed significant light on the functioning and dynamics of the tabanid larval food web and expose the effect of an ecosystem stressor on a marsh food web.

1.11 Deciphering food webs

Methods to decipher food webs have evolved drastically over time (Sheppard and Harwood, 2005). Initial dietary studies of insects involved visual observation of gut contents (Sheppard and Harwood, 2005, Ingerson-Mahar, 2002, Pierce and Boyle, 1991). In the nineteenth century, the diet of coleopteran predators was studied by visually identifying gut contents (Forbes, 1883). However, the major limitation was the degradation of gut contents due to digestion such that visual recognition was difficult (Forbes, 1883, Pearson, 1966). In addition, studying the diets of liquid feeding insects was impossible (Forbes, 1883).

Dietary analysis using fecal samples had similar limitations (Pearson, 1966). In the past, use of radioisotopes, chromatographic analysis of prey pigments, polyclonal antibodies to detect prey pigments and electrophoretic detection of prey isozymes were relied on for identifying trophic links to decipher food webs (Pompanon et al., 2012). In 1997, the first study of identification of prey using DNA found in predator guts was published and started a new era in food web studies (Asahida et al., 1997). DNA barcoding dietary studies of animals including arthropods gained popularity as an accurate, rapid and dependable way forward (Soininen et al., 2009). DNA

barcoding has transformed the field of biodiversity science (Cristescu, 2014) and changed our understanding of quantified webs of ecological interactions (Wirta et al., 2014).

The scope of DNA barcoding in environmental samples and dietary analysis has been recognized as an important asset for ecologists (Valentini et al., 2009b). For example, molecular gut content analysis showed that larval woodborers of the *Monochamus spp*. are facultative intraguild predators of bark beetle larvae proving that PCR based methods are useful in identifying insect interactions and mechanisms driving population fluctuations (Schoeller et al., 2012). DNA barcoding complements molecular phylogenetics, population genetics and taxonomy (Hajibabaei et al., 2007).

1.12 Current methods for environmental monitoring

Many methods are used for environmental monitoring. Surveys are conducted to monitor birds (Seaside and Nelson's Sparrows, water birds, Reddish Egrets, Black Rail), insects (different species of butterflies) and reptiles such as the Mississippi Diamondback Terrapin (Reed and Wilson, 2004) to determine effects of environmental stressors. Biosensors that detect pollutants or toxic compounds and bioanalytical assays that detect cytotoxicity, genotoxicity, biological oxygen demand and pathogenic bacteria are used for environmental monitoring (Rogers, 2006). Bioindicators can be monitored using traditional surveying of known bioindicator species. However, classical taxonomic methods can be cumbersome and often require a high level of expertise to identify organisms to the species level. In addition, identifying morphologically cryptic species is difficult. These limitations can be overcome by using DNA identification. DNA barcoding and community sequencing using next generation sequencing to detect bioindicators is gaining importance. For example, Chironomidae is a family of bioindicator insects of pollution that have been detected using next generation sequencing. Bioindicators can

be detected to provide efficient assessment and monitoring of marsh health (Carew et al., 2013, Bongers and Ferris, 1999).

1.13 DNA metabarcoding and Next Generation Sequencing (NGS)

DNA barcoding in combination with high throughput sequencing used for targeted gene amplification for multiple species in environmental DNA is known as DNA metabarcoding and widely used in biodiversity studies (Cristescu, 2014, Mason et al., 2012, Berry et al., 2017). DNA metabarcoding is performed by amplifying single or multiple regions of the DNA of interest. Commonly used molecular markers with respective regions for animals, plants, protists and fungi are COI-barcodes (Mitochondria), 16S-rRNA genes (Nucleus), cytb (Mitochondria), ITS1-rDNA (Nucleus), ITS2-rDNA (Nucleus), 18S-rRNA gene (Nucleus). The rbcL (Plastid) gene can be used for autotrophic protozoa and plants. DNA metabarcoding in biodiversity studies can be performed using universal primers (Blankenship and Yayanos, 2005) and/or group specific primers (Jarman et al., 2004). Universal primers cover a wider range of diversity in comparison to group specific primers, which amplify targeted taxa of interest (Blankenship and Yayanos, 2005).

Metagenomic studies including DNA metabarcoding on community DNA via high-throughput next-generation sequencing is gaining importance in a wide array of fields including ecology (Bass et al., 2015, Mason et al., 2012, Berry et al., 2017). For example, DNA barcoding combined with high throughput sequencing has been used to identify diets of mammals (Valentini et al., 2009b), birds, molluscs and insects (Valentini et al., 2009a). In addition, effects of human activities on ecosystems have been recognized by studying eukaryotic assemblages (Chariton et al., 2010). The strength of DNA metabarcoding is that multiple organisms can be identified from a bulk sample or degraded samples, which can be ancient or modern (Taberlet et

al., 2012). DNA metabarcoding fulfills the ecologist need for high throughput taxon identification (Taberlet et al., 2012).

Next Generation Sequencing technology has revolutionized the field of genomics because it can produce millions of reads in a single sequencing run (Shokralla et al., 2012). Ion Torrent's PGM, Pacific Biosciences' RS, Illumina MiSeq, and Illumina HiSeq are fast turnaround sequencing platforms in the sequencing world (Quail et al., 2012). Ion Torrent's PGM detects signals as protons are released during incorporation of bases in sequencing, whereas Pacific Biosciences' RS detects fluorescence signals generated during incorporation of bases in real time (Quail et al., 2012). However, Illumina has been at the forefront in the recent years dominating the sequencing industry (Quail et al., 2012). Illumina technology utilizes clonally amplified DNA templates which are immobilized to an acrylamide coating on the surface of a glass flowcell followed by detection of fluorescently labelled reversible-terminator nucleotides (Quail et al., 2012). In addition, Illumina has lower error rates and fewer false positives than Ion Torrent's PGM and Pacific Biosciences' RS (Quail et al., 2012). Illumina MiSeq v3 reagents are capable of producing up to 15 GB of output consisting of 25 million sequencing reads with 2 × 300 bp read lengths (Quail et al., 2012) while Illumina HiSeq produces 375 million sequencing reads with 2 × 150 bp read lengths (Quail et al., 2012). The latest on the sequencing front is Illumina NovaSeq 6000 S4 which generates 10,000 million sequencing with 2×150bp read lengths (https://genohub.com/ngs-instrument-guide/).

Even though Next Generation Sequencing technology has revolutionized sequencing itself, sequence abundance, sequence quality, sequencing artifacts and sequence contamination still haunt accurate downstream analysis (Schmieder and Edwards, 2011, Bokulich et al., 2013). Problems of read abundance and high quality read length can be overcome by using abundance

cutoff for reads (Bokulich et al., 2013). Accurate taxonomic assignments and diversity analysis improve by removing sequences with low abundance from datasets by minimizing spurious reads (Bokulich et al., 2013). Issues related to misassembly and drawing erroneous conclusions can be dealt by using freely available quality control softwares such as NGS data QC tools, including QC-Chain, FastQC, Fastx_Toolkit, NGS QC Toolkit and PRINSEQ (Zhou et al., 2014, Patel and Jain, 2015, Schmieder and Edwards, 2011, Bokulich et al., 2013).

Chapter 2. Introduction

Coastal marshlands are an integral component of the coastal ecosystem supporting magnitudes of diversity and innumerably valuable ecological and anthropological services (Shepard et al., 2011). Salt marshes of Louisiana are tidal marsh areas within 15 m from the shoreline and dominated by the plant *Spartina alterniflora* (Boesch et al., 1994).

The productive salt marshes of Louisiana provide habitats for various birds, reptiles and butterfly species, nurseries for the larval stages of commercially viable fish and shrimp species, increase water quality by acting as a phosphorus and nitrogen sink, protect from storms and hurricanes, and act as a water reservoir, among other important functions (White et al., 1978, Boorman, 1999).

Threats to the salt marshes of Louisiana are similar to most wetlands and are most often a combination of both natural and anthropogenic stressors leading to habitat destruction (O'Riordan et al., 2000). Natural threats in this region mainly include inundation due to storms, loss of land due to salt water intrusion (sea level rise), erosion and subsidence, and invasive species (Orth et al., 2006, Barbier et al., 2011). Anthropogenic stressors include pollution due to chemical run offs and oil spills, construction of levees, dykes, water ways, roads and pipelines, restoration efforts, and commercial and industrial development (Bowen and Valiela, 2001, Kirwan and Megonigal, 2013).

A need for quick and reliable tools to assess marsh health is required in the face of the main pollution threats and physical changes mentioned above. Current methods involve surveying of endangered species, and determination of abundance of commercial and native species; and whether they show signs of genotoxicity , classical taxonomic identification of known

bioindicators, counting microscopic species abundance, fluorescent antibody detection and/or enzymatic assays to detect bacterial bioindicators, community sequencing and targeted sequencing of bioindicators (Reed and Wilson, 2004, Rogers, 2006).

Insects are preferred as bioindicators for environmental monitoring due to their sensitivity to environmental changes facilitated by their acute sensory system and ability to perceive and quantify changes in the environment (Oliveira et al., 2014). For example, Environmental monitoring of freshwater ecosystems targeting *Cytochrome oxidase I* (COI) and *Cytochrome B* (CytB) genes of the chironomid species using Next Generation Sequencing technology in Australia underlines the potential of routine insect species-level diagnostic monitoring of chemical pollution (Carew et al., 2013). Chironomids are used as bioindicators in paleoenvironmental and biogeographical studies (Porinchu and MacDonald, 2003). The cosmopolitan nature of the chironomids along with their ability to tolerate gradients of pH, dissolved oxygen, depth, salinity and temperature and the presence of haemoglobin make them suitable indicators of freshwater environmental monitoring (Porinchu and MacDonald, 2003).

The Deepwater Horizon Oil Spill (DHOS) was the largest man-made marine oil spill where 4.9 million barrels of oil continuously gushed for 4 months into the Gulf of Mexico (Crone and Tolstoy, 2010a, McNutt et al., 2012). The DHOS occurred on April 20th contaminated shorelines including salt marshes along the Gulf coast and presented a unique opportunity to study the impact of oiling on invertebrates and search for bioindicator species of oil contamination, marsh health decline and recovery.

Moderate to heavy oiling was observed along 75 km in which East Louisiana coastal marshes were severely affected (Silliman et al., 2012, Michel et al., 2013) albeit with a highly patchy distribution (Turner et al., 2014).

The effect of the DHOS was extensive, damaging vegetation, vertebrate and invertebrate communities in the coastal and marine ecosystems (Silliman et al., 2012). Vertebrate communities highly affected by the DHOS included marine mammals, birds such as Seaside Sparrows, sea turtles and large fish species (Bentivegna et al., 2015, Rabalais and Turner, 2016). Vegetation health, productivity, photosynthetic rate, biomass, stem density and regrowth was reduced in the Louisiana marshes post the DHOS (Hester et al., 2016, Lin and Mendelssohn, 2012) . The dominant species of vegetation in the coastal marshes of Louisiana is *Spartina alterniflora* also known as saltmarsh cordgrass was almost eliminated in areas which experienced heavy oiling in the Louisiana marshes (Lin and Mendelssohn, 2012). This (Lin and Mendelssohn, 2012)

Invertebrate species associated with *Spartina alterniflora* were suppressed by 50% in 2010 in oiled sites after the DHOS (McCall and Pennings, 2012). Terrestrial arthropods were affected by oiling even in regions where plants were unaffected : therefore this group has been suggested to be useful indicators of oils spills (Pennings et al., 2014). In particular, the over 100 species of arthropods that are associated with *Spartina alterniflora* contain bioindicators of marsh health (Wimp et al., 2010, Pfeiffer and Wiegert, 1981) . Soil dwelling dipteran larvae found in salt marshes also are considered dependable bioindicators due to their abundance and distribution (Frouz, 1999).

An insect species that was adversely affected by the DHOS was the greenhead horse fly, *Tabanus nigrovittatus* Macquart (Diptera: Tabanidae) (Husseneder et al., 2016). The greenhead horse fly, is native to the coastal marshes of Louisiana from Texas to Nova Scotia (Hansens, 1979). Larvae of the greenhead horse fly are apex invertebrate predators in the coastal marshes (Magnarelli and Stoffolano Jr, 1980). Tabanid larvae develop for long periods of time in the

Spartina marsh sediments (3-9 months) and their development is dependent on the presence of a healthy food web (Magnarelli and Stoffolano Jr, 1980). Due to their position at the top of the invertebrate food web in the marsh soil and to the characteristics of the soil dwelling dipteran larvae mentioned above *T. nigrovittatus* larvae are invaluable in studying in salt marsh ecosystems (Frouz, 1999).

The overall hypothesis for the study is that the tabanid larvae and their associated food web can be used for monitoring the health of salt marshes. A chance to test this hypothesis presented itself after the Deepwater Horizon Oil Spill of 2010.

After the DHOS, effective population size of adult greenhead horse flies, number of breeders and gene flow was reduced and genetic bottlenecks were detected in oiled locations of East Louisiana (Husseneder et al., 2016). Total adult and larval counts were low in the oiled areas of East Louisiana in comparison to West Louisiana (Husseneder et al., 2016).

We hypothesized that the low larval population in East Louisiana would indicate missing components in the food web of the greenhead horse fly larvae. To test this hypothesis, larvae and surrounding sediments were collected from 11 sites in West Louisiana and 14 sites in East Louisiana in 2011. DNA was extracted, amplified and sequenced from the larval gut contents and the sediments to identify the larval food web components using 18S metabarcoding in the sediments and guts. Metabarcoding is a combination of high throughput sequencing and DNA taxonomy to identify diversity in environmental samples.

In this study, we used the 18SrRNA gene as a taxonomic marker due to the following reasons: 1. 18S is present in tandem repeats, has multiple copies and undergoes concerted evolution. These characteristics facilitate DNA amplification from among different species and conserved within

species making 18S an ideal region for diversity analysis across a wide range of taxa (Creer et al., 2010, Drummond et al., 2015). 2. There is a large number of taxonomically described 18S sequences identified to the genera and species level in public repositories such as NCBI and SILVA (Creer et al., 2010, Fonseca et al., 2014) to which sequences can be compared for taxa identification.

The aim of this study was to identify as many components of the larval food web as possible and hence we chose 18S universal primers that amplify a wide range of taxonomic groups. We used the primer NF1 and 18Sr2b because they amplify a short fragment of the V9 region of the 18SrRNA gene, which has been shown to be best suited for biodiversity studies as it contains a 60 basepair long variable region in the center of the region (O'Rorke et al., 2012, Hadziavdic et al., 2014). This variable region provides good resolution across a broad group of organisms/taxa. In addition, amplicons generated using these primers are sufficiently divergent to serve as diagnostic tools in metabarcoding studies (Bik et al., 2012, Porazinska et al., 2009a).

Although food webs in saltmarshes and their dynamics reflecting environmental changes has received recent attention, a deficit in knowledge of food webs of insects in salt marshes remains (Bergeon Burns et al., 2014). We chose to investigate the food web of *T.nigrovittatus* larvae since they are top predators in the Louisiana marsh soil and, therefore, represent the top of the invertebrate food pyramid in the marsh soil.

Comparison of food web components between sediments where larvae are present and absent could be used to determine bioindicators of a healthy food web that sustains top invertebrate predators.

After identifying the components of the food web, several factors that might have an impact on the taxa composition of the food web were assessed. Toxicity of oil on invertebrate taxa has been

observed previously (Mendelssohn et al., 2012). Therefore, relative abundance of taxa in sediments at oiled and unoiled sites (defined by presence of >700ppb of oil at the time of collection) within the same region (Grand Bayou) was compared to assess the influence of oil on food web composition.

However, as oiling was extremely patchy (Turner et al., 2014) and neither Alpha-diversity of taxa (variance within samples) nor Beta-diversity (variance among samples) could be explained by oil alone, we considered the effect of other factors in the sediments that might have an effect on the larvae and/or their food web. The first factor was an array of sediment chemistry parameters.

Previous evidence suggests that sediment chemistry affects food webs in coastal ecosystems (Humborg et al., 2000). Hence, measuring sediment chemistry levels and evaluating their effect on the taxa in the sediments and larval guts was crucial.

The second factor was the toxicity of the sediments on the larvae and food web elements, since sediment toxicity due to pollutants affects insect larval development (Ristola et al., 1999).

To determine if sediments could have had a toxic effect on the tabanid larvae causing larval population crashes as observed previously (Husseneder et al., 2016), a standardized sediment toxicity assay (Nebeker and Miller, 1988) (https://www.astm.org/Standards/E1367.htm) was performed using amphipods as an invertebrate model organism known to be sensitive to soil toxicity.

The overall goal was to identify bioindicator taxa that sustain a viable food web for the development of tabanid larvae by using high-throughput sequencing in both larval guts and sediments collected from oil contaminated and unaffected regions.

2.1 Objectives of the study

1. Describe the components of the food web of tabanid larvae in the Louisiana marshes.
2. Determine predictors of larval presence or bioindicator taxa sustaining healthy food web by comparing sediments where larvae are present/absent.
3. Determine if oil content has an effect on the abundance of taxa at oiled and unoiled collection sites in Grand Bayou.
4. Determine if sediment chemistry levels have an effect on relative abundance of taxa.
5. Perform a sediment toxicity assay to determine lethality of the sediments.

Chapter 3. Material and Methods

3.1 Site selection

Post the Deepwater Horizon oil spill but prior to land fall, we chose four locations along the Louisiana coast that were predominantly *Spartina* marshes (Figure 3.1) and selected multiple sites within each location to begin surveillance for adult tabanids. Sites had been selected based on availability of access, presence of *Spartina*, and absence of confined livestock. As predicted, the two locations in Western Louisiana (Rockefeller Wildlife Refuge and Cypremort Point) were not affected by oil while areas within both of the Eastern locations (Grand Isle and Grand Bayou) were oiled. The locations described as Eastern locations were not actually in East Louisiana but were east of the western locations.However, oiling was extremely heterogeneous in the Eastern locations (see literature review for details). The *Spartina* habitat at Rockefeller Wildlife Refuge is Gulf of Mexico tidal shoreline, Cypremort and Grand Bayou are fragmented marsh islands whereas Grand Isle is a barrier island.

3.2 Collection of sediments

In 2011, sediment samples were taken from transects that were approximately 5 m long from each site at the high tide mark. Vegetation was removed prior to sampling each transect with an Echo® SRM – 280 T gas trimmer to expose the soil. Then, a 40 cm ditch bank blade was used to mark the boundaries of a 0.5 m^2 transect by scoring the soft ground. Approximately 10 cm of the upper substratum along each transect was removed using a 10 cm x 30 cm ditch shovel and placed in buckets. The shovel was thoroughly rinsed with water in between sampling. One voucher specimen was collected from each transect and placed in a 9.5 liter bucket on ice until stored in a freezer at – 20 degrees Celsius. The remaining total volume, approximately 56 liters,

of the substratum from each transect was placed in three 18 Liter buckets and transported to the LSU AgCenter St. Gabriel Research Station, St. Gabriel, Louisiana, and subsequently searched for tabanid larvae.

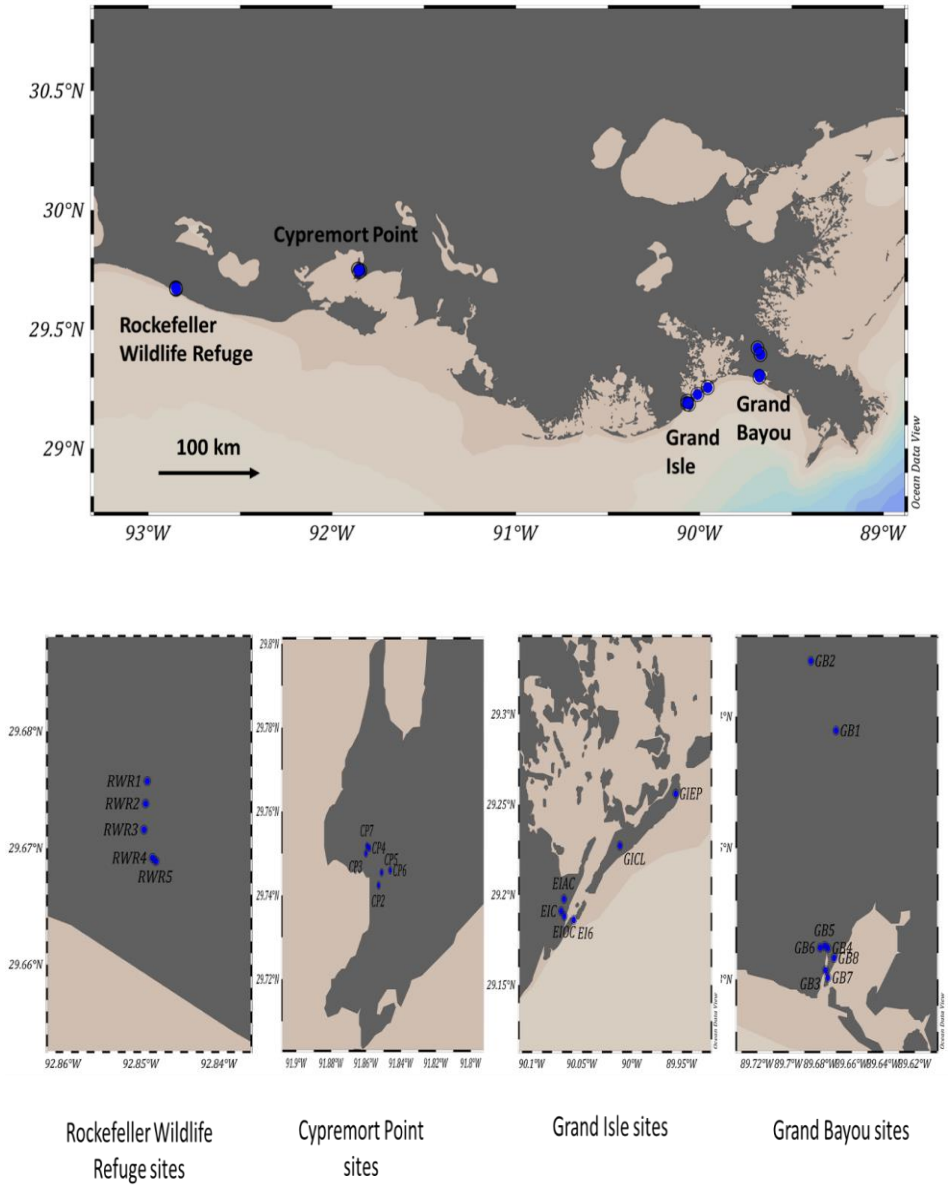

Figure 3.1. Map of sampling locations in West Louisiana (Rockefeller Wildlife Refuge and Cypremort Point) and East Louisiana (Grand Isle and Grand Bayou) of larvae and sediments samples in the Gulf of Mexico. Collections are from four locations, 5-7 sites per location. Sampling sites from which larvae and sediments were collected are marked by balloons. GPS coordinates for each site are available in Table 3.1. Map plotted in Ocean Data Viewer (v3).

3.3 Larval collection

Collection permits for horse fly larvae and adults were obtained from the Louisiana Department of Wildlife and Fisheries (permit numbers LNHP-10-074 and LNHP-11-092). Tabanid larvae were collected from five to seven marsh substrate samples taken from each of the four study locations from 25 August 2011 to 28 September 2011. A flotation technique was used to separate tabanid larvae from the substrate (Mullens and Rodriguez, 1984). First, the sample was removed from the buckets, broken into small handfuls and divided among nine or ten 38.8 liter (88.27 cm L. x 41.91 cm W. x 15.24 cm H.) storage containers (Sterlite Corp. Model # 19608006). Then, the containers were filled with water, and approximately 600 ml of water saturated with rock salt was added to the water in each container to increase buoyancy. The final salt concentration was approximately 40 ppt in the containers. The components (plant material, organic and inorganic matter, and organisms) of the substrate in the containers were broken into smaller pieces in the salt water solution by kneading and larger pieces, e.g. of plant material, were discarded after careful washing. The containers were searched twice, for ten minutes each time, and floating tabanid larvae and pupae were collected.

3.4 Tabanid larval identification

Each larva and pupa was examined visually under an Automontage system from Leica (Leica Z16 APO, DFC 450, 10447367, Leica, Buffalo Grove, IL) to confirm that it belongs to the family Tabanidae by morphological identification (Merritt and Cummins, 1996). Tabanid specimens found in the samples, were preserved in 95 % ethanol. A list of the number and sizes of larvae collected and their locations is compiled in Table 3.1.Steps 3.1 to 3.4 were done before this thesis and 3.5 onwards was done for this thesis.

Table 3.1. List of samples collected in 2011: Collection sites and sample names of sediments and larvae collected in the study along with GPS coordinates of each site. Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name .LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered. Size of larvae was measured. Locations from where larvae were collected can be found in the Figure 3.1.

Region	Site	Date	Larvae	Co-ordinates	
Unaffected				LAT	LONG
Rockefeller Wildlife Refuge	1	8/25/2011		29.675732°	-92.849224°
Rockefeller Wildlife Refuge	2	8/25/2011	4	29.673793°	-92.849425°
Rockefeller Wildlife Refuge	3	8/25/2011		29.671567°	-92.849640°
Rockefeller Wildlife Refuge	4	8/26/2011		29.669144°	-92.848543°
Rockefeller Wildlife Refuge	5	8/26/2011	10	29.668882°	-92.848130°
Cypremort Point	2	9/8/2011	2	29.742382°	-91.852816°
Cypremort Point	3	9/9/2011		29.750014°	-91.860089°
Cypremort Point	4	9/8/2011		29.751322°	-91.858228°
Cypremort Point	5	9/8/2011	2	29.745467°	-91.851100°
Cypremort Point	6	9/8/2011		29.745976°	-91.846179°
Cypremort Point	7	9/9/2011		29.751742°	-91.859423°
Possible Oil contaminated areas					
Grand Isle	GI East Park	9/16/2011		29.256008°	-89.956286°

(Table 3.1 continued)

Region	Site	Date	Larvae	Co-ordinates	Region
Grand Isle	GI West Cherry Lane	9/16/2011		29.227229°	-90.012126°
Grand Isle	EI C	9/15/2011		29.190957°	-90.070921°
Grand Isle	EILS 6	9/16/2011		29.186114°	-90.058188°
Grand Isle	EI Old Camp	9/15/2011		29.188118°	-90.067555°
Grand Isle	EI Air Control	9/16/2011		29.197690°	-90.068050°
Grand Bayou	1	8/30/2011		29.394719°	-89.669994°
Grand Bayou	2	8/30/2011		29.421217°	-89.685967°
Grand Bayou	3	8/30/2011		29.303427°	-89.676619°
Grand Bayou	4	8/31/2011		29.311935°	-89.675197°
Grand Bayou	5	8/31/2011	1	29.312764°	-89.677129°
Grand Bayou	6	8/31/2011		29.312081°	-89.680138°
Grand Bayou	7	9/28/2011		29.300680°	-89.675161°
Grand Bayou	8	9/28/2011		29.308156°	-89.671232°

3.5 Generating a molecular identity for *Tabanus nigrovittatus* Macquart

Adult horse fly females were collected and stored at -80°C. The flies were morphologically identified to the species level as *Tabanus nigrovittatus* (Sofield et al., 1984). Since no DNA records of *T. nigrovittatus* could be found in NCBI GenBank (7/12/2015), species specific sequences were generated to allow future identification of larva. Five flies collected using a canopy trap at the St.Bernard Drawbridge were washed with sterile water to remove any surface contaminants and their legs were removed. Legs were lysed overnight according to the instructions in the Dneasy Blood & Tissue kit (Qiagen Cat No. /ID: 69506, Qiagen, Germantown, MA) to extract DNA according to instructions of the manufacturer. Three regions of mitochondrial and genomic DNA commonly used for species identification were amplified by polymerase chain reaction (PCR) to generate a molecular identity for *T. nigrovittatus* (Cywinska et al., 2010, Whiting et al., 1997, Porazinska et al., 2009b) . The three regions targeted (Table 3. 2) were the mitochondrial cytochrome oxidase c 1 (COI, 687bp) using primers

LCO1490/HC02198 (Cywinska et al., 2010), a region of the 18S rRNA gene (982bp) using primers 18S ai/bi (Whiting et al., 1997) and another region of the 18S rRNA gene (412bp) using primers NF1/18Sr2b (Porazinska et al., 2009b). Primers were synthesized at Eurofins, New Orleans, LA.

Each PCR reaction was performed using One*Taq*® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA) with the standard reaction setup of a total volume of 25 µl recommended by the manufacturer. Initial denaturation was done at 94°C for 30 seconds followed by 35 cycles consisting of a denaturation step at 94°C for 30 seconds, an annealing step at primer specific temperatures and time for LCO1490/HC02198 gene (55°C for 1 min); 18S ai/bi (58°C for 1.5 min); NF1/18Sr2b (58°C for 30 seconds) and an elongation step at 68°C for 30 seconds.

Table 3.2. Primers used for DNA barcoding: Primer sequences and target regions of the primer pairs used in DNA barcoding (1-3) and metabarcoding (2).

No	Name of primer pair	Sequence	Region
1.	18Sai/ 18Sbi	5’CCTGAGAAACGGCTACCACATC-3’/ 5’GAGTCTCGTTCGTTATCGGA3’	391-1421 nucleotides of 18S rRNA gene
2.	NF1/ 18Sr2b	5’GCCTCCCTCGCGCCATCAGGGTGGTG CATGGCCGTTCTTAGTT-3’/ 5’GCCTTGCCAGCCCGCTCAGTACAAAG GGCAGGGACGTAAT-3’	400 bp from the 3’ end of 18S rRNA gene
3.	LCO1490/ HC02198	5'- GGTCAACAAATCATAAAGATATTGG-3'/ 5'- TAACTTCAGGGTGACCAAAAAATCA-3'	1490-2198 nucleotides of mitochondrial cytochrome oxidase I gene

A final extension at 68°C for 5mins was added. A 1% agarose gel was used to check the presence of the PCR products. After PCR purification, bidirectional Sanger sequencing was carried out for all primer pairs by GeneWiz, South Plainfield, NJ. Forward and reverse sequences were aligned using ChromasPro® 2.6.4 (from Technelysium Pty Ltd, available at http://technelysium.com.au/wp). A consensus sequence for each of the three target regions was generated and submitted to GenBank (NCBI). The sequences of *T. nigrovittatus* in GenBank can be accessed using the following accession numbers; KT381971 (mitochondrial cytochrome oxidase c 1 gene-687), KT222915 (18S gene-982bp) and KU321600.1 (18S gene-412bp).

3.6 Tabanid larval dissection, DNA extraction and amplification

Nineteen larvae were collected in 2011 (Table 3.1), stored in 95% ethanol, morphologically identified as tabanids, and then dissected under disinfected conditions. The dissection pan with wax (Thermo Fisher Scientific, Wilmington, DE) and dissection tools were wiped clean with 4% sodium hypochlorite solution prior to each dissection. Between larval dissections all the instruments and surfaces were wiped with Clorox bleach until visibly clean and free of stains and tissues. The larvae were pinned on the sterilized dissection pan and washed with 70% ethanol. Fine insect pins were used to pin the anterior and posterior ends of larvae with the dorsal side of the larvae facing upwards. Microdissection scissors (Fine Science Tools, Foster City, CA) were used to cut the cuticle at the ventral midline near the posterior end and all the way through to the anterior end. The lateral edges of the cut cuticle were pinned to the dissection pan exposing the gut. The fat body covering the gut was removed and the gut was removed. The gut lining was cut and pinned to the dissection pan and gut contents were pipetted into a 1.5ml microcentrifuge tube containing lysis buffer (ATL) from the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA). DNA was extracted from the gut contents according to the manufacturer's instructions. DNA

quantity (>10ng) and purity (260/280 ratio of ~1.8) was measured using NanoDrop® ND-1000, Spectrophotometer. DNA was amplified using illustra™ Ready-To-Go™ GenomiPhi™ HY DNA Amplification Kit (GE Healthcare, Thermo Fisher Scientific, Wilmington, DE) according to the manufacturer's General Protocol available on GE health care Life Sciences website (http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences-us/29108039).The amplified DNA was purified using the QlAquick® PCR Purification Kit protocol PCR using the primer pair NF1 and 18Sr2b (Table 3.2) of the 18SrRNA gene was done with cycling conditions mentioned above. Agarose gels of 1% (Sigma Aldrich, Saint Louis, MO) were used to check the amplified bands using the GeneRuler 1kb ladder (Thermo Fisher Scientific, Wilmington, DE). A band with an approximate size of 400 bp was considered as evidence for successful amplification.

3.7 Molecular identification of tabanid larvae

DNA from the remaining carcasses of 19 tabanid larvae was extracted using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA). After DNA extraction, Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE) was used to determine quantity (>10ng) and quality (260/280 ratio of ~1.8) of the DNA. The three regions described above were amplified via PCR as described in section 3.5. PCR products were checked on a 1% agarose gel, purified and submitted for Sanger sequencing in both forward and reverse directions (GeneWiz, South Plainfield, NJ). Forward and reverse sequences for each larva were aligned as described in 3.5. The aligned sequences were matched to the NCBI database using the BLASTn program for species level identification. Larvae that showed ≥ 99% match to a species in the database were assigned to that species.

3.8 Cloning to detect extent of tabanid DNA contamination in the gut contents

PCR products obtained using the primer set NF1/18Sr2b were cloned into One Shot® Competent *E. coli* (Life Technologies™, Thermo Fisher Scientific, Wilmington, DE) using the TOPO®TA Cloning® Kit (Invitrogen, Life Technologies™, Thermo Fisher Scientific, Wilmington, DE). A ligation reaction was set up using 2 µl of the DNA product with a concentration in the range of 10-100 ng, 2 µl of water, 1 µl of salt solution provided with the kit and 1µl of the TOPO® vector. A 15 minute incubation period at room temperature allows the ligase enzyme, which is linked to the vector, to insert the PCR product into the plasmid vector. The ligation mixture was purified using ice cold absolute ethanol precipitation. After an overnight incubation at -20 ºC precipitated DNA was pelleted down the following day by centrifugation (Centrifuge 5418, Eppendorf, Hauppauge, NY) at 10,000 rpm for 20 minutes at room temperature. DNA pellets were dried by allowing the ethanol to volatize from the tubes at room temperature, and then dissolved in 20 µl of nuclease free water (Ambion, Thermo Fisher Scientific, Wilmington, DE). Ligated DNA products were transformed into One Shot® TOP 10 Chemically Competent *E. coli* (Life Technologies™, Thermo Fisher Scientific, Wilmington, DE) according to manufacturer's instructions (available at https://tools.thermofisher.com/content/sfs/manuals/topota_man.pdf).

Plates containing Luria Bertani Agar with kanamycin (100 ug/ul) were prepared. X-gal (Thermo Fisher Scientific, Wilmington, DE) at a concentration of 20ug/ml was spread on the plates with a spreader and left to dry in the incubator. Cloned PCR products were then spread on the plate and incubated overnight. Untransformed cells did not grow on the plates containing kanamycin as the vector containing the resistance conferring gene was absent in these cells. Cloned colonies were selected using blue-white screening. In this method of screening, bacterial cells successfully transformed with the plasmid vector containing the insert (PCR product) cannot

degrade the X-gal using the beta galactosidase enzyme and form white colonies. This occurs because the beta galactosidase enzyme function is disrupted due to the presence of the insert (PCR product). However, cells that do not contain the insert, contain a functional beta galactosidase enzyme, degrade X-gal and consequently form blue colonies. Colony PCR was performed with 20 white colonies of each PCR product in a 96-well plate with a reaction setup in accordance to the One*Taq*® 2X Master Mix with Standard Buffer (New England Biolabs® Inc.). Twenty white colonies were picked using a sterile pipette tip and dipped in the reaction mixture. PCR was done according to manufacturer's protocols using M13 primers. Cells were lysed at 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 45°C for 30 seconds and 68°C for 30 seconds. A final extension of 5 minutes at 68°C was added. After confirming the presence of the PCR product on an agarose gel (1%), the products were sequenced in the forward orientation by GeneWiz®. VecScreen on the NCBI website was used to remove the portion of the vector in the DNA sequence. The remaining portion of the sequence without the vector was compared to the NCBI nucleotide database using BLAST® to identify the species of origin. If the sequence matched with ≥ 99% to a sequence in the database it was considered as the same species.

3.9 DNA extraction from voucher sediments

DNA was extracted from the voucher sediments collected in 2011 from 25 sites in East and West Louisiana (From 3.2). The samples were thawed and DNA was extracted from six parts of each sediment sample using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Qiagen, Germantown, MA) following the manufacturer's protocol. DNA from different parts of the same sample was pooled and quantified using Nanodrop. Sediment DNA was sent to MR DNA (www.mrdnalab.com, Molecular Research LP, Shallowater, TX) for next-generation sequencing.

3.10 DNA amplification and sequencing

DNA amplification and sequencing was done at MR.DNA, Shallowater, Texas with a procedure developed for diversity high-throughput amplicon sequencing (Dowd et al., 2008a, Dowd et al., 2008b). In the first step, amplification of DNA using PCR with the primers NF1 and 18Sr2b was done for individual samples. During this PCR, the forward primer was barcoded for individual samples. PCR was performed using HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, a final elongation step at 72°C for 5 minutes. PCR products were checked on a 2% agarose gel to ensure amplification and relative intensity of bands

In the second step, all barcoded samples were pooled together in equal proportions based on their molecular weight and DNA concentrations followed by purification using calibrated Ampure XP beads. In the third step, pooled and purified PCR products were used to prepare a DNA library by following Illumina TruSeq DNA library protocol (Illumina, San Diego, CA).

In the fourth step, sequencing was performed using the Illumina MiSeq (2x300) paired end sequencing platform following the manufacturer's guidelines.The fifth step, is a quality filtering step in which sequenced amplicons in the files r1 and r2 are trimmed using the phred quality score of 25 removing ambiguous bases.

Next, the trimmed ends are joined and reoriented in the 5' to 3' direction. The resulting fasta and qual files after joining and reorienting are the text versions of the sequence data and quality scores, respectively. Fasta and qual files still contain primers and barcodes. A mapping file also was provided by MR.DNA which includes sample names, barcode information, primer information and project name. This mapping file was updated with all metadata collected for

sediments such as sediment biochemistries, Polycyclic Aromatic Hydrocarbons (PAH's), toxicity, location, geography and presence/ absence of larvae.

3.11 Bioinformatic and statistical analyses

After the steps carried out at MR.DNA mentioned above, further data analysis was carried out using QIIME (v.1.9.1) (Caporaso et al., 2010) accessible on a server provided by Hubbard Center for Genome Studies, University of New Hampshire.

Fasta and qual files containing reads that were joined and reoriented were converted into a Fastq file using the Python script *convert_fastaqual_fastq.py* in QIIME. The script *extract_barcodes.py* created two files namely barcodes. fastq (sequences with barcodes) and reads. fastq (sequences without barcodes). Using the output files of *extract_barcodes.py* and the mapping file, sequences were demultiplexed (divided into separate samples based on barcodes) with *split_libraries_fastq.py* and a minimum Phred quality score of 33 to remove ambiguous bases. Operational Taxonomic Units (OTUs) were clustered using the *pick_open_reference_otus.py.* The reference database for OTU picking was SILVA 119 (accessed on 1/23/2017) (Pruesse et al., 2007). Taxonomic classification was assigned at 99% clustering for OTU picking.

After taxonomic classification, an OTU table was created using the *make_otu_table.py.* Chimeras were identified using *identify_chimeric_sequences.py* and filtered from the OTU table using the *filter_otus_from_otu_table.py*. Pynast was used to align sequences to SILVA 119 using the *align_seqs.py*. The pairwise alignment method selected was UCLUST and the minimum percent identity to the closest blast hit to be included in the alignment was 70%. The gaps in the alignment were removed using the *filter_alignment.py*.

The 18S rRNA gene is less variable compared to other marker regions and, thus, higher clustering cutoff values are commonly used in sequencing studies using this gene (Bik et al.,

2016, Hadziavdic et al., 2014, Caron et al., 2009). After using *filter_alignment.py* to remove chimeras and alignment failures, a 0.05% abundance cutoff was applied to each sample separately to remove all OTUs with ≤ 0.05% abundance in each single sample. The 0.05% cutoff was applied to make data more meaningful and downstream analysis more reliable. In addition, quality filtering to remove OTUs with an abundance of a less stringent filter than we applied, ≤ 0.005% improves diversity estimates by minimizing spurious additional OTUs (Bokulich et al., 2013). Each sample was filtered from the OTU table using the script *filter_samples_from_otu_table.py* and the cutoff was applied using the script *filter_otus_from_otu_table.py specifying* the --min_count_fraction 0.0005 (converting percent 0.05% to fraction). After applying the abundance cutoff, computation of OTU tables and all downstream analysis was done separately for larval gut samples and sediment samples because they are two different sample types. The OTU table of sediment samples was generated by combining all the individual sediment OTU tables obtained after applying the 0.05% cutoff using the script *merge_otu_tables.py*. All larval gut samples were combined in a similar fashion. Final number of sequences in each sample was obtained from OTU tables computed using the script *make.otu.table.py* for larval guts and sediments separately.

Estimate S was used for rarefaction analyses on OTU tables after cutoff for overall sediments and larval gut samples using the default settings of EstimateS with 100 randomizations without replacement. Mao Tao estimator was used to compute observed OTU richness (number of OTUs) in each larval gut content and sediment sample. Sampling coverage in terms of capturing the majority of OTUs in sediments and guts was assessed by computing rarefaction curves of the number of observed OTUs captured at different sample sizes and extrapolation.

Shannon diversity index, which measures OTU diversity in the sample by taking into account both abundance and richness, was calculated. Shannon H and Shannon exponential were both computed using the above settings in Estimate S. Shannon H is not linear and hence Shannon exponential is used to compare between communities. Shannon exponential (true diversity) is linear implying that when the value for Shannon exponential is double, the diversity is actually double (Magurran, 2013).

A second diversity index, Simpson Inverse D (Magurran, 2013), which differs from the Shannon index in that it is largely dominance based, was obtained in Estimate S using the above settings. For easier interpretation, Simpson Inverse D was converted to Simpson D in Microsoft Excel 2010. Simpson D measures the probability that two OTUs randomly selected from the sample will be the same.

After the cutoff to filter out extremely rare OTUs ($\leq 0.05\%$) was performed OTU diversity was assessed in QIIME using the *core_diversity_analyses.py* script. Prior to using this script, a phylogenetic tree of the sequences of the OTUs with > 0.05% abundance was constructed using the *make_phylogeny.py* script.

The relative abundances at different taxonomic levels defined by the SILVA 119 taxonomy file (L2 –"Phyla" to L6 "Genera") were computed for OTUs (> 0.05% in each gut and sediment sample and taxonomic bar charts based on percent relative abundances were generated.

Manual BLAST (Johnson et al., 2008) against the NCBI database was done for the 52 insect OTUs identified to the species level by the SILVA 119 database. OTUs belonging to insect species were picked from the OTU table imported into Excel and OTU sequences were retrieved from the fasta file containing sequences after quality filtering.

Each of the 23 OTU sequences were manually blasted to the nucleotide database in NCBI and the top five blast hits were recorded.

Alpha diversity between groups of samples based on the two sample t-test was determined. The *compare_alpha_diversity.py* script in QIIME (v1.9) was used to make box plots based on the alpha diversity of observed OTUs. This script uses the non-parametric Monte Carlo method with 999 permutations. The mapping file mentioned in section 3.11 was used to group samples based on categories such as location, geography, presence/absence of oil, presence/absence of larvae and larval diets based on location.

Variation in OTU composition in larval guts across locations was determined in R studio version 3.4.1 using the package Vegan. Vegan provides tools for community ecology including diversity analysis, community ordination and dissimilarity analysis (Dixon, 2003). Data was transformed by $X^{0.19}$ to avoid analytic bias caused by highly abundant taxa. The distance matrix used for computation was the Bray-Curtis-Distance, which takes into account the abundances of OTUs (Beals, 1984). A Non-metric multidimensional scaling plot (NMDS) was created based on the Bray-Curtis-Distance matrix. A NMDS plot is a visualization method, which represents original position of communities (consisting of OTUs in our study) in a multidimensional space (CLARKE, 1993). An ordination spider was constructed, which connected all samples to their group means with non-dotted lines using the function *ordispider* (Oksanen et al., 2007). Ordination hulls to denote different groups being compared in the NMDS plot by dotted lines were plotted using the function *ordihull* (Oksanen et al., 2007). Ordination hulls can be used to compare groups and non-overlapping ordination hulls indicate significant difference among groups (Ramette, 2007).

Permutational multivariate analysis of variance (PERMANOVA) is a method for non-parametric multivariate analysis of variance (Anderson, 2001). In this method, data is randomly shuffled (permutations) and an F-ratio is determined for each shuffle. F-ratios of randomized data are then compared to F-ratios of original data. The p value is the fraction of F-ratio that is higher than the F-ratio of the original data. Adonis is a function in the package Vegan which analyses and partitions sums of squares using semimetric and metric distance matrices (Oksanen et al., 2007). PERMANOVA with the function Adonis in Vegan was used to determine if the differences in the OTU composition in the groups compared in the NMDS plot are statistically significant.

OTU abundances of taxa at multiple taxonomic levels as determined by the SILVA 119 database were converted into their relative abundances using the script *summarize_taxa.py.* Samples were grouped based on the presence/absence of larvae, geography, location, lightly oiled and unoiled sites of Grand Bayou and larval diets. A linear discriminant analysis effect size (LEfSe) was carried out to determine which taxa had significantly higher relative abundance in one group of samples when compared to the other. The non-parametric factorial Kruskal-Wallis (KW) sum-rank test was used to determine significant differential abundance between the specified groups of samples. After uploading the files on the Galaxy website of the Huttenhower Lab available at this link (https://huttenhower.sph.harvard.edu/galaxy/) default parameters in the LEfSe modules was used to conduct analysis and plot results.

Comparison of taxa between sediments where larvae were present and absent was done by comparing the percent read abundance between these two groups of sediments. Percent read abundance of larval gut samples also was computed to determine if there was a pattern connecting the larval guts and the corresponding sediment they were collected from. Horizontal bar charts of percent read abundance of taxa at taxonomic levels defined by SILVA as “Phylum”,

"Class", "Order", "Family" and "Genera" based on a combined OTU table of all larval guts and sediment samples were visualized in PHINCH using the Taxonomy Bar Chart option. Bar charts of individual sediment and larval guts were assembled and labelled in Microsoft Excel 2010 for easier visualization and interpretation.

Variation in OTU composition of sediments where larvae were present versus sediments where larvae were absent and variation in sediment OTUs across locations also were determined by the NMDS plot and PERMANOVA using the functions in Vegan as described above. However the transformation of data was by $X^{0.20}$ to reduce the effect of highly abundant taxa.

3.12 Measuring Sediment PAH levels

Total Polycyclic Aromatic Hydrocarbon (PAH) content in sediments in East Louisiana where oil made landfall was measured using Gas Chromatography-Mass Spectrometry (GC-MS) using the SIM acquisition mode. This method targeted 28 alkanes and 18 parent aromatic hydrocarbons based on EPA SW-846 methods (2000).

PAH measurements were conducted at the Department of Environmental Sciences by Scott Miles. Detailed description of the extraction and analysis of PAHs can be found in a previous publication of Scott Miles (Turner et al., 2014).

Relationship of OTUs in the oiled and unoiled sediments across all locations and relationship of OTUs in the oiled and unoiled sediments in Grand Bayou were computed using a NMDS plot and PERMANOVA with functions in Vegan as described above.

Variation in OTU composition between sediments from East and West Louisiana were computed using NMDS and Principal Co-ordinate Analysis (PCoA) plots. The NMDS plot was computed

as described above and PCoA plot was computed in QIIME (v1.9) using the *beta_diversity_through_plots.py* script and visualized in Emperor.

3.13 Measuring Sediment chemistry Parameters

Sediment chemistry parameters were measured at the Soil Testing and Plant Analysis Laboratory at the LSU AgCenter, Baton Rouge. The parameters measured in the sediment were Calcium, Chloride, Conductivity, Magnesium, Salts, SAR (Sediment accumulation rate), Sodium, Sulfur, Copper, pH, Phosphorus, Potassium, Zinc, Iron, Manganese, Organic content, Boron, Aluminum and Nitrogen using methods routinely used for measuring these parameters in Louisiana sediments. A detailed description of methods is available at http://www.lsuagcenter.com/portals/our_offices/departments/spess/servicelabs/soil_testing_lab.

Effect of sediment chemistry on OTU composition was determined using the script *compare_distance_matrices.py*. First, a distance matrix of the OTU composition based on UniFrac distances was computed using the *core_diversity_analysis.py* script. Next, metadata for all sediments in the mapping file (mentioned in 3.11) were updated with the results from the biochemistry analysis. Then, a Euclidean distance matrix was created for each of the 19 parameters measured above. The distance matrix of each biochemical parameter was compared to the distance matrix of the OTUs. This script determines if the similarity of a biochemical parameter among sediments correlates with similarity of OTU composition using the Mantel correlation. The sediment chemistry parameter pH had an effect on the OTUs and to determine which OTUs correlate to pH the *observation_metadata_correlation.py* script was used. This script determined the correlation of the OTUs from OTU table to the pH values in the mapping file. The Pearson's score was used to determine correlation and the Bootstrapping method was used to determine p value.

3.14 Sediment Toxicity assay using the model organism *Hyallela azteca*

A 10 day sediment toxicity assay was conducted using modified American Society for Testing and Materials (ASTM) procedures with amphipods. Sediments were collected in 2011 from East and West Louisiana and stored at minus 20 degrees Celsius for 6 years as described in section 1.1. Clean and dry lipless glass beakers of 100 ml (Cat.no. 13920-012, VWR, Houston, TX) were used as test chambers for the assay (Burton Jr et al., 2001). Test chambers were rinsed with tap water, followed by a careful rinse with diluted Hydrochloric Acid to remove metals and bases. Next, chambers were rinsed with deionized water followed by an acetone wash to remove organic compounds and, finally, rinsed again with deionized water. All the test chambers were then autoclaved to eliminate any biological contamination.

Sediments were thawed overnight. The following day, 10 grams of sediment was transferred to individual test chambers; there were three replicates for each of the vouchers. Reconstituted water was prepared as recommended in the ASTM procedure (Smith et al., 1997), i.e. 5 grams of CaSO4, 5 grams CaCl2, 3 g of MgSO4, 9.6 g NaHCO3 and 0.4 g KCl was dissolved in 100L of deionized water and aerated (Smith et al., 1997).

Reconstituted water was added to each test chamber until the 60 ml mark was reached by gently pouring the water down the sides of the beaker to ensure minimal disturbance of the sediment. Amphipods (*Hyallela azteca*) (obtained from Sachs Systems Aquaculture Inc., St. Augustine, FL) were transferred to a 10 gallon aquarium containing reconstituted water and kept overnight in the aquarium to acclimatize to the reconstituted water. Amphipods were collected in a sieve from the aquarium and 5 amphipods were introduced gently into each test chamber below the air-water interface using blunt forceps. Dissolved oxygen and temperature were measured using Pro DSS Multiparameter Water Quality Meter (YSI Gulf Coast, Baton Rouge, LA) in test

chambers and their levels were maintained under recommended conditions. The recommended conditions were a minimum of 2.5 mg/L of dissolved oxygen and a temperature range between 22 to 24℃ throughout the experimental period (Plumb Jr, 1981). Yeast Cerophyll Trout chow medium (1 ml) was fed to the amphipods in the test chambers every day for 10 days. This medium was prepared in the lab from Trout Chow Pellets (Kruse Feed Supply, La Habra, CA), yeast (Fleischmann's) and Aldon™ Innovating Science™ Cereal Grass Medium (Fischer Scientific, Hampton, NH) using the procedure described in the ASTM 2010 manual (Adams and Rowland, 2003). In short, 5 grams Trout Chow Pellets were blended, suspended in 1L of deionized water and digested for a week separately without the other components of the YCT medium. At the end of the week, 5 grams of yeast and 5 grams of Cereal Grass medium were dissolved each in 1L of water. The digested trout chow, yeast and Cereal Grass medium were mixed and the undissolved components were filtered. The medium was divided into aliquots and stored at -20℃.

Two volumes of water were added per day to the test chambers. Each day three test chambers/replicates per sediment were examined and the number of live/dead amphipods was recorded. The chambers were numbered from 1 to 30 for each sediment sample and selection of chambers was predetermined. The sediment in each test chamber was emptied onto a fine sieve and the sediment was washed off with water to expose the amphipods that were retained on the sieve. Live amphipods were counted as dead based on unresponsiveness of the amphipods to probing with the forceps. Data were recorded and percent survival was calculated at the end of the experiment.

Two different concentrations of Water Accommodated Fraction (WAF) of Mississippi Canyon 252 Weathered Crude Oil (Louisiana Light Sweet Crude) were obtained by weighting eight and

13 grams of crude oil and adding 100 ml of reconstituted water. The mixture of crude oil and water was kept overnight on a Thermo Forma orbital shaker (Thermo Fisher Scientific, Wilmington, DE) at 200 rpm for maximum emulsification of oil in water.

Negative control 1 for the experiment was a test chamber containing 10 grams of Quickrete play sand (available at Home Depot) with 60 ml of reconstituted water. Negative control 2 contained 60 ml of reconstituted water without sand. For Positive Control 1 (with 10 grams of Quickrete play sand) and Positive Control 2 (without sand), 100 ml of WAF obtained from eight grams of oil was added. For Positive Control 3 (without sand), 100 ml of WAF from 13 grams of crude oil used (Saco-Álvarez et al., 2008).

Shapiro Wilks test was used to determine normality and Levene's test was used to calculate variance. Kruskal Wallis test and post hoc Kruskal-Nemenyi were used to calculate the effect of days on toxicity. Welch's t-test assumes non-normal data with unequal variances and this test was used to compare each sediment to the negative control to determine toxicity.

Chapter 4. Results

4.1 Molecular identification of tabanid larvae

All larvae were morphologically identified to the genus Tabanus. The larval barcoding BLAST results against DNA from an adult specimen morphologically identified as *T. nigrovittatus* showed varying similarities (Table 4.1). Larvae RWR5.1.LG, RWR5.2.LG, RWR5.7.LG, RWR5.8.LG, RWR5.10.LG, RWR5.12.LG, RWR5.13.LG, RWR5.16.LG, RWR5.18.LG, RWR2.3.LG, RWR2.4.LG, RWR2.5.LG, RWR2.6.LG and CP5.1.LG with an identity in all three regions of ≥ 97% to the reference sample likely are the same species. Larvae with lower matches to the *T. nigrovittatus* reference at the most variable locus (COI) (92% to 95%) nevertheless likely to belong to the *T. nigrovittatus* species complex, such as GB5.1.LG, RWR5.9.LG, CP2.1.LG and CP2.2.LG. The only larva showing similarity values less than 95% to the T. nigrovittatus reference was CP5.2.LG, and might, thus, represent a different species, most likely *T. hinellus*. The mitochondrial cytochrome oxidase subunit 1- 5' sequences of the adult reference specimen of *T. nigrovittatus* from Louisiana marshes identified by morphological measurements (Sofield et al., 1984) differed by 5% from a previously submitted *T. nigrovittatus* from Florida.

4.2 Tabanid DNA and preliminary data on food web components in larva gut contents

A pilot study using cloning, sequencing and blasting of 18S-3'end region with the primer pair NF1 and 18Sr2b (details in Materials and methods Table 3.2) amplicons from larval gut contents against the NCBI database determined the proportions of DNA from tabanids and other taxa in the 19 larval guts to aid in selection of larvae for next generation sequencing.

Table 4.1 Larval species ID: Percent identity match of morphologically identified tabanid larvae to DNA from an adult specimen of *Tabanus nigrovittatus* at three regions of the genome, Mitochondrial CO1 (GenBank Accession no KT381971), 18S-5' (GenBank Accession no KT222915) and 18S-3' (GenBank Accession no KU321600). Abbreviations of samples and locations from where larvae were collected can be found in the Figure 3.1.

Tabanid larvae	% Identity match to mtCO1 region of *T. nigrovittatus*	% Identity match to 18S-5' (~1000bp) region of *T. nigrovittatus*	% Identity match to 18S-3' (last 400bp) region of *T. nigrovittatus*
GB5.1.LG	94%	100%	100%
RWR5.1.LG	99%	99%	100%
RWR5.2.LG	98%	99%	100%
RWR5.7.LG	97%	99%	100%
RWR5.8.LG	99%	99%	100%
RWR5.9.LG	95%	99%	100%
RWR5.10.LG	99%	97%	100%
RWR5.12.LG	97%	98%	100%
RWR5.13.LG	97%	99%	100%
RWR5.16.LG	98%	99%	100%
RWR5.18.LG	99%	99%	99%
RWR2.3.LG	99%	99%	100%
RWR2.4.LG	99%	99%	99%
RWR2.5.LG	98%	99%	100%
RWR2.6.LG	99%	99%	100%
CP2.1.LG	92%	98%	98%
CP2.2.LG	92%	95%	98%
CP5.1.LG	99%	99%	100%
CP5.2.LG	92%	70%	91%

About half the larvae showed presence of *T. nigrovittatus* DNA in varying proportions. Three larvae (RWR2.6.LG, CP2.1.LG and CP2.2.LG) had exclusively *T. nigrovittatus* DNA in their

guts, with all 20 clones showing an identity match from 98% to 100% to this species. Since no other food web component was found, these larvae were not used for deep sequencing. Larva RWR5.2.LG showed a 95% (19 of the 20 clones) dominance of *T. nigrovittatus* DNA. More than half but less than 95% of the clones of RWR5.1.LG, CP5.1.LG, RWR5.13.LG and RWR5.18.LG match *T. nigrovittatus*. RWR.8.LG, RWR5.9.LG, RWR5.12.LG, RWR2.3.LG show low proportions of *T. nigrovittatus* DNA in the range of 5% to 35%. Larvae GB5.1.LG, RWR5.10.LG, RWR2.4.LG, RWR2.5, LG, RWR2.16.LG, CP5.1.LG, CP5.2.LG, CP5. 2. LG had no clones matching *T. nigrovittatus*.

Cloning results indicate that the gut contents of the larva in the East (GB5.1.LG) demonstrated a presence of *Aedes sp.* (11/20 clones with 98%-100% identity) while 12 of the larvae in the West showed a prominence of fungi. *Malassezia* isolates were the most common followed by various uncultured fungus clones. Stramenopiles were present in 4 larvae (RWR5.8.LG, RWR2.3.LG, RWR2.4.LG, and RWR2.5.LG) whereas a protozoan *Hymenostomatia sp*. and a single celled alga *Ostreococcus lucimarinus* were present in RWR5.8.LG and CP5.1.LG, respectively. Insects from the order Hemiptera (matching closely to *Pentatoma japonica, Laodelphax striatellus,* and *Paracopta maculata*) were present in multiple larvae (CP5.2.LG, CP5.1.LG, RWR2.3.LG, RWR2.4.LG, RWR5.7.LG, RWR5.9.LG, RWR5.12.LG, RWR5.13.LG and RWR5.16.LG). The gut contents of one larva (CP5.2.LG) found at Cypremort Point showed one clone matching a parasitic mermithid nematode (*Hexamermis albicans*).

The results from this pilot study led to the selection of 16 larval guts containing DNA from other species besides *T. nigrovittatus* for next-generation sequencing.

4.3 General assessment of next generation sequence data

Location and collection details of all 43 sediment and gut content samples used in this study have been deposited in the NCBI database as individual BioSamples and can be accessed under the BioProject PRJNA408157. These also can be accessed on the GRIIDC website using (https://data.gulfresearchinitiative.org/dataset-submission?regid=R5.x277.000:0006).

Raw Illumina Miseq sequence data for all BioSamples has been deposited in the Sequence Read Archive (SRA) under BioProject PRJNA408157 and has been released with SRA study number SRP118538 titled "18S metabarcoding of greenhead horse fly food web: Larval gut contents and sediments**."** A total of 5,605,043 reads after joining forward and reverse sequences were obtained using QIIME (v.1.9).

Quality filtering removed 6,858 reads as they were too short after quality truncation. The number of resulting demultipexed reads was 5,598,185 with a median sequence length of 367 base pairs. Taxonomy assignment using SILVA 119 based on clustering of sequences with 99% similarity generated 36,907 Operational Taxonomic Units (OTUs.)

Pairwise alignment method with PyNAST in UCLUST was performed for all 36,907 OTU sequences where each OTU sequence was blasted against the SILVA 119 database to provide a higher taxonomic resolution than without aligning clustered sequences. A total number of 8,699 chimeric OTUs were filtered from the OTU table. After filtering, 28,208 OTUs comprising of 5,303,923 sequences with no chimeras and alignment failures were obtained. After removing OTUs with an abundance of ≤0.05% from each sample separately, 2,279,638 sequences remained in the sediments comprising 1,415 OTUs and 2,258,540 sequences remained in the larval guts comprising 225 OTUs.

4.4 Assessment of sample sizes and OTU capture

Rarefaction curves for OTU richness depending on the number of samples after applying the 0.05% cutoff in sediments and larval guts indicated that an increase in sample size would have allowed the capture of additional OTUs in larval guts and sediments (Figure 4.1).

Doubling of the 16 samples of the guts would increase the number of OTUs by 68 thereby increasing overall OTU richness by approximately 30% in the guts (Figure 4.1A).

After the extrapolation the rarefaction curves started to level off, indicating that doubling the sample size would have captured the majority of larval gut diversity. Similarly, doubling of sediment samples by an additional 25 samples would increase OTU richness by 601 which represent approximately 42% of the 1,412 OTUs found in the 25 sediment samples (Figure 4.1B).

Even after extrapolation the curves did not reach an asymptote. This indicates that the vast diversity of organisms in the marsh soil was not entirely captured by the sample size used in this study.

However, the primary objective of this study was to describe the food web of tabanid larvae based on comparison of organisms found in the larval gut to those found in surrounding sediment and did not require a description of the entire eukaryotic diversity in sediments. Nevertheless, future diversity studies should make an effort to increase sample size and take spatio-temporal variation into account.

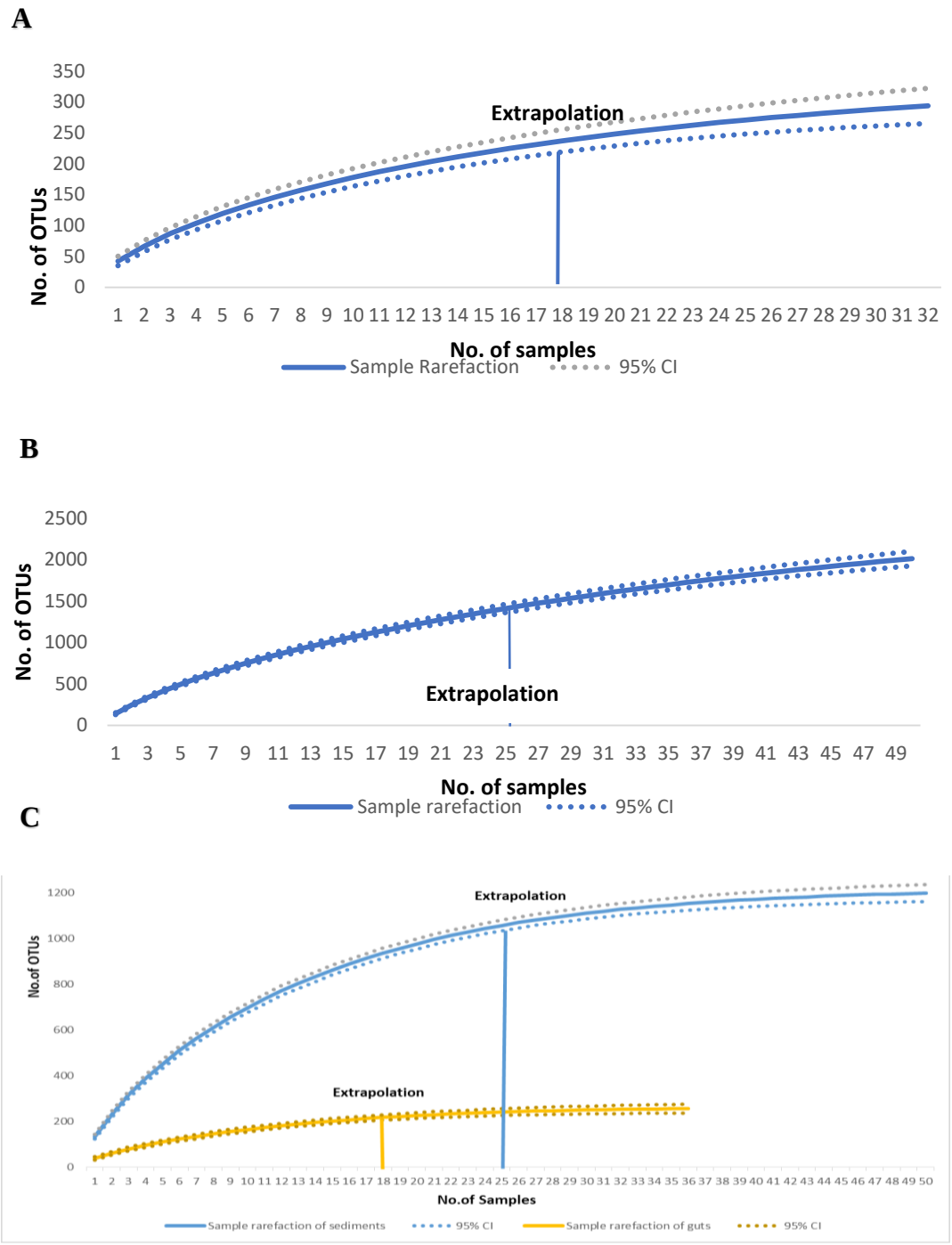

Figure 4.1.Rarefaction curves of OTUs depending on the number of samples: A. Rarefaction curve of OTUs in larval guts. B. Rarefaction curve of OTUs in sediments C. Rarefaction curve of OTUs in sediments and larval guts.

4.5 OTU richness

The 95% confidence intervals (CI) of the sediments and guts did not overlap indicating that OTU richness was significantly greater in sediments than the larval guts by approximately 6.3 times (Figure 4.1C). Observed OTU richness, i.e., number of different OTUs, was 225 (95% CI, between 207.82 and 242.18) across all 16 larval gut samples and 1,412 (95% CI, between 1,360.96 and 1,463.04) across all 25 sediment samples.

Table 4.2 shows the breakdown for individual samples. The OTU richness in individual sediment samples ranged from 228 at Grand Bayou 5 (GB5) to only 49 at Grand Bayou 6 (GB6), 49 at Cypremort Point 2 (CP2) and 49 at Rockefeller Wildlife Refuge (RWR5). In the larval guts the OTU richness ranged from 26 in GB5.1.LG to 55 in RWR5.16.LG. On the other hand, the number of sequences was highest in the larval gut GB5.1.LG (199,384) and over a hundred thousand in other guts indicating that even though OTU richness in the guts was low, the OTUs were represented by a large number of sequences (Table 4.2).

Table 4.2 Number of sequences and OTU richness in individual sediment and larval gut samples: The sample names are defined by their location. Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name .LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered. Abbreviations of samples and locations from where larvae were collected can be found in the map (see Figure 3.1).

		General information	
Location	Sample name	No. of sequences	OTU richness
Rockefeller Wildlife Refuge	RWR1	106,019	157
	RWR2	90,278	212
	RWR2.3.LG	111,374	49
	RWR2.4.LG	91,505	51
	RWR2.5.LG	87,686	61
	RWR3	117,709	121
	RWR4	102,966	173

(Table 4.2 continued)

Location	General information		
	Sample name	No. of sequences	OTU richness
Cypremort Point	RWR5	175,813	49
	RWR5.1.LG	158,576	28
	RWR5.2.LG	120,416	38
	RWR5.7.LG	119,565	50
	RWR5.8.LG	144,720	28
	RWR5.9.LG	149,218	51
	RWR5.10.LG	154,892	52
	RWR5.12.LG	174,985	37
	RWR5.13.LG	166,508	27
	RWR5.16.LG	133,952	55
	RWR5.18.LG	153,707	37
	CP2	165,088	49
	CP3	77,019	156
	CP4	94,938	132
	CP5	155,130	54
	CP5.1.LG	155,532	55
	CP5.2.LG	136,520	35
	CP6	85,231	123
	CP7	75,910	113
Grand Isle	GIEP	57,595	220
	GICL	71,279	133
	EIOC	62,694	194
	EIAC	63,991	177
	EIC	74,808	149
	EI6	77,183	156
Grand Bayou	GB1	67,858	193
	GB2	66,701	150
	GB3	88,574	86
	GB4	67,527	226
	GB5	61,097	228
	GB5.LG	199,384	26
	GB6	80,683	49
	GB7	87,236	118
	GB8	106,311	96

4.6 OTU diversity analysis in sediments and guts

Table 4.3 represents the OTU diversity in individual sediments and gut samples. Shannon diversity (Shannon H) ranged from 1.66 in GB6 to 4.5 in GB4 and the effective number of species (H exponential) was in the range of 5.24 in GB6 to 89.78 in GB4 in the sediments with a mean of 4.35 (Shannon H) and 77. 27 (H exponential). In the larval guts, Shannon H was in the range of 0.33 in RWR5.1.LG to 1.61 in RWR5.7.LG and the effective number of species (H exponential) was in the range of 1.38 in RWR5.1.LG to 5.25 in RWR2.3.LG for guts with a mean of 2.45 (Shannon H) and 11.63 (H exponential). Simpson index (D) in the sediments ranged from 0.02 in GB4 to 0.3 in GB6 and in the guts from 0.29 in RWR5.7.LG to 0.9 in RWR5.1.LG suggesting that two randomly selected OTUs from the guts have a higher probability of being the same than two randomly selected OTUs from the sediments.

Table 4.3 Diversity indices: Shannon H, H Exponential and Simpson diversity indices of OTUs in individual sediment and larval gut samples: The sample names are defined by their location. Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name.LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered. Abbreviations of samples and locations from where larvae were collected can be found in the map (See Figure 3.1).

Location	Sample name	Shannon H	H Exponential	Simpson (D)
Rockefeller Wildlife Refuge	RWR1	2.97	19.48	0.15
	RWR2	3.82	45.47	0.07
	RWR2.3.LG	1.66	5.25	0.31
	RWR2.4.LG	1.18	3.27	0.56

(Table 4.3 continued)

Location	Sample name	Shannon H	H Exponential	Simpson (D)
	RWR2.5.LG	1.72	5.6	0.36
	RWR3	2.68	14.65	0.17
	RWR4	3.68	39.7	0.06
	RWR5	1.77	5.9	0.24
	RWR5.1.LG	0.33	1.38	0.9
	RWR5.2.LG	1.31	3.69	0.46
Rockefeller Wildlife Refuge	RWR5.7.LG	1.61	4.99	0.29
	RWR5.8.LG	1.36	3.89	0.33
	RWR5.9.LG	1.44	4.23	0.43
	RWR5.10.LG	1.48	4.39	0.35
	RWR5.12.LG	0.94	2.56	0.62
	RWR5.13.LG	0.96	2.61	0.52
	RWR5.16.LG	1.38	3.97	0.41
	RWR5.18.LG	1.43	4.16	0.4
Cypremort Point	CP2	1.84	6.32	0.24
	CP3	3.13	22.94	0.18
	CP4	3.39	29.61	0.07
	CP5	2.01	7.46	0.2
	CP5.1.LG	1.51	4.52	0.43

(Table 4.3 continued)

Location	Sample name	Shannon H	H Exponential	Simpson (D)
Cypremort Point	CP5.2.LG	0.55	1.73	0.83
	CP6	2.76	15.76	0.16
	CP7	2.87	17.58	0.12
Grand Isle	GIEP	4.08	59.11	0.05
	GICL	2.59	13.38	0.28
	EIOC	3.86	47.39	0.07
	EIAC	3.5	32.95	0.11
	EIC	3.28	26.48	0.15
	EI6	3.73	41.84	0.05
Grand Bayou	GB1	3.93	50.96	0.04
	GB5.1.LG	1	2.71	0.46
	GB2	3.07	21.56	0.14
	GB3	2.18	8.86	0.24
	GB4	4.5	89.78	0.02
	GB5	4.04	56.88	0.06
	GB6	1.66	5.24	0.3
	GB7	3.46	31.69	0.06
	GB8	2.56	12.88	0.15

4.7 Diet of the top invertebrate predator (tabanid larvae) in Louisiana marsh soil

Relative abundance of the larval gut OTUs after applying the 0.05% cutoff was computed in QIIME as described previously (Section 1.6 of Materials and Methods) generating bar charts at 5 taxonomic levels including “Phylum”, “Class”, “Order”, “Family”, “Genera”. The SILVA database that was used to assign OTUs in this study organizes organisms to taxonomical categories that are not always congruent with the classical biological taxonomy and, therefore, used with quotation marks.

For example, Insecta is considered as a Class in classical taxonomy but in the SILVA taxonomy it is considered as a “Genus”. Hence, the bar graphs (Figure.4.2, Figure 4.3, Figure 4.4, Figure 4.5, and Figure 4.6) below were computed based on the relative abundance of taxa at consecutive levels of classification in SILVA, i.e. Figure.4.2 was computed on the “Phyla” and Figure 4.6 on the “Genera” level of taxonomy.

Metagenomic sequencing using primers amplifying a portion of the 18S rRNA gene covered the eukaryotic diversity (Kingdom Eukaryota) in the larval guts. Super group Opisthokonta (ranging from 24.2% in RWR5.8.LG to 99.4% in GB5.1.LG) had the highest relative abundance in the larval guts (overall 91%, Figure 4.2).

Opisthokonta are a super group within the Kingdom Eukaryota comprising a broad group of eukaryotes including eukaryotic microorganisms, fungi and metazoa. Super group SAR (Stramenopiles-Alveolates-Rhizaria) was the second most abundant group with an overall abundance of 7.3% in the larval guts.

SAR is a metagroup within Kingdom Eukaryota covering all photosynthetic eukaryotes. SAR was found in higher abundance in four larvae at Rockefeller Wildlife Refuge site 2 [RWR2.3.LG

(13.8%), RWR2.4.LG (8.3%), RWR2.5.LG (17.4%)] and one larvae at site 5 [RWR5.8.LG (74.6%)] in comparison to the rest of the larvae (ranging from 0.2% to 0.4%; Figure 4.2).

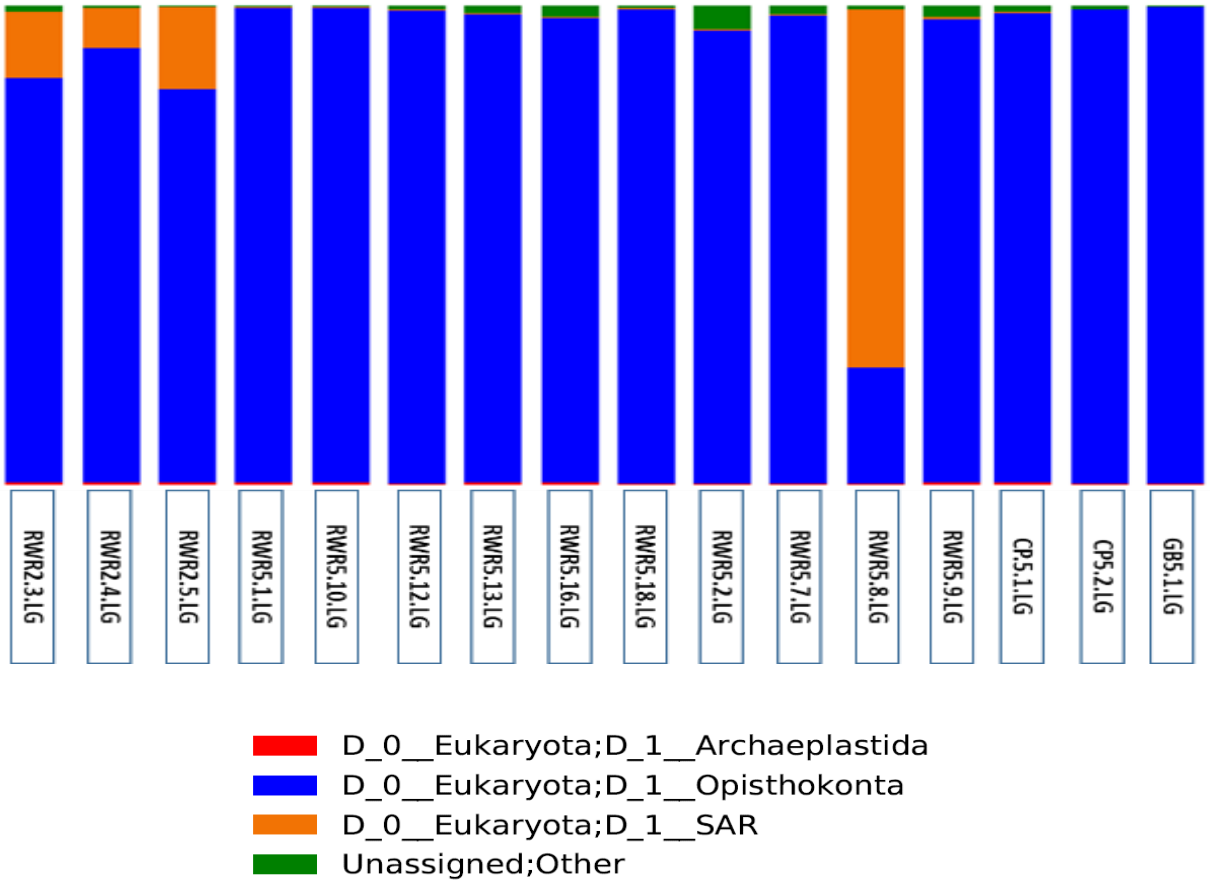

Figure 4.2 Relative abundance of OTUs at the "Phylum" level in the larval guts showing super group Opisthokonta as the major group and SAR as the minor group

The third most abundant phylum found in the larval guts was Archeoplastida (0.4%) comprising plants (Figure 4.2). A minor fraction of 1.2% of organisms could not be assigned to any taxa because of a lack of a match in the SILVA 119 database (Figure 4.2) Point and Grand Bayou exhibited a low presence of Alveolata in the range of 0.1% to 0.2%. However, there was variation of Alveolata among RWR5 larvae. A high proportion of Alveolata (49%) was found in a single sample (RWR5.9.LG) whereas all other samples, including those from the same location

(RWR5.1.LG, RWR5.2.LG, RWR5.7.LG, RWR5.8.LG, RWR5.10.LG, RWR5.12.LG, RWR5.13.LG, RWR5.16.LG, RWR5.18.LG) contained Alveolata in the range of 0.1% to 0.2%. Stramenopiles were found in all the three larvae from RWR2 (RWR2.3.LG; 13.7%, RWR2.4.LG; 8.2% and RWR2.5; 17.3%) and one larvae from RWR5 (RWR5.8; 25.5%) with proportions higher than the rest of the larvae (RWR5.1.LG, RWR5.2.LG, RWR5.7.LG, RWR5.9.LG, RWR5.10.LG, RWR5.12.LG, RWR5.13.LG, RWR5.16.LG, RWR5.18.LG, GB5.1.LG, CP5.1.LG and CP5.2.LG) which displayed a relative abundance of Stramenopiles in the range of 0.1% to 0.2% (Figure 4.3). At the "Class" level, Opisthokonta was split into two clades Holozoa (ranging from 3.8% in RWR2.5.LG to 98.5% in GB5.1.LG) and Nucletmycea (ranging from 0.9% in GB5.1.LG to 84.8% in RWR5.10.LG). Holozoa (total relative abundance = 54.6%) is a clade of Opisthokonta which includes metazoa (multicellular eukaryotes) but not fungi (Adl et al., 2012). Nucletmycea (total relative abundance = 37.3%) is another clade of Opisthokonta comprising primarily fungi but not metazoans (Adl et al., 2012). Holozoa had a relative abundance of over 90% in RWR5.1.LG, RWR5.12.LG, RWR5.13.LG, CP5.2.LG, and GB5.1.LG, encompassing multiple locations (Figure 4.3).

These six larval guts had less than 4% relative abundance of Nucletmycea (Figure 4.3). However, Nucletmycea had a relative abundance of over 70% in RWR2.4.LG, RWR2.5.LG, RWR5.10.LG, RWR5.9.LG and CP5.1.LG larvae (Figure 4.3). A high variation in Holozoa and Nucletmycea was seen in RWR5 larvae where Nucletmycea was the main component in RWR5.9.LG (76.2%), RWR5.10.LG (84.8%), and RWR5.16.LG (64.6%) whereas the relative abundance of Holozoa was maximum in RWR5.1.LG, RWR5.2.LG, RWR5.12.LG, RWR5.13.LG and RWR5.18.LG in the range of 79.3% to 97.9%. Larvae from Rockefeller Wildlife Refuge site 2 (RWR2) had a distinct pattern in comparison to larvae from other

locations namely RWR5, CP5 and GB5 with low among site variation with Nucletmycea being most abundant (55% to 78%), followed by Holozoa (3.8% to 28.8%) and lastly Stramenopiles (8.2% to 17.3%).

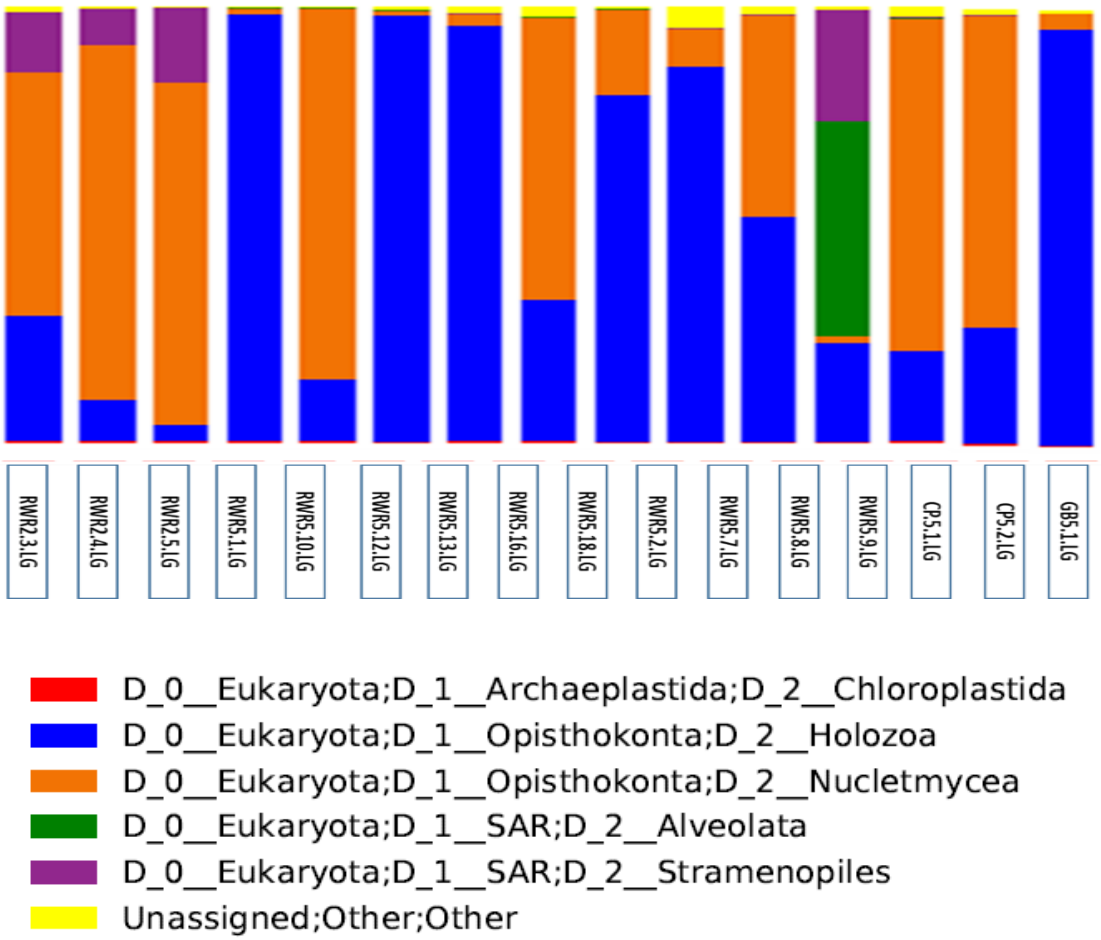

Figure 4.3 Relative abundance of OTUs at the "Class" level in the larval guts showing Holozoa and Nucletmycea as the major group; Alveolata and Stramenopiles as the minor group

A varied distribution of Holozoa and Nucletmycea was seen in Cypremort Point samples with Nucletmycea dominating CP5.1.LG (71.4%) while Holozoa dominated CP5.2.LG (95.3%). Besides Holozoa and Nucletmycea, representatives of the SAR super group namely Alveolata (2.8%) and Stramenopiles (3.7%) were next in line with respect to relative abundance.

Alveolates are a clade comprised of protists and Stramenopiles are comprised of diatoms and algae. Larvae from most sites of Rockefeller Wildlife Refuge, all sites of Cypremort

The distribution of the relative abundance of the “Order” was similar to the “Class”. However, the predominant Opisthokonta clades Holozoa and Nucletmycea were classified further into Metazoa and Fungi, respectively (Figure 4.4).

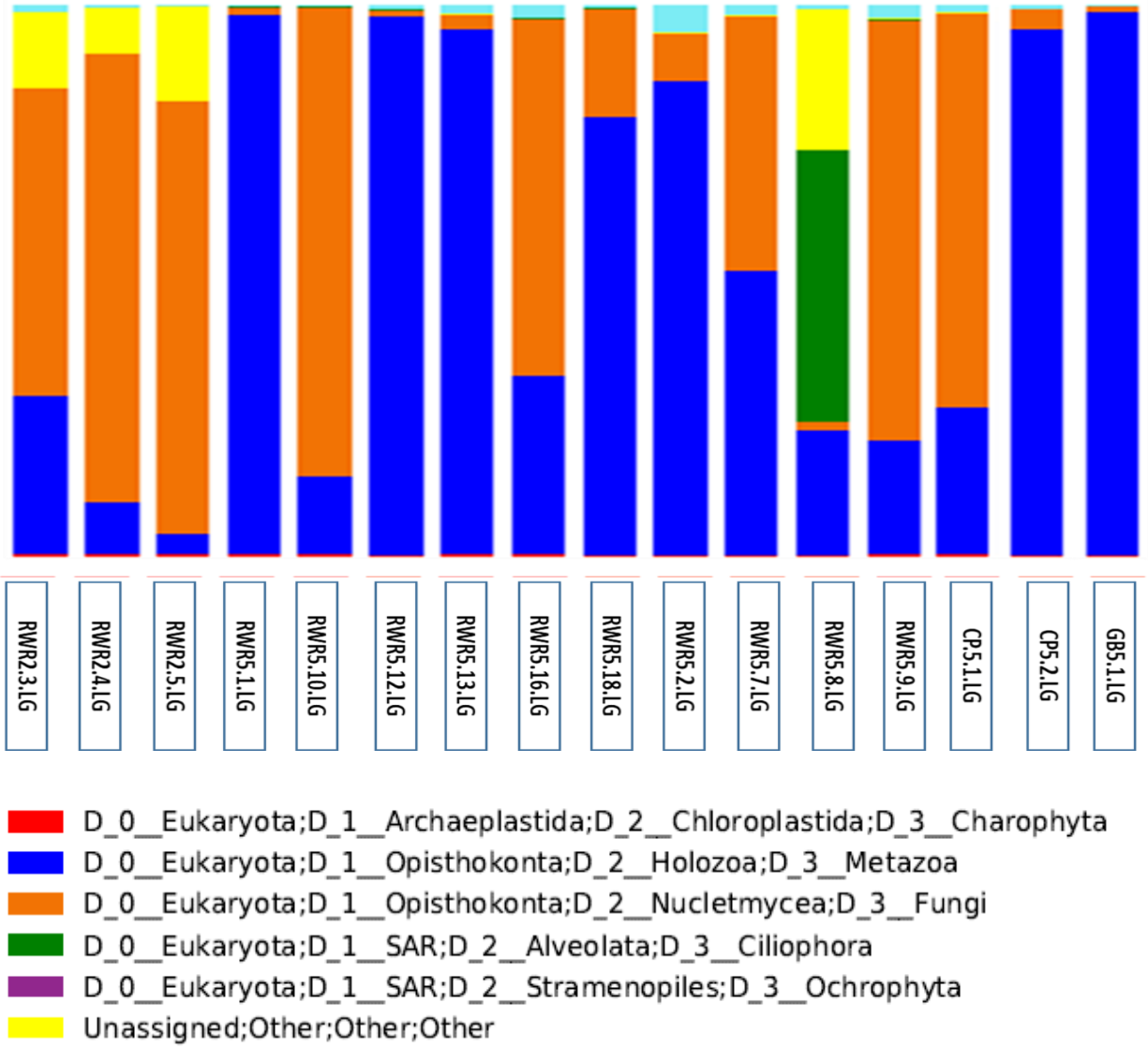

Figure 4.4 Relative abundance of OTUs at the “Order” level in the larval guts showing Metazoa and Fungi as the major groups; Ciliophora and Ochrophyta as the minor group

Relative abundance was calculated for the “Family” level in the SILVA database. The highest relative abundance across all samples was Hexapoda (36.2%) closely followed by unidentified fungi (32.8%). Other metazoa contributed to 17.2% of total relative abundance. Hexapods were present in all samples albeit in a high variation. The variation in Hexapoda ranged from 1.7% (RWR5.16.LG) to 97.3% (RWR5.1.LG) in larvae from Rockefeller Site 5, from 1.4% (RWR2.4.LG) to 17.8% in RWR2 (RWR2.3.LG) in larvae from Rockefeller Site 2, and from 1.3% (CP5.2.LG) to 21.6% (CP5.1.LG) in larvae from Cypremort Point 5. The sole larva from Grand Bayou 5 (GB5.1.LG) contained almost exclusively (98.1%) Hexapoda. Dothideomyceteceae (3%), Microstromataceae (0.3%), and Saccharomycetaceae (0.1%) were identified to be a minor part of the larval gut content together with unidentified fungi (Figure 4.5). Ciliophora (from SAR) present primarily in RWR5.9 was further classified as Hymenostomatia with a total relative abundance of 2.8% (Figure 4.5). The other member of the SAR super group was Chrysophyceae (3.6%) found primarily in RWR2.3, RWR2.4 and RWR2.5 (Figure 4.5).

Relative abundances were computed at the “Genus” level in the SILVA database. The major group of hexapods in the larval guts was the class Insecta (36.2%). Class Insecta was present in all larval guts but with high variation among samples ranging from 1.2% (CP5.2.LG) to 98.1% (GB5.1.LG). Relative abundance of insects above 60% was seen in RWR5.1.LG, RWR5.16.LG, RWR5.18.LG and GB5.1.LG (Figure 4.6). The next highly abundant taxa was the fungal genus Malassezia (29.1%) found in over 50% in seven larvae across locations and in low proportions (ranging from 0.9% to 19.6%) in 11 larval samples (Figure 4.6). Genus *Tetrahymena* (belonging to SAR) was present in higher abundance (49 %) in RWR5.9.LG in comparison to the rest of the larvae across locations where *Tetrahymena* was present in the range from 0.1% to 0.2%.

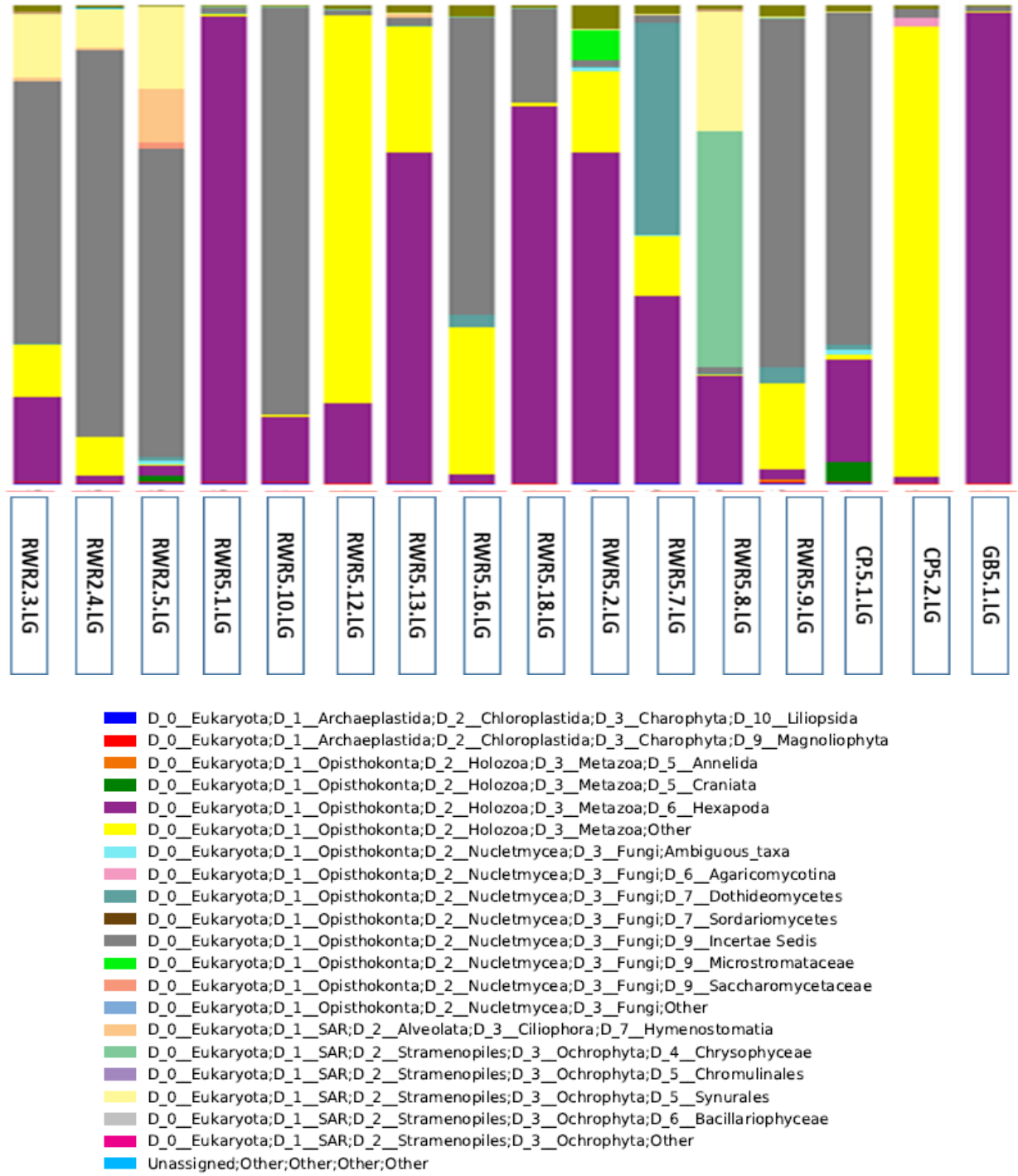

Figure 4.5 Relative abundance of OTUs at the “Family” level in the larval guts showing Hexapods and unidentified fungi as the major group; Hymenostomatia and Chysophyceae as the minor group

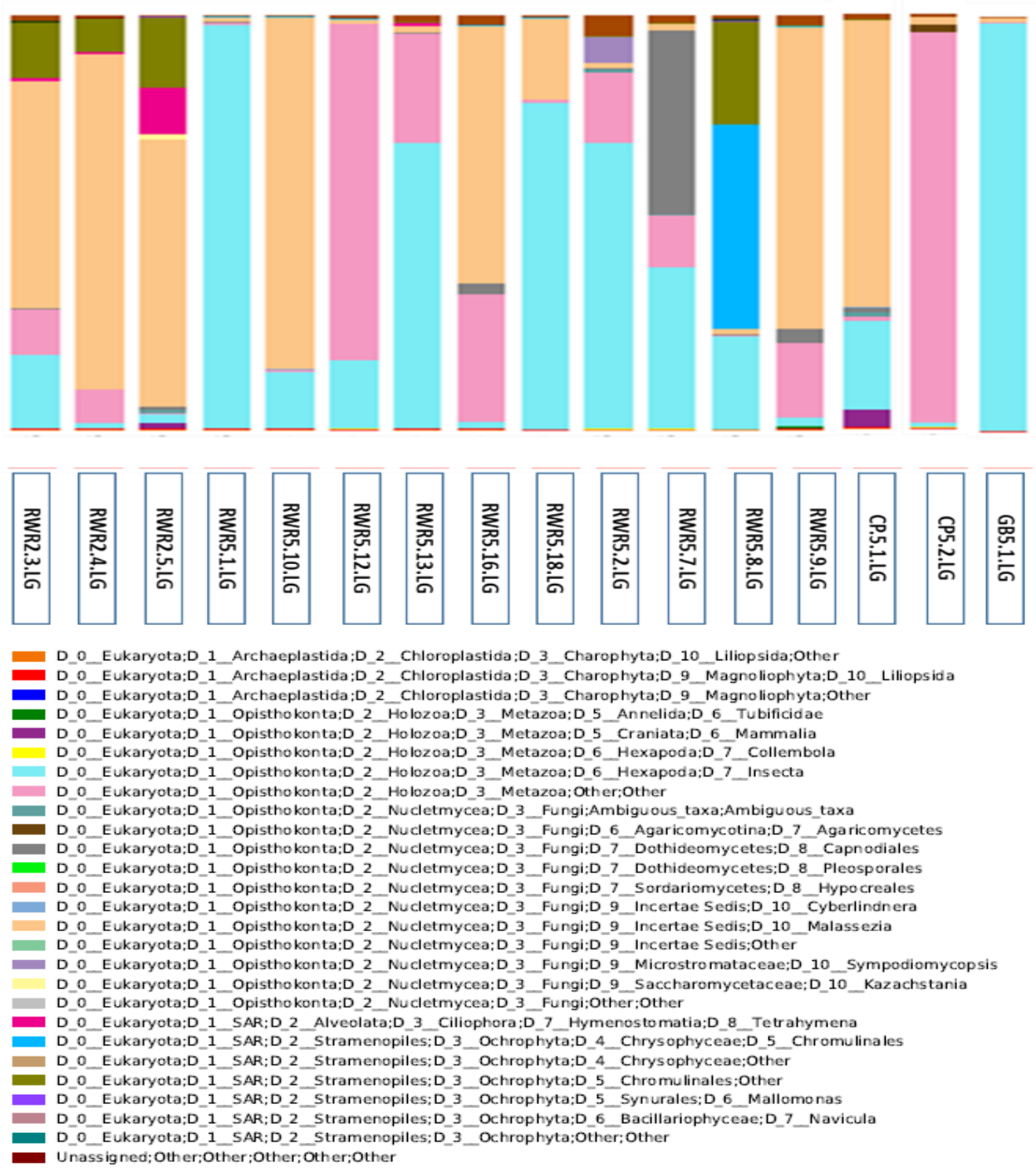

Figure 4.6 Relative abundance of OTUs at the “Genus” level in the larval guts showing Insecta and Malassezia as the major group; *Tetrahymena* and Chromulianes as the minor group

4.8 Identification of insect species in the tabanid larval gut

Insects were a major component of the diet of the tabanid larvae (Results 4.7) with the highest relative abundance of 36.2% in the larval guts belonging to Class Insecta. The SILVA 119 database identified 52 OTUs from the larval guts in this study as belonging to the Class Insecta but only 23 were further classified to the species level. The rest of the 29 OTUs were not classified beyond Insecta in the SILVA 119 database. The top matches for the species identified by the SILVA 119 database were two mosquito species *Aedes aegypti and A. albopictus*, the fruit fly *Drosophila sechellia*, the horse fly *Haematopota pluvialis* and the parasitic wasp *Leptopilina heterotoma*. The reliability of the species assignment by SILVA was subsequently checked by manual blasting of the sequences against the NCBI database.

BLAST results showed matches that can be described in various categories. Firstly, high reliability of assignment (Identity match of 99% to 100% and E-value of zero) was seen for OTU sequences identified as *Drosophila sechellia* in SILVA when matched to *Drosophila melanogaster* in the NCBI database, confirming the genus. One OTU sequence belonging to *H. pluvialis* was identified (100%) as *T. nigrovittatus* by NCBI. Secondly, *A. albopictus* and *A. aegypti* showed matches to multiple species within the Genus Aedes with a 98% to 100% match and a zero E-value. Hence, OTU sequences preliminarily identified as *A. aegypti* and *A. albopictus* by SILVA belong to closely related species of the same genus. Thirdly, other *H. pluvialis* than the one matching 100% to *T. nigrovittatus* showed matches to multiple species with a low identity match (86% to 97%). However, at least three top BLAST hits of each OTU that belonged to *H. pluvialis* were within the family Tabanidae. Fourthly, no clear taxonomic assignment in the NCBI database was provided for the OTU sequences identified as the parasitic wasp *L. heterotoma* by SILVA. Top blast hits of the *L. heterotoma* sequences matched

Drosophila melanogaster and the water bug *Paravelia bullialata* at low identity values (90% to 97%) with E-values below zero. The NCBI database lacks sequences for 18SrRNA gene region for *L. heterotoma*. However, Drosophila is a known host of the *L. heterotoma* and thus the match was to a similar species or genus of a wasp that infected either the *Drosophila melanogaster* or the water bug *Paravelia bullialata*. The match was not to the same species as the identity values were low.

Overall, the results indicate that the main dietary components of the tabanid larvae that were identified to the species level by SILVA belong to the genera *Drosophila, Aedes* and the Family Tabanidae. However, the matches are to be viewed with caution for reasons to be discussed in detail (see Chapter 5. Discussion).

4.9 Variation in tabanid larval diets across locations

Larval diets in Rockefeller Wildlife Refuge, Cypremort Point and Grand Bayou were not significantly different across larvae due to high variation within locations.

OTU richness (box plots and t-test) from Figure 4.7, OTU composition (indicated by NMDS and confirmed by PERMANOVA) from Figure 4.8 and relative abundance of OTUs (determined by LEfSe) from Figure 4.9 was not significantly different among all three locations.

However, the fungus Agaricomycetes (Agaricomytina) was present in significantly higher proportions in the larvae from Cypremort Point in comparison to larvae from Rockefeller Wildlife Refuge as indicated by LEfSe.

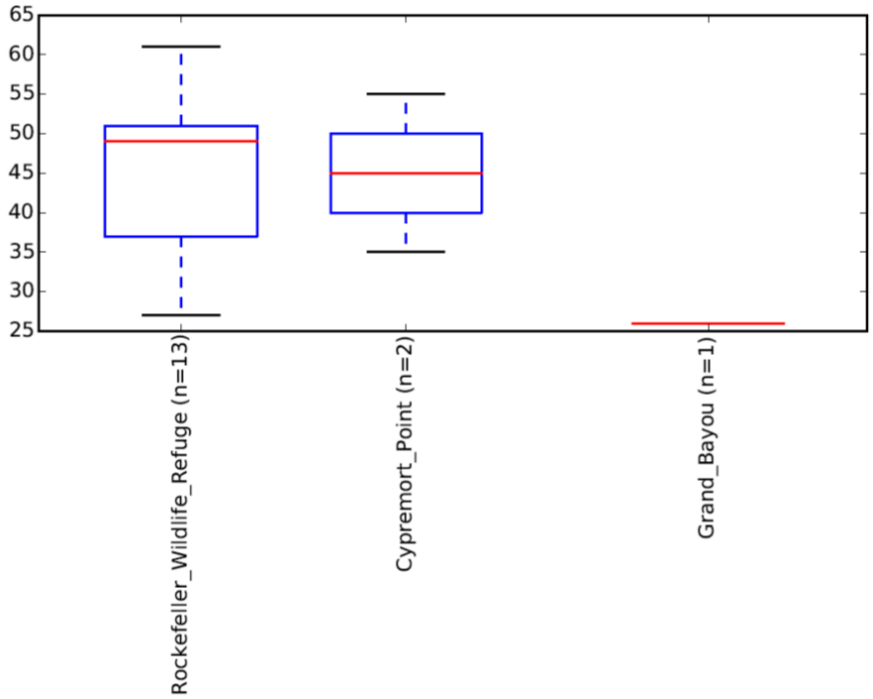

Figure 4.7 Box plots displaying diversity of OTUs in larval guts within and between locations. X-axis represents the number of OTUs and Y-axis denotes the location. Red lines indicate the median for each location, the top of the box denotes the upper and lower quartile and the whiskers show the maximum and the minimum (excluding outliers). A non-parametric t-test did not show significant differences in the OTU richness of larval diets between Rockefeller Wildlife Refuge, Cypremort Point and Grand Bayou.

Total variation of OTU richness was high among sites of Rockefeller Wildlife Refuge as the number of observed OTUs ranged from approximately 26 to 62. This could be because Rockefeller Wildlife Refuge had the maximum number of larvae (n=13) in comparison to Cypremort Point (n=2) and Grand Bayou (n=1). Comparing the middle 50% (2^{nd} and 3^{rd} quartiles) of OTU richness of larvae at Rockefeller Wildlife Refuge and Cypremort Point indicated almost similar distribution of OTU numbers of the two groups. This observation was supported by a non-parametric t-test based on 999 Monte Carlo permutations ,which indicated that there was no significant difference in the observed number of OTUs in the larval diets between Rockefeller Wildlife Refuge and Cypremort Point (P = 1). Similarly, OTU richness between the sites of Rockefeller Wildlife Refuge and Grand Bayou (P=0.4), and of Cypremort

Point and Grand Bayou (P = 1) was not significantly different either, despite the low number of OTUs in the larval gut from Grand Bayou (26). This result is to be viewed with caution since sample sizes were low. The lack of significant differences in OTU richness alone does not allow conclusions towards OTU composition and, therefore additional tests were performed taking into account OTU relative abundance and shared OTUs.

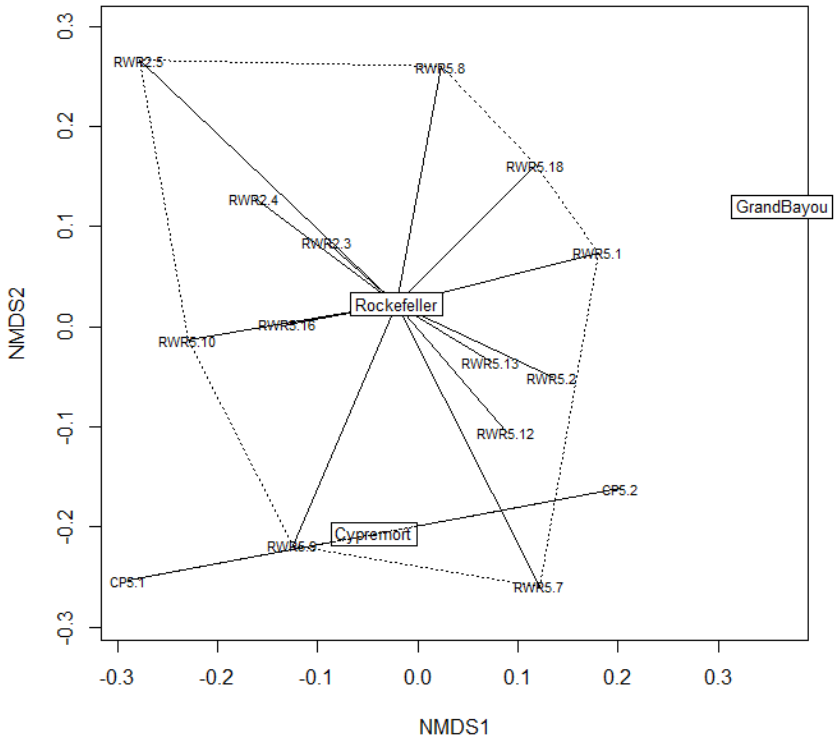

Figure 4.8 Non-metric multidimensional scaling plot (NMDS) based on Bray Curtis distances of composition of OTUs in the larval guts grouped based on sites. Ordination hulls denoted by dotted lines indicate the groups compared: 1. Larvae found at Rockefeller Wildlife Refuge (Rockefeller) 2. Larvae found at Cypremort Point (Cypremort) and 3. Larva found at Grand Bayou (Grand Bayou). Non-dotted lines denote distance of the larval gut sample from the group mean. Abbreviations of samples and locations from where larvae were collected can be found in the Figure 3.1.

Non-metric multidimensional scaling plot (NMDS, Figure 4.8) showed no significant difference in the composition of OTUs between larval guts collected from Rockefeller and Cypremort Point

as the non-dotted line connecting CP5.1 and CP5.2 to their group mean intersects the ordination hull of Rockefeller Wildlife Refuge. The sample from Grand Bayou is an outlier but since it was the sole sample from this location, variance could not be established for this location. The Permutational multivariate analysis of variance (PERMANOVA) with the function Adonis found a marginal but not significant difference in the composition of OTUs between Rockefeller Wildlife Refuge, Cypremort Point and Grand Bayou (P = 0.073).

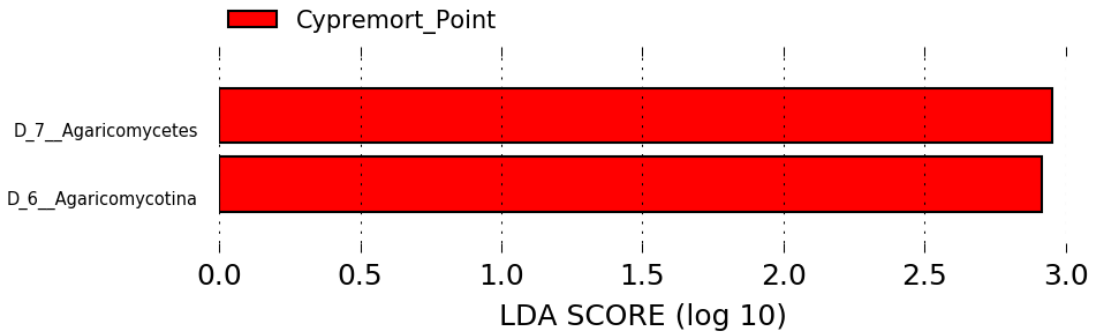

Figure 4.9 LEfSe (Linear discriminant analysis Effect Size) with enriched taxa found in the larvae from Cypremort Point in comparison to larvae from Rockefeller Wildlife Refuge.

Larval gut contents from Cypremort Point showed enrichment of two fungal classes compared to those from Rockefeller Wildlife Refuge. “Species” Agaricomycetes which is a Class in classical taxonomy belongs to the fungal division Basidiomycota and is a known member of the Louisiana saltmarsh sediment community (Roy et al., 2010). “Genera” Agaricomycotina is a yeast and has many known associations with insects such as providing habitats to insects and as a food source while in other cases it acts as an entomopathogen (Roy et al., 2010).

4.10 Comparison of OTU composition in sediments and larvae

A higher number of larvae were found in the West in comparison to the East despite similar sampling methods and a greater sampling effort in the East. In the West, larvae were always present where a higher abundance of Hexapoda and fungi was present. However, this trend is not consistent in the East where a high abundance of Hexapoda and fungi did not guarantee larval presence. This inconsistent trend in the east could be due to the observed population crashes of the adult tabanid in the eastern areas. Proportional split of "Phyla" from Figure 4.10, "Classes" from Figure 4.11 and "Families" from Figure 4.12 was different in the larval guts and their corresponding sediments.

Locations in the West (Rockefeller Wildlife Refuge and Cypremort Point) had a high number of larvae (18) but only at 1/3 of the sites. Larval gut content of the three larvae collected from CP2 was not included in next-generation sequencing analysis because the pilot study (see 4.2) showed the gut content consisting 100% of *T. nigrovittatus* DNA. Nevertheless, the larvae were counted as present for this analysis. The four sites in the West where larvae were found (RWR2, RWR5, CP2 and CP5) had the most Opisthokonta (> 57%) in their respective regions. This suggests a higher likelihood of finding larvae at sites with high Opisthokonta in the West. Although the sampling method used in the East and West was the same and more sampling effort was undertaken (more sites) in the East, only one larva was retrieved from Eastern regions (GB5). This larva was not found in sediments with particularly high Opisthokonta (only 34%). Even in sediments (GB3, GB6, GB8 and GICL), which had Opisthokonta percent reads comparable to the sites in the West where larvae were present (> 67% in comparison to >57% in the West), Eastern sites were devoid of larvae.

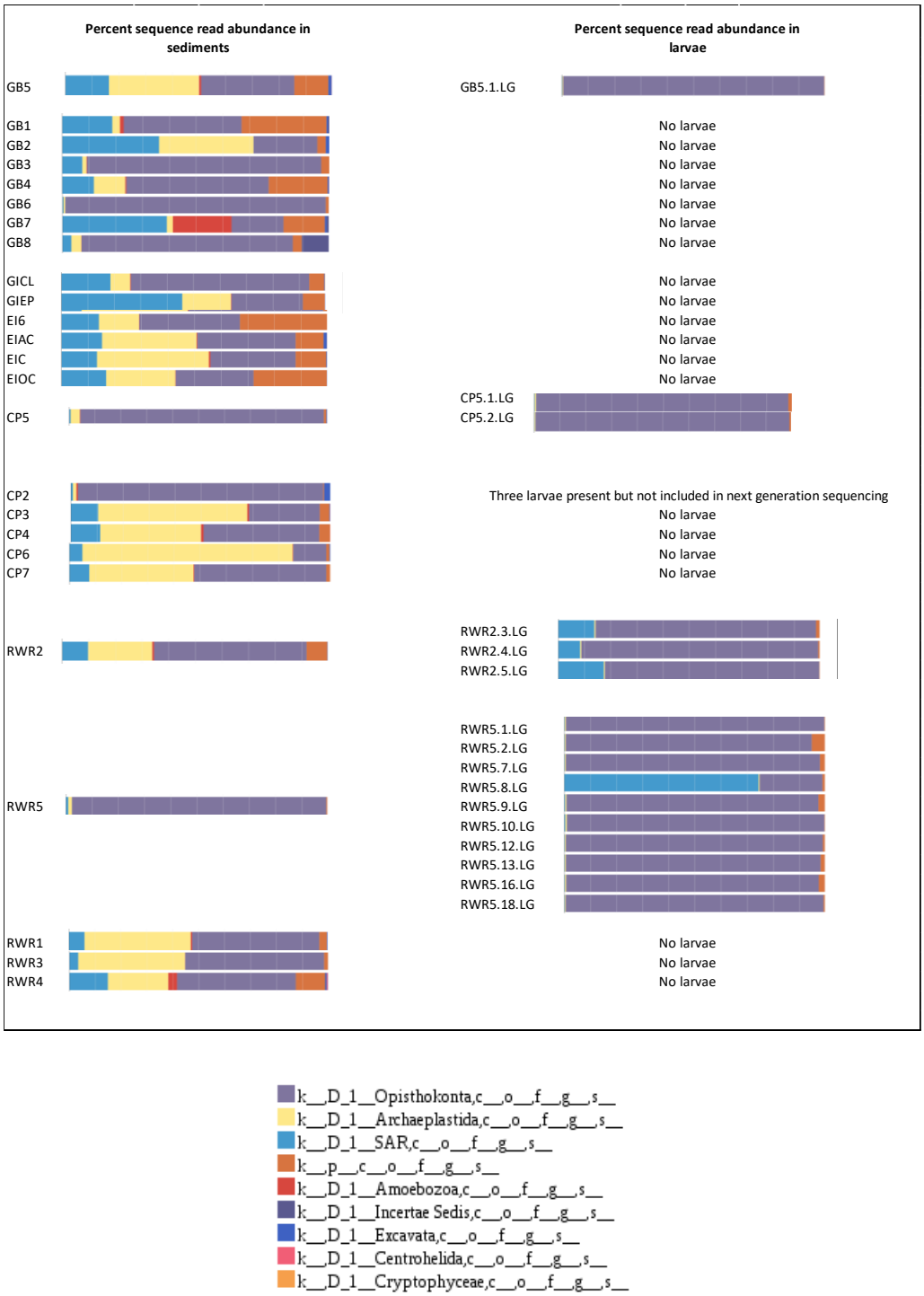

Figure 4.10 Percent sequence read abundance of "phyla" in sediments and larval gut samples: The sample names are defined by their location (see Figure 3.1). Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name. LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered.

Opisthokonta had the largest abundance in the majority of larval guts (> 82 %) except RWR5.8.LG which had only 24%. Opisthokonta were present in all sediment samples with moderate to high abundance in the range of 34.94% (GB5) to 97.34% (RWR5). The Eastern sample (GB5) represented the lowest abundance of Opisthokonta in the sediment where at least one larva was found. High Opisthokonta abundance is a predictor for larval presence in the West, since larvae were found in all sediment samples with > 57% Opisthokonta. However, this trend was not seen in the East as larvae were absent even in sediments with > 67 % Opisthokonta.

As mentioned in detail in the previous section (4.7) Holozoa and Nucletmycea were present in all larval guts but in varied proportions. Proportional split of Holozoa and Nucletmycea in the larval guts varied widely even among larvae from the same site (CP5, RWR5). Only larvae from the RWR2 sediment had similar proportions of Holozoa and Nucletmycea. Proportional split of Nuclemycea and Holozoa in larval gut did not necessarily reflect proportions in sediment. High and even proportions of Holozoa and Nucletmycea were a strong predictor in the Western sites that larvae would be found. The majority of sediments where no larvae were found had a large proportion of other orders such as Chloroplastida, Stramenopiles and Alveolata and less than 60% of Holozoa and Nucletmycea combined. However, no such predictions could be made for Eastern sites. The one larva found in the East (GB5.1.LG) was found in sediment with comparatively low Holozoa and Nucletmycea content (GB5 = 35%). Although other sediments in the East did have high proportions of Holozoa and Nucletmycea such as GB3, GB6, GB8 and GICL (> 60 %), no larvae were found in these sediments. Sediments also contained varying proportions of Chloroplastida, Stramenopiles and Alveolata, but only Stramenopiles and Alveolata showed up in 4 larvae in (RWR2.3.LG, RWR2.4.LG, RWR2.5.LG and RWR5.8.LG).

Figure 4.11 Percent sequence read abundance of "classes" in sediments and larval gut samples: The sample names are defined by their location (see Figure 3.1). Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name. LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered.

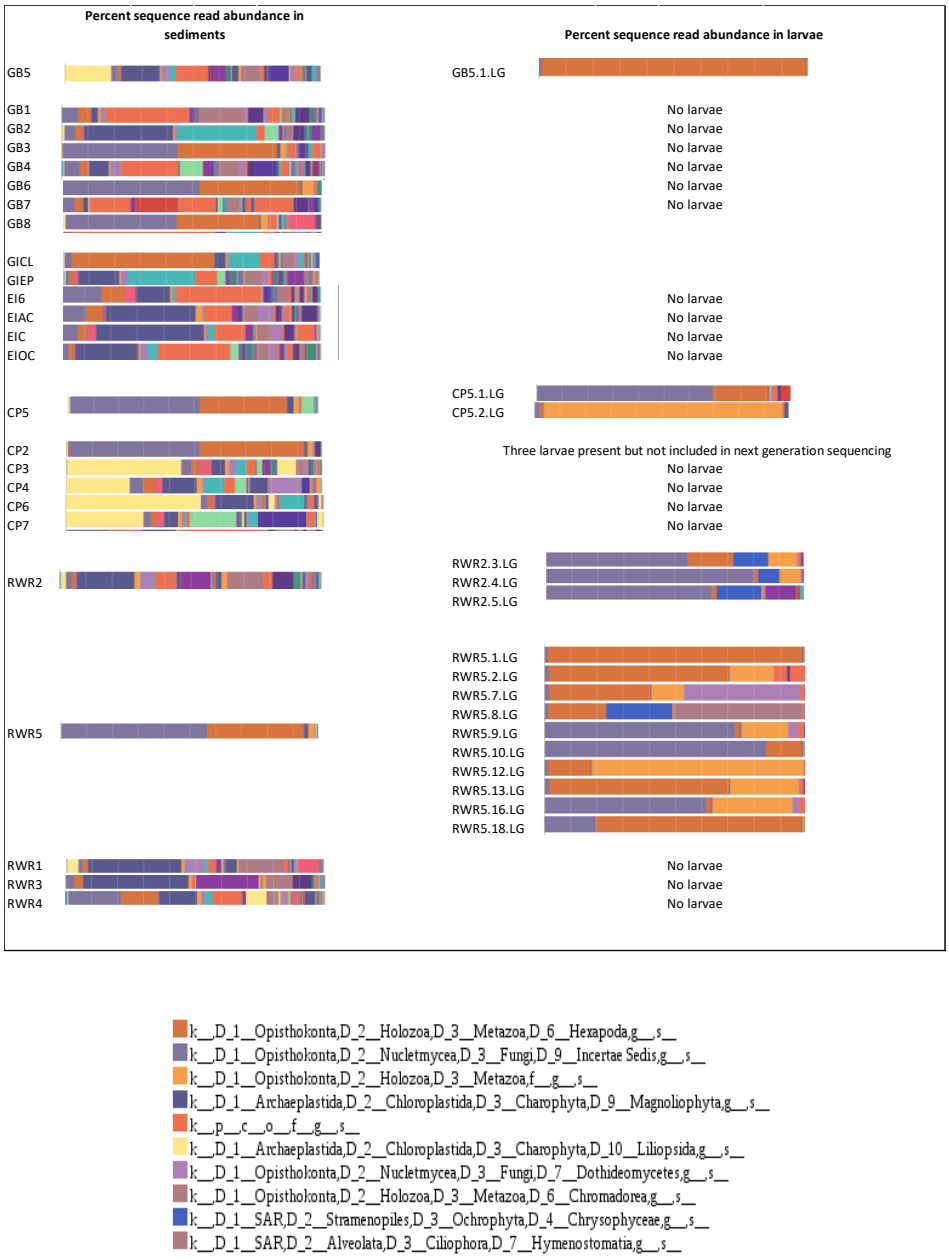

Figure 4.12 Percent sequence read abundance of "families" in sediments and larval gut samples: The sample names are defined by their location (see Figure 3.1). Gut content samples of larvae found at the same site as a sediment sample were designated with sediment sample name. LG. If multiple larvae were retrieved from the same sediment sample site, they were individually numbered.

Hexapoda, unclassified Metazoa and fungi were found in varying proportions in all larval guts

(described in section 4.7) collected from Western sites. As with the "Phylum", "Class" and

"Order" levels before, proportions in larval gut do not reflect proportions in sediment at the "Family" level. For example, Hexapoda and unclassified fungi were dominant and almost equally proportional in the CP5 sediment but the larvae found in CP5 had varied proportions of Holozoa and unclassified fungi such that one larva (CP5.1.LG) had a higher abundance of unclassified fungi (70%) while the other larva (CP5.2.LG) was dominated by unclassified metazoa and had < 4% of Holozoa and unclassified fungi combined.

Similarly, RWR2 sediment had only 2% abundance of unclassified fungi while > 60% abundance of unclassified fungi was found in the larvae from this sediment. "Family" diversity and proportions of "families" in sediments do not predict larval presence, since larvae were found equally in sediments dominated by family Hexapoda and Unclassified Fungi (RWR5, CP5) but also in sediments with diverse families (RWR2, GB5) where Hexapods and Unclassified Fungi were not abundant. In the East, GB3, GB6, GB8 and GICL had "family" proportions of Hexapods and unclassified fungi (> 60%) yet no larvae were found in these sediments.

4.11 Variation in sediment OTU composition across locations

A high variation in OTU composition as seen in Figure 13 among sediments from Rockefeller Wildlife Refuge, Cypremort Point, Grand Isle and Grand Bayou was observed from the PCoA plot and alpha and beta diversity analysis below. Rockefeller Wildlife Refuge and Cypremort Point had significantly different OTU composition with *Spartina* (Liliopsida), Crustacea and Annelids enriched in sites at Cypremort Point and Rhizaria enriched in those of Rockefeller Wildlife Refuge.

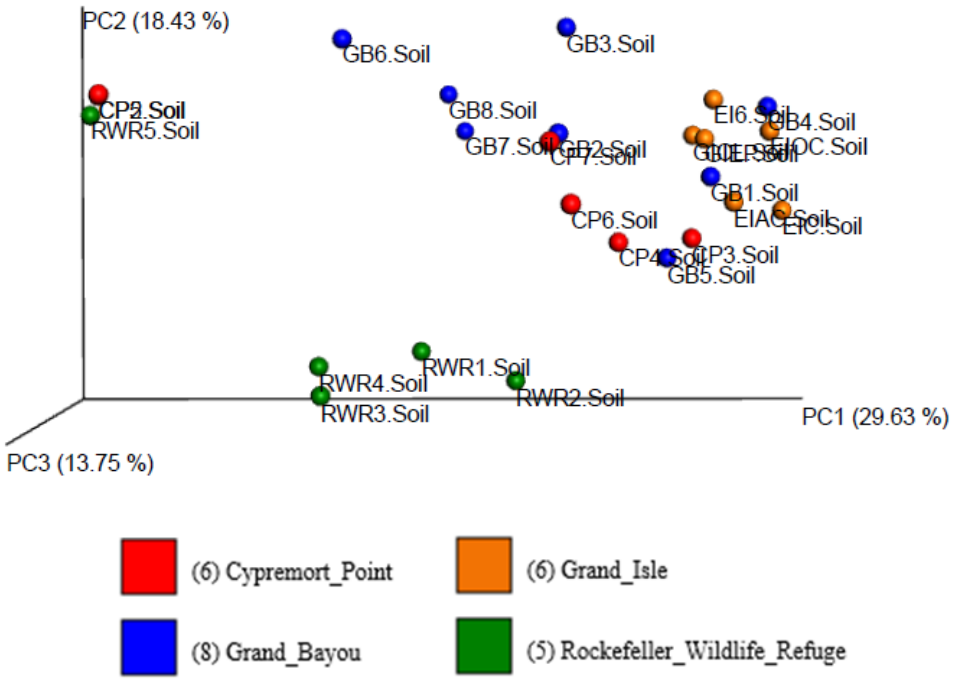

Figure 4.13 Principal Co-ordinates Analysis (PCoA) multidimensional scaling plot based on unweighted UniFrac distances of OTU composition. Each circle in the plot represents a sediment sample collected at a site from Rockefeller Wildlife Refuge, Cypremort Point (both West LA), or Grand Isle and Grand Bayou (both East LA) denoted by different colors.

The above Principal Coordinates Analysis Plot showed that sediments from Grand Isle clustered together based on similarity of their OTU composition, but they did not separate from samples collected at Grand Bayou and Cypremort Point. The OTU compositions of most samples from Rockefeller Wildlife Refuge separated somewhat from the other three regions along the second Principal Coordinate. Interestingly, the three outliers whose OTU composition differed from other samples at their respective locations (RWR5, CP2 and 5) were three of the four sites in the West where the majority of larvae was found. The only site in the East with a larva present did not separate from the other sites in the same location based on similarity in OTU composition.

4.12 Alpha and Beta diversity among sediments based on locations with statistical tests

As seen in Figure 4.14, the median and range of OTU richness among sites within locations was similar for Rockefeller Wildlife Refuge (median 160, range: 50 to 220) and Grand Bayou (median 125, range: 50 to 230). Samples from Cypremort Point had the lowest median of OTU numbers median 110, range: 50 to 150) and Grand Isle sites had the highest (median 175, range: 140 to 225). Overall, results of the t-test showed no significant difference in the number of OTUs among all locations (P > 0.2 and P = 0.07 between Cypremort Point and Grand Isle).

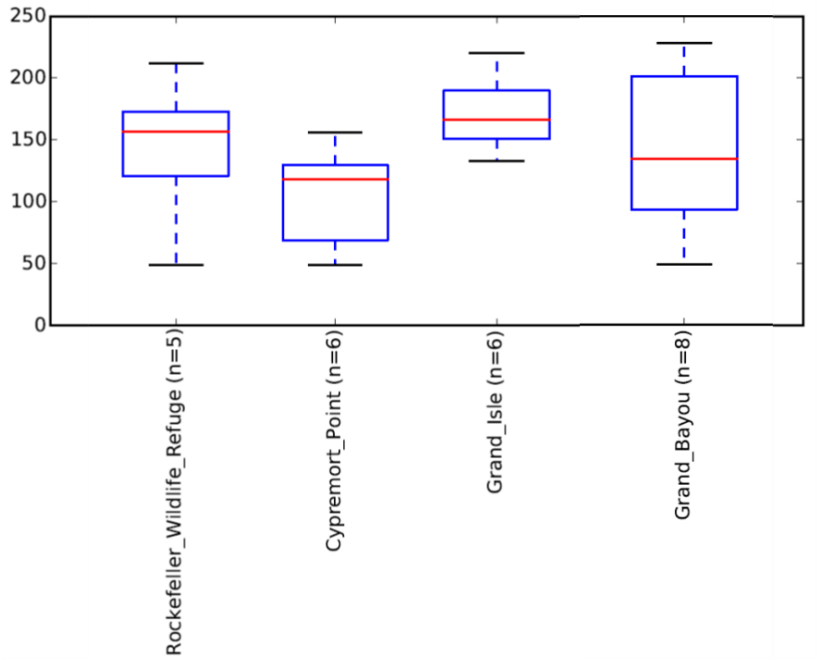

Figure 4.14 Box plots displaying diversity of OTUs in sediments within and between locations. X-axis represents the number of OTUs and Y-axis denotes the location. Red lines indicate the median for each location, the top of the box denotes the upper and lower quartile and the whiskers show the maximum and the minimum (excluding outliers). A non-parametric t-test did not show significant differences in the OTU richness in sediments between Rockefeller Wildlife Refuge, Cypremort Point, Grand Isle and Grand Bayou.

Non-metric multidimensional scaling plot (NMDS, Figure 4.15) showed no significant difference in the composition of OTUs among sediment collected from different sites at Rockefeller Wildlife Refuge (RWR), Grand Isle (GI) and Grand Bayou (GB) indicated by overlapping ordination hulls. However, there was a significant difference in the OTU composition between Cypremort Point (CP) and Rockefeller Wildlife Refuge (RWR) as the ordination hulls between these sites did not overlap. The Permutational multivariate analysis of variance with the function Adonis confirmed that there was a significant difference in the composition of OTUs between Rockefeller Wildlife Refuge and Cypremort Point (P = 0.021).

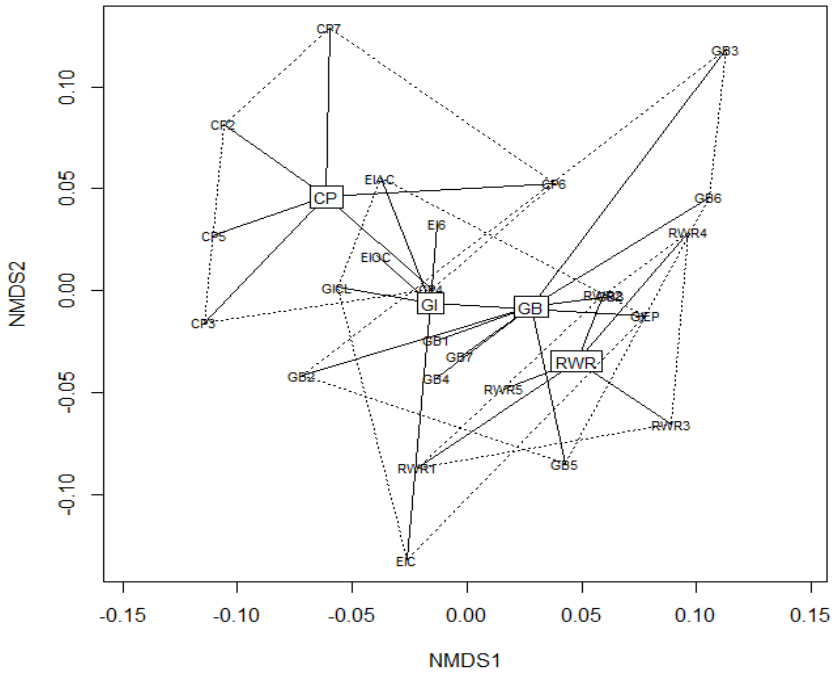

Figure 4.15**.** Non-metric multidimensional scaling plot (NMDS) based on Bray Curtis distances of composition of OTUs in sediments based on geographical location. Ordination hulls denoted by dotted lines indicate the groups compared namely sediments from sites at 1. Rockefeller Wildlife Refuge (RWR), 2. Cypremort Point (CP), 3. Grand Isle (GI) and Grand Bayou (GB). Non-dotted lines denote distance of the sediment sample from the group mean. Abbreviations of samples and locations from where sediments were collected can be found in the Figure 3.1.

LEfSe (Linear discriminant analysis Effect Size) denoted by bar chart in Figure 4.16 showed 43 differentially abundant taxa found in sediment at sites collected from Cypremort Point and Rockefeller Wildlife Refuge. Majority of the taxa found in Rockefeller Wildlife Refuge (10/23) belonged to the group Rhizaria from the SAR supergroup (mostly unicellular eukaryotes). Minority of taxa were fungi (5/23) followed by Nematodes (3/23), ciliated protozoa (2/23), amoeba (1/23), unicellular flagellate (1/23) and heterotrophic nanoflagellate, *Halocafeteria* (1/23) known to feed on prokaryotes. None of these taxa were found in the larval guts and hence their role (if any) in the tabanid food web is unknown. Of the 20 taxa enriched at Cypremort Point, Liliopsida and Annelida were groups that also were found in larval guts. However, larval diets from Cypremort Point did not display a significantly high occurrence of Liliopsida and Annelida in comparison to Rockefeller Wildlife Refuge (see 4.9).

Spartina alterniflora, the most dominant vegetation in the marshes belongs to the family Liliopsida and, therefore, the increased abundance of Liliopsida in the sediment from Cypremort Point was most likely due to *Spartina*.

Other taxa enriched in Cypremort Point include green algae and diatoms which belong to the phytoplankton layer, the basal trophic level in marsh food webs.

4.13 Differences in sediment OTU composition between the East and West

OTU richness and composition was not significantly different between sites from the East and the West as pointed by the Box plots from Figure 4.17 and NMDS from Figure 18 below. However, a higher relative abundance of Liliopsida (most likely *Spartina*), Maxillopoda (crustaceans), and Cyrtolophosidida (ciliates) in the West in comparison to the East and a higher

relative abundance of Rhizaria and Alveolata in the East in comparison to the West was observed in Figure 19.

OTU richness was not significantly different between sites in East and West Louisiana (P = 0.14).

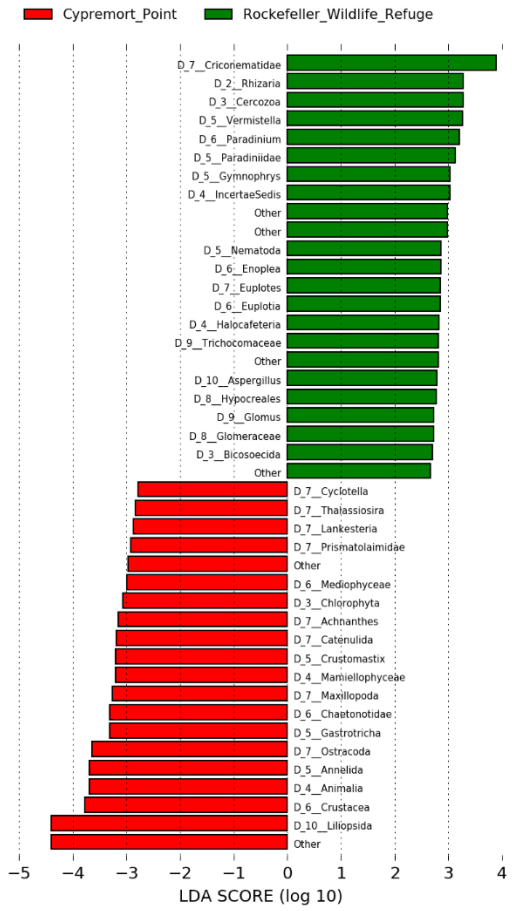

Figure 4.16 LEfSe (Linear discriminant analysis Effect Size) with differentially abundant taxa found in Cypremort Point and Rockefeller Wildlife Refuge. Bars in red denote “genera” that are significantly more abundant in sediments collected from Cypremort Point in comparison to Rockefeller Wildlife Refuge and bars in green denote vice versa.

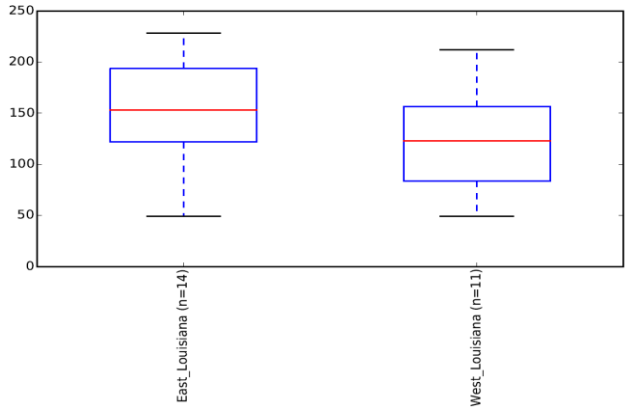

Figure 4.17 Box plots displaying diversity of OTUs in sediments within and between East and West Louisiana. X-axis represents the number of OTUs and Y-axis denotes the geography. Red lines indicate the median for each location, the top of the box denotes the upper and lower quartile and the whiskers show the maximum and the minimum (excluding outliers). A non-parametric t-test did not show significant differences in the OTU richness in sediments between East and West Louisiana.

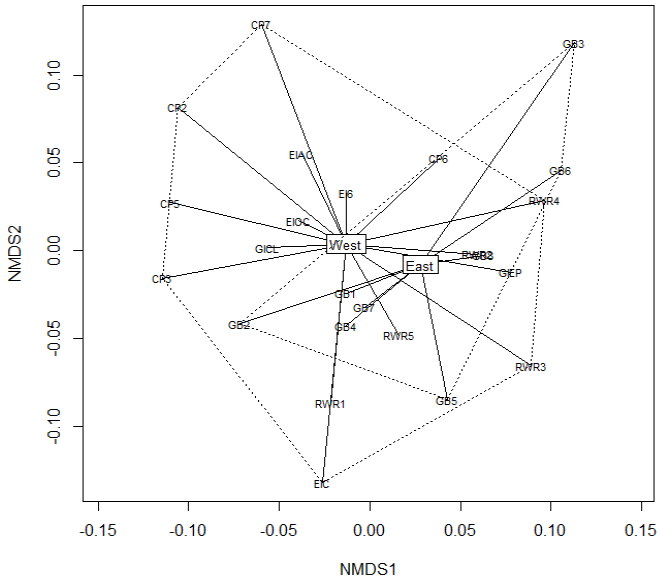

Figure 4.18 Non-metric multidimensional scaling plot (NMDS) based on Bray Curtis distances of composition of OTUs in sediments from East and West Louisiana. Ordination hulls denoted by dotted lines indicate the group of sediments which were collected from sites in East Louisiana (East) and sediments which were collected from sites in West Louisiana (West). Non-dotted lines denote distance of the sediment from the group mean. Abbreviations of samples and locations of sites from which sediment was collected can be found in the Figure 3.1.

The Non-metric multidimensional scaling plot (NMDS, Figure 4.18) showed that there was no significant difference in the overall composition of OTUs in the sediments from the East and West, which also was confirmed by PERMANOVA with function Adonis (P = 0.165). Although the overall OTU composition did not show significant differences between Eastern and Western sites due to high variation among sites within each region, a detailed analysis of relative abundance of taxa using LEfSe revealed 24 taxa that were enriched in sediments from West Louisiana and 46 that were enriched in sediments from East Louisiana.

In East Louisiana, Rhizaria and Alveolata from the SAR super group were significantly more abundant in comparison to West Louisiana. Besides these major groups, the abundances of Breviata which are amoeboid eukaryotes and of green alga (Chlorophyta) also were higher in East in comparison to West Louisiana.

In the West, Liliopsida (*Spartina*), Maxillopoda (crustaceans), Cyrtolophosidida (ciliates) were notable groups as members of these groups were found in the tabanid larval guts.

4.14 Difference in OTU composition of sediments where larvae were present vs sediment where larvae were absent

OTU composition was not significantly different in both these groups as seen in Figure 20. Opisthokonta, specifically Nucletmycea, were significantly enriched in sediments where larvae were found in comparison to sediments where larvae were not found.

Stramenopiles (Ochrophyta) and Alveolates were more abundant in the sediments where larvae were absent in comparison to sediments where larvae were present as seen in Figure 21.

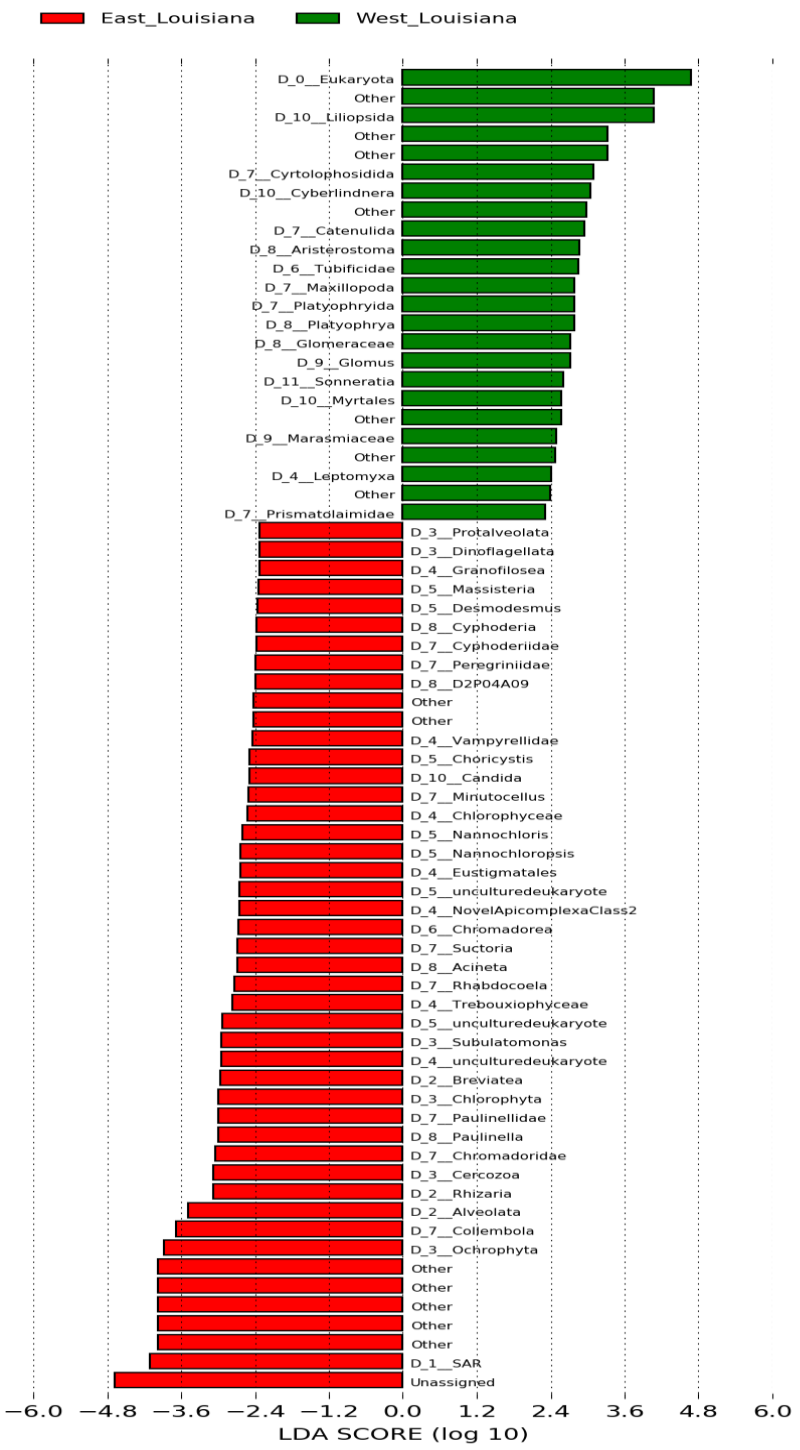

Figure 4.19 LEfSe (Linear discriminant analysis Effect Size) denoted by bar charts with significantly abundant taxa found between East and West Louisiana. Index in red denotes "genera" that are significantly abundant in East Louisiana in comparison to West Louisiana and index in green denotes vice versa.

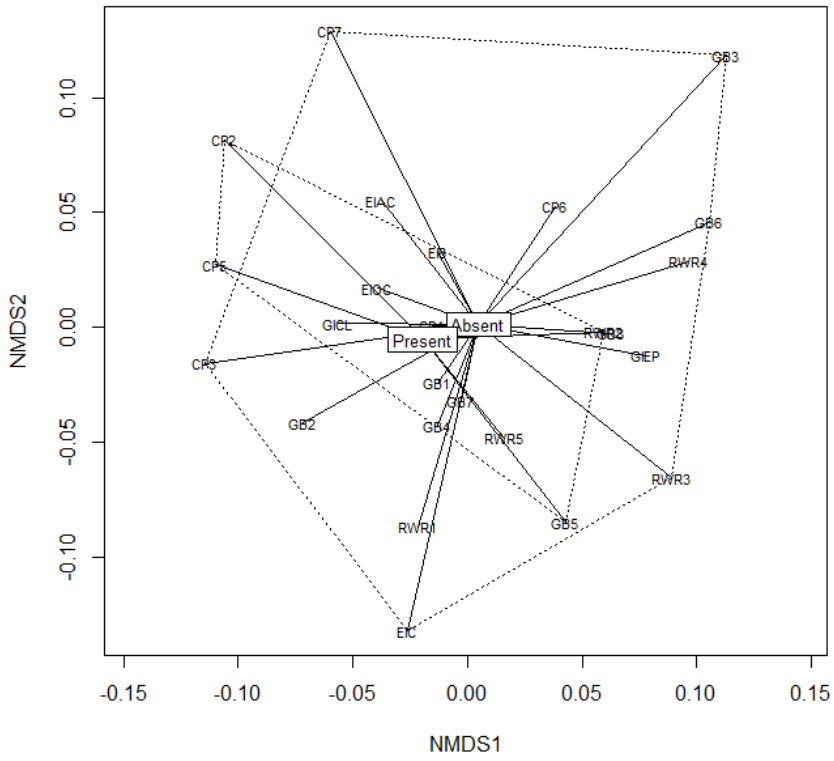

Figure 4.20 Non-metric multidimensional scaling plot (NMDS) based on Bray Curtis distances of composition of OTUs in sediments grouped on the basis of presence/absence of larvae. Ordination hulls denoted by dotted lines indicate the two groups compared: 1. Present = Sediments in which larvae were found (RWR2, RWR5, CP2 (not sequenced), CP5, GB5) and 2. Absent = Sediments in which larvae were not found (see Material and methods Table 3.1). Non-dotted lines denote distance of the sediment composition from the group mean. Abbreviations of samples and locations of sites from which sediment was collected can be found in the Figure 3.1.

The non-metric multidimensional scaling plot (NMDS, Figure 4.20) showed no significant difference in the composition of OTUs determined by shared OTUs between sediments in which larvae were present and sediments in which larvae were absent based on the overlapping ordination hulls. Further evaluation, using the Permutational multivariate analysis of variance (PERMANOVA) with the function Adonis confirmed that there was no significant difference in the composition of OTUs between the groups of sediments where larvae were present and those without larvae (P = 0.9).

In sediments where larvae were present, Opisthokonta and Nucletmycea, the major branch of Opisthokonta comprising fungi, were significantly enriched.

This confirms these taxa as predictors for the presence of larvae as previously suggested (see Results 4.10). The main component in the sediments where larvae were absent was the Stramenopiles and Alveolates of the SAR supergroup. However, Placididae a non-photosynthetic eukaryote from the Stramenopiles was significantly abundant in sediments where larvae were present. Members of both Alveolates and Stramenopiles specifically Ochrophyta were found in the larval guts. This indicates that these taxa are part of the tabanid food web and are not detrimental to larval survival.

4.15 Total Polycyclic Aromatic Hydrocarbon (PAH) content in sediments

As seen in Figure 4.22 total Polycyclic Aromatic Hydrocarbon (PAH) content in sediments showed that GB2 (Total PAHs = 980 ppb) and GB3 (Total PAHs = 1,219 ppb) were above the baseline of 700 ppb determined by the EPA. This PAH content classifies the sites as minimally oiled as the baseline for lightly oiled was 6.2 mg/g i.e.6,200,000 ppb (Fleeger et al., 2015), and for moderately oiled sites was or 70mg/g i.e.70,000,000 ppb (McCann et al., 2017). Oil from our study sites has the signature of South Louisiana Crude, which points to DWH as the likely origin. However, since biomarkers change due to degradation, the origin cannot be determined with absolute certainty. The other sites in East Louisiana had Total Polycyclic Aromatic Hydrocarbons (PAH) lower than the EPA baseline and were in the range of 5.85 ppb at the site Elmer's Isle Air Traffic Control to 447.017 ppb at the site Elmer's Isle C. Our data also indicates that the oiling was patchy, which is supported by previous observations (see Chapter 1 Literature review).

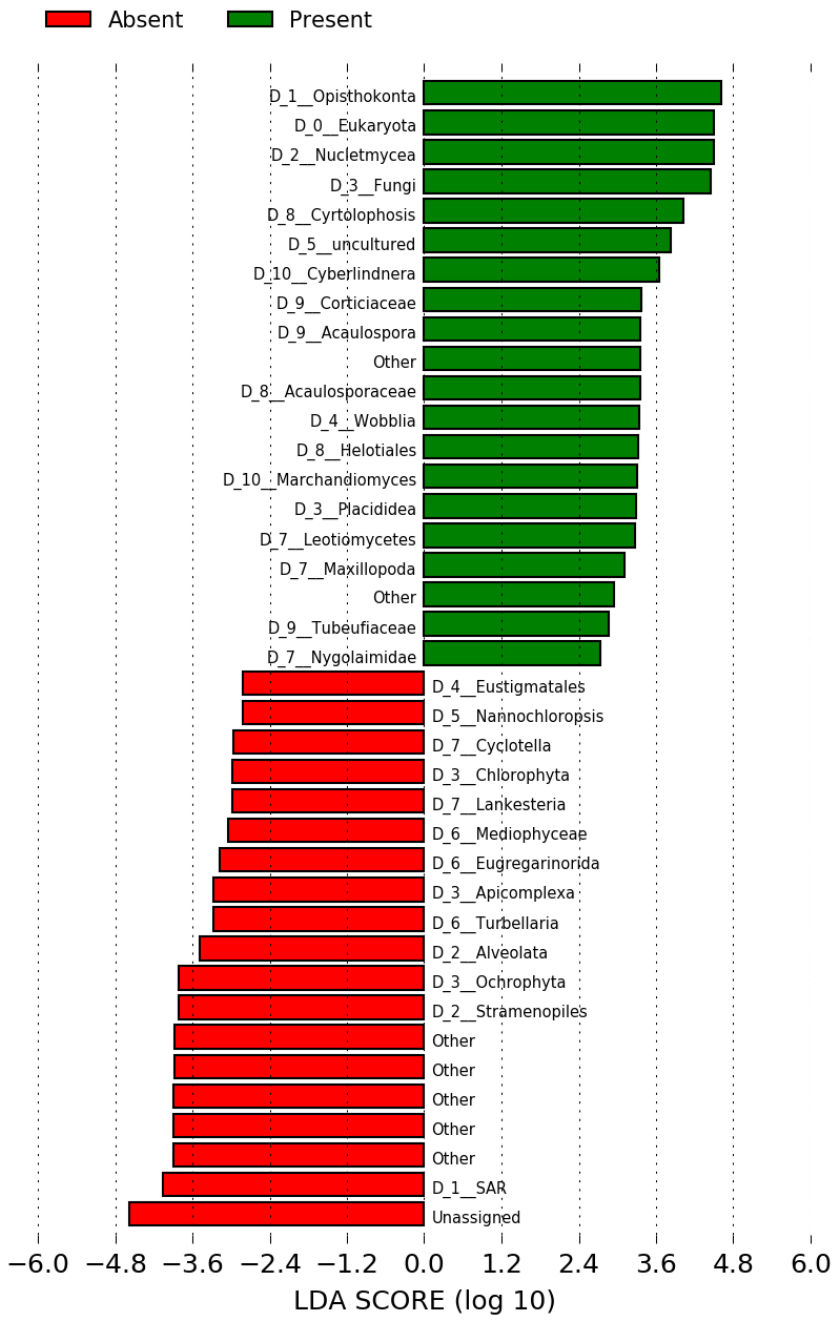

Figure 4.21 LEfSe (Linear discriminant analysis Effect Size) denoted by bar charts with significantly abundant taxa found between sediments where larvae are present and sediments where larvae are absent. Index in red denotes “genera” that are significantly abundant in sediments with larvae in comparison to sediments without larvae and index in green denotes vice versa.

PAH	GB1	GB2	GB3	GB4	GB5	GB6	GB7	GB8	EIOC	GICL	GIEP	EIC	EIAC	EI6
Naphthalene	4.196	23.194	2.373	2.176	1.426	2.996	<MDL	<MDL	2.305	3.126	2.443	2.002	<MDL	<MDL
C-1 Naphthalene	8.473	18.979	7.236	6.757	4.370	7.221	<MDL	<MDL	6.224	7.034	7.665	4.042	<MDL	ND
C-2 Naphthalene	4.472	6.53	4.959	3.414	2.918	4.000	ND	ND	3.233	3.622	3.774	2.360	<MDL	ND
C-3 Naphthalene	3.064	4.479	5.958	2.501	1.878	2.814	<MDL	ND	2.698	3.018	3.611	1.773	<MDL	<MDL
C-4 Naphthalene	2.27	3.921	5.503	1.852	1.243	2.077	<MDL	<MDL	1.564	1.460	2.161	1.184	<MDL	<MDL
Fluorene	4.434	13.03	2.764	3.469	2.549	1.699	<MDL	<MDL	3.425	1.683	5.194	3.783	<MDL	<MDL
C-1 Fluorene	4.971	10.362	11.571	3.46	1.960	7.388	<MDL	<MDL	4.293	4.361	7.982	2.899	ND	<MDL
C-2 Fluorene	15.139	146.172	30.918	9.992	5.633	24.609	ND	ND	8.583	10.507	32.647	8.992	<MDL	<MDL
C-3 Fluorene	18.263	31.038	39.51	12.253	5.846	14.874	ND	ND	10.347	13.704	18.029	4.524	ND	ND
Dibenzothiophene	2.766	4.359	3.845	1.39	0.781	1.927	<MDL	ND	1.600	2.812	4.120	4.275	<MDL	<MDL
C-1 Dibenzothiophene	5.062	7.758	11.919	2.789	1.480	3.799	<MDL	<MDL	3.080	4.906	4.651	3.539	<MDL	<MDL
C-2 Dibenzothiophene	8.174	9.236	37.97	3.666	1.773	5.276	ND	ND	3.562	6.654	5.954	4.144	ND	ND
C-3 Dibenzothiophene	5.719	7.618	54.014	3.261	2.399	14.189	ND	ND	3.127	3.440	7.422	3.829	ND	ND
Phenanthrene	18.486	57.521	18.033	6.541	4.458	11.148	<MDL	<MDL	10.298	13.589	18.442	121.934	<MDL	<MDL
C-1 Phenanthrene	27.98	291.442	122.875	18.26	4.504	123.640	ND	ND	12.612	22.803	117.199	28.902	ND	ND
C-2 Phenanthrene	17.065	18.736	130.404	9.171	5.590	9.571	ND	ND	11.226	12.461	12.478	13.241	ND	ND
C-3 Phenanthrene	8.968	11.583	116.705	5.079	3.228	5.543	ND	ND	5.191	6.809	7.508	4.664	ND	ND
C-4 Phenanthrene	5.083	9.836	76.702	3.309	2.791	5.139	ND	ND	3.885	3.411	4.052	2.518	ND	ND
Anthracene	3.531	8.419	6.137	2.383	1.014	2.298	0.007	ND	2.643	13.409	5.545	7.576	ND	<MDL
Fluoranthene	24.754	59.432	29.38	16.488	1.50	3.91	<MDL	<MDL	6.365	21.493	15.920	100.243	1.24	ND
Pyrene	19.098	43.508	30.801	33.818	1.39	2.47	0.757	<MDL	7.090	18.619	10.661	44.307	<MDL	ND
C-1 Pyrene	10.717	20.283	28.006	11.833	1.675	1.830	<MDL	<MDL	4.081	10.069	4.343	6.257	ND	ND
C-2 Pyrene	10.766	24.626	29.968	13.297	1.590	2.986	ND	ND	4.933	7.943	7.197	8.965	ND	ND
C-3 Pyrene	7.166	18.904	27.099	8.532	1.788	2.715	ND	<MDL	2.889	4.647	3.202	2.676	ND	ND
C-4 Pyrene	5.506	9.928	28.886	6.089	1.246	2.631	ND	ND	2.284	3.493	2.211	2.535	ND	ND
Napthobenzothiophene	2.807	4.13	5.545	1.44	0.219	0.330	ND	ND	0.686	1.694	0.969	1.284	ND	ND
C-1 NBT	5.873	9.652	31.524	6.273	1.273	2.027	ND	ND	2.014	2.502	2.978	1.744	ND	ND
C-2 NBT	4.274	5.617	32.475	3.605	0.725	1.290	ND	ND	1.044	1.507	1.235	1.029	ND	ND
C-3 NBT	3.82	5.003	16.883	4.662	1.075	1.776	ND	<MDL	1.159	1.470	1.938	1.211	ND	ND
Benzo(a)Anthracene	5.494	8.127	11.447	7.169	0.79	0.96	1.41	1.61	3.125	8.105	2.738	1.614	<MDL	ND
Chrysene	25.929	12.767	36.56	5.286	0.67	1.09	<MDL	1.46	4.166	14.541	3.889	13.725	<MDL	10.9
C-1 Chrysene	7.811	11.613	63.346	7.013	1.286	1.741	ND	1.33	2.765	7.155	2.536	2.627	ND	ND
C-2 Chrysene	5.673	11.217	50.368	6.991	1.650	2.577	<MDL	ND	2.352	3.725	4.615	2.043	ND	ND
C-3 Chrysene	6.14	8.085	27.508	7.623	1.419	1.813	ND	ND	1.566	2.369	1.644	1.802	ND	ND
C-4 Chrysene	9.124	16.896	6.188	14.986	2.032	3.564	ND	ND	2.535	2.442	3.142	3.047	ND	ND
Benzo (b) Fluoranthene	3.336	6.253	20.477	3.56	1.42	2.75	2.54	1.42	5.497	32.054	7.267	7.890	1.77	ND
Benzo (k) Fluoranthene	1.276	3.546	7.012	1.78	0.52	0.79	1.23	<MDL	2.146	6.802	1.963	3.412	1.11	ND
Benzo (e) Pyrene	2.061	4.22	15.362	2.533	0.83	1.52	1.88	1.35	2.789	15.790	3.398	4.095	1.74	<MDL
Benzo (a) Pyrene	1.477	2.559	7.491	2.022	0.544	1.016	<MDL	<MDL	2.084	11.490	2.542	1.490	<MDL	1.05
Perylene	5.169	6.043	14.53	9.045	5.13	4.36	1.69	1.84	9.836	4.251	5.359	4.957	<MDL	12.9
Indeno (1,2,3 - cd) Pyrene	1.072	1.734	4.044	1.009	0.43	0.582	<MDL	<MDL	1.231	6.974	1.303	1.761	<MDL	<MDL
Dibenzo (a,h) anthracene	0.116	0.334	1.14	0.218	0.095	0.173	<MDL	<MDL	0.437	1.551	0.457	0.431	<MDL	1.21
Benzo (g,h,i) perylene	0.797	1.555	3.85	0.78	0.55	0.617	<MDL	<MDL	0.946	4.006	1.256	1.694	<MDL	<MDL
Total PAHs (ppb)	338.375	980.242	1219.285	277.775	85.7	295.9	9.50	9.00	173.915	333.502	363.639	447.017	5.85	26.1

Figure 4.22 Total Polycyclic Aromatic Hydrocarbon (PAH) content of sediments in East Louisiana as analyzed by GC-MS/SIM. Concentrations are in ppb of 18 PAH families. ND is Not Detected and MDL is Maximum Detection Limit. Abbreviations of samples and locations from where sediment was collected can be found in the Figure 3.1

4.16 Comparison of OTUs in sediments with PAH content above vs. below the baseline in Grand Bayou

No significant difference in OTU richness and composition was observed between the minimally oiled and sediments of Grand Bayou with a PAH content below the established baseline as seen in Figures 4.23, 4.24. Nevertheless, Hymenostomatia (*Tetrahymena*), Bivalvia (mollusks), Maxillopoda (Crustacea) and Peronosporomycetes (fungus like eukaryotes) were significantly enriched in the minimally oiled sediments of Grand Bayou as seen in Figure 4.25.

Although the numeric median and range of observed OTUs was higher at sites with PAH content below the baseline than the values at the minimally oiled sites of Grand Bayou, OTU richness was not significantly different (P = 0.568).

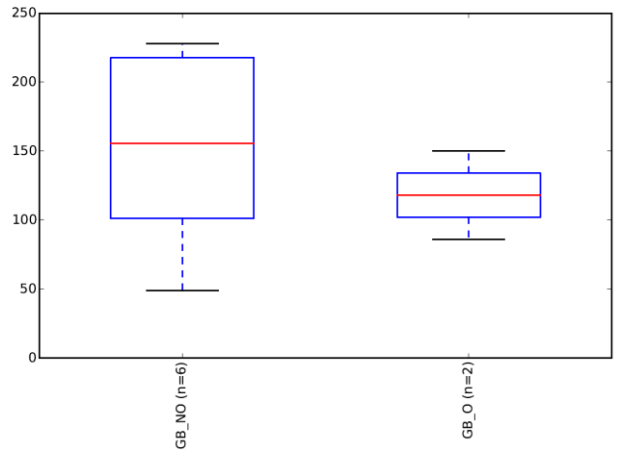

Figure 4.23 Box plots showing diversity of OTUs between sediments which were minimally oiled and unoiled sediments. X-axis represents the number of OTUs and Y-axis denotes the oiled and unoiled locations of Grand Bayou. Red lines indicate the median for each location, the top of the box denotes the upper and lower quartile and the whiskers show the maximum and the minimum (excluding outliers). A non-parametric t-test did not show significant differences in the OTU richness in in minimally oiled and unoiled sediments.

Non-metric multidimensional scaling plot (NMDS, Figure 24) showed that minimally oiled sediment GB3 was within the ordination hull of the group of non-oiled sediments. This overlap between the two groups indicates that there was no significant difference in the composition of OTUs between minimally oiled and unoiled sediments. In addition, permutational multivariate analysis of variance (PERMANOVA with function Adonis) confirmed that there was no significant difference in the overall composition of OTUs between minimally oiled locations of Grand Bayou and those with PAH below the baseline (P = 0.631). However, the non-significant

result should be viewed as preliminary since the low sample size of oiled sediment might have limited the power of the statistical test.

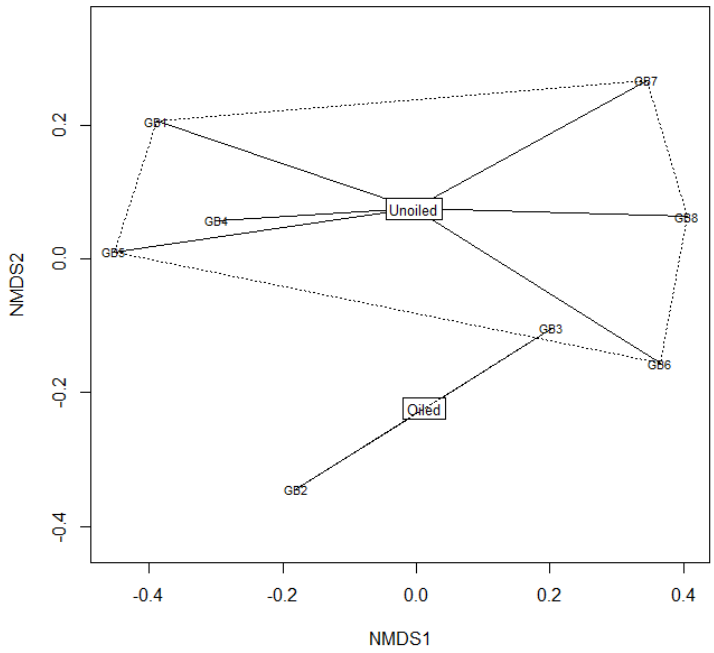

Figure 4.24 Non-metric multidimensional scaling plot (NMDS) based on Bray Curtis distances of composition of OTUs in sediments grouped on the presence/absence of oil in Grand Bayou. The ordination hull denoted by dotted lines indicates the group of sediments with a Total Polycyclic Aromatic Hydrocarbon (PAH) content <700 ppm (Unoiled). Sediments where Total Polycyclic Aromatic Hydrocarbon (PAH) content >700 ppm; GB2 and GB3 = Oiled). Non-dotted lines denote distance of the sediment from the group mean. Abbreviations of samples and locations of sites from which sediment was collected can be found in the Figure 3.1.

Although NMDS and PERMANOVA did not show significant differences in overall beta diversity based on OTU composition between minimally oiled and sites with PAH < 700ppb, a detailed breakdown of the relative abundance differences of "genera" using LEfSe did show enrichment of a number of taxonomic groups. This difference occurs because NMDS is based on Bray Curtis distances which measure numbers of shared OTUs whereas Kruskal Wallis test used for LEfSe computes differences between groups based on relative abundances of OTUs.

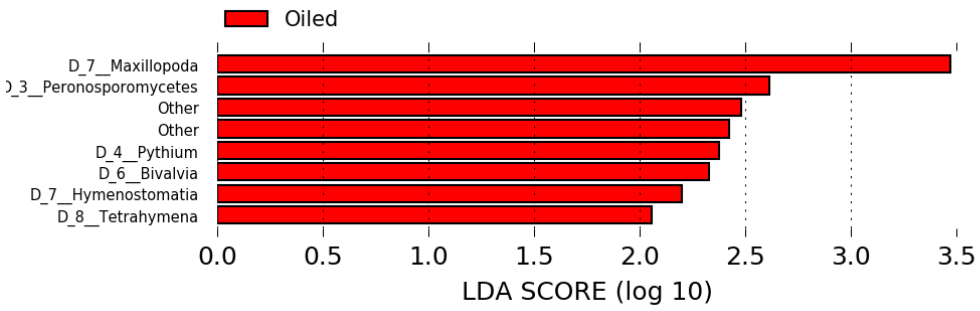

Figure 4.25 LEfSe (Linear discriminant analysis Effect Size) results show significantly enriched "genera" in the minimally oiled locations in Grand Bayou in comparison to locations with PAH content below 700 ppb.

Relative abundance is the percent composition of an organism relative to the total number of organisms making LEfSe more sensitive than NMDS. Hymenostomatia (*Tetrahymena*), Bivalvia (mollusks), Maxillopoda (Crustacea) and Peronosporomycetes (fungus like eukaryotes) were significantly enriched in lightly oiled locations of Grand Bayou. Enrichment of these groups in the minimally oiled locations indicate that they were either resistant to oil, possess the ability to degrade oil or the oiling was not at lethal levels.

4.17 Effect of sediment chemistry on OTUs in the sediments and larval guts

The sediment chemistry parameter pH had an effect on sediment OTUs and showed a positive correlation to Liliopsida and Malassezia whereas a negative correlation of pH was observed with Gregarina, Aplanochytrium, Pseudocohnilembus, Euplotes from the SAR supergroup, the fungus *Leptosphaeria maculans*, the Nematode Nygolaimidae and the Annelid Enchytraeidae.

4.18 Effect of sediment chemistry parameters on sediment OTUs

Correlation of distance matrices for each sediment chemistry parameter to the UniFrac distance matrix of sediment OTUs indicates that similarity of most sediment biochemistries did not

influence similarity of OTU composition significantly, because the Mantel r statistic is close to zero and the p value is > 0.05 as seen in Table 4.4. However, a slightly positive and statistically significant correlation is detected between similarity in pH and sediment OTUs because the Mantel r statistic is 0.22 and the p value is 0.01. Similarity in Phosphorus and Manganese contents of sediments had a marginal impact on similarity in OTU composition ($p<0.10$).

Table 4.4. Correlation of Euclidean distance matrices of sediment parameters with the UniFrac distance matrix of sediment OTUs in QIIME v1.9. Mantel r statistic denotes the strength of the correlation and p value indicates the significance of the Mantel test. Number of permutations used for all correlations = 999.

Sediment chemistry	Mantel r statistic	p-value
Calcium	0.13034	0.3
Chloride	0.11965	0.348
Conductivity	-0.04835	0.561
Magnesium	0.13931	0.235
Salts	-0.04835	0.569
SAR	-0.05364	0.776
Sulfur	0.12273	0.315
Copper	0.05424	0.53
pH	0.22117	0.012
Phosphorus	0.17093	0.095
Potassium	-0.03602	0.595
Copper	-0.00227	0.989
Zinc	-0.15455	0.133
Iron	0.04679	0.849
Manganese	0.14146	0.063
Organic content	0.06864	0.545
Boron	-0.0876	0.662
Aluminium	0.0665	0.81
Nitrogen	0.02607	0.797
Toxicity	0.05943	0.629

4.19 OTUs affected by pH

As similarity of OTUs in sediments was influenced by similarity in pH a correlation analysis was done in QIIME v1.9 to determine which OTUs' abundances are significantly correlated to pH-levels.

No.	Taxonomy	Test stat.	pval
OTU1	D_0__Eukaryota; D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_9__Magnoliophyta; D_10__Liliopsida	0.5106	0
OTU2	D_0__Eukaryota; D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_9__Magnoliophyta; D_10__Liliopsida	0.494981	0
OTU3	D_0__Eukaryota; D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_9__Incertae Sedis; D_10__Malassezia; D_11__uncultured fungus	0.425305	0
OTU4	D_0__Eukaryota; D_1__SAR; D_2__Alveolata; D_3__Apicomplexa; D_6__Eugregarinorida; D_7__Gregarina	-0.58372	0
OTU5	D_0__Eukaryota; D_1__SAR; D_2__Stramenopiles; D_3__Labyrinthulomycetes; D_4__Thraustochytriaceae; D_5__Aplanochytrium	-0.47462	0
OTU6	D_0__Eukaryota; D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_5__Annelida; D_6__Enchytraeidae	-0.45951	0
OTU7	D_0__Eukaryota; D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_7__Scuticociliatia; D_8__Pseudocohnilembus	-0.45235	0
OTU8	D_0__Eukaryota; D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_6__Euplotia; D_7__Euplotes; Ambiguous_taxa	-0.45235	0
OTU9	D_0__Eukaryota; D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_6__Enoplea; D_7__Nygolaimidae	-0.45235	0
OTU10	D_0__Eukaryota; D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_7__Eurotiomycetes; D_8__Onygenales; D_9__Leptosphaeria maculans JN3	-0.45235	0
OTU11	D_0__Eukaryota; D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_7__Eurotiomycetes; D_8__Onygenales; D_9__Leptosphaeria maculans JN3	-0.45235	0
OTU12	Unassigned	0.513751	0
OTU13	Unassigned	-0.51108	0
OTU14	Unassigned	-0.61916	0
OTU15	Unassigned	0.445015	0
OTU16	Unassigned	-0.45235	0
OTU17	Unassigned	-0.45235	0

Figure 4.26 Taxonomic classification of OTUs whose abundance is correlated with pH with correlation statistic and p-value determined using bootstrapping and Pearson correlation.

Liliopsida and uncultured fungus (Malassezia) are positively correlated with pH with a Pearson correlation statistic value of 0.4 and 0.5, respectively, indicating that the abundance of Liliopsida (*Spartina*) and Malassezia increase/ decrease with an increase/decrease of pH (Figure 4.26). On the other hand, Gregarina, Aplanochytrium, Pseudocohnilembus, Euplotes from the SAR supergroup, the fungus *Leptosphaeria maculans JN3*, the Nematode Nygolaimidae and the Annelid Enchytraeidae are negatively correlated to pH in the range of (-0.4) to (-0.5). In addition five unassigned taxa also are affected by the pH but could not be identified because there was no

match in the SILVA 119 database. Liliopsida and Malassezia were found in the tabanid larval gut indicating that pH has an influence of the tabanid larval food web.

4.20 Toxicity assay

As seen in Figure 4.27, sediments CP2, GB3, GIEP and RWR4 showed significantly higher toxicity against amphipoda in comparison to the Negative control with sediment NC1 ($P<0.05$).

Sediment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
RWR1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	86 ± 23	100 ± 0	100 ± 0
RWR2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	86 ± 23	100 ± 0	93 ± 11	93 ± 11
RWR3	100 ± 0	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11
RWR4	100 ± 0	100 ± 0	83 ± 28	100 ± 0	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	100 ± 0
RWR5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
CP2	100 ± 0	100 ± 0	73 ± 46	100 ± 0	100 ± 0	86 ± 11	100 ± 0	93 ± 11	93 ± 11	66 ± 23
CP3	100 ± 0	100 ± 0	93 ± 11	93 ± 11	86 ± 11	93 ± 11	100 ± 0	86 ± 11	100 ± 0	86 ± 11
CP4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	86 ± 11	93 ± 11	100 ± 0
CP5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11
CP6	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	86 ± 23	100 ± 0
CP7	100 ± 0	93 ± 11	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	80± 0	80± 0	93 ± 11
EI-ATC	100 ± 0	100 ± 0	100 ± 0	86 ± 23	100 ± 0	100 ± 0	60± 34	93 ± 11	100 ± 0	86 ± 11
EI-OC	100 ± 0	86 ± 11	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0	100 ± 0	100 ± 0	100 ± 0
EI6	95 ± 8	95 ± 8	100 ± 0	86 ± 11	100 ± 0	100 ± 0	93 ± 11	80 ± 20	100 ± 0	93 ± 11
EIC	94 ± 9	94 ± 9	100 ± 0	100 ± 0	100 ± 0	100 ± 0	86 ± 11	93 ± 11	100 ± 0	100 ± 0
GIEP	93 ± 11	86 ± 11	93 ± 11	93 ± 11	80 ± 20	86 ± 11	100 ± 0	93 ± 11	80 ± 20	100 ± 0
GICL	100 ± 0	100 ± 0	93 ± 11	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11
GB1	100 ± 0	100 ± 0	100 ± 0	93 ± 11	93 ± 11	86 ± 11	100 ± 0	93 ± 11	93 ± 11	86 ± 11
GB2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	80 ± 20	100 ± 0	93 ± 11	100 ± 0	100 ± 0	100 ± 0
GB3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0	80 ± 20	93 ± 11	100 ± 0
GB4	86 ± 11	86 ± 11	100 ± 0	93 ± 11	93 ± 11	93 ± 11	93 ± 11	100 ± 0	100 ± 0	100 ± 0
GB5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	86 ± 11	100 ± 0	86 ± 11	93 ± 11	80 ± 20
GB6	100 ± 0	93 ± 11	86 ± 11	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0
GB7	100 ± 0	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0	100 ± 0	93 ± 11	100 ± 0	80 ± 20
GB8	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0
Negative-1 (with sediment)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Negative-2 (no sediment)	100 ± 0	93 ± 11	100 ± 0	100 ± 0	93 ± 11	93 ± 11	100 ± 0	86 ± 11	93 ± 11	93 ± 11
Positive-1 (with sediment)	100 ± 0	100 ± 0	100 ± 0	86 ± 11	40± 20	93 ± 11	66± 11	33± 41	46± 23	60± 20
Positive-2 (no sediment)	93 ± 11	73± 11	53± 11	53± 11	46± 11	53± 30	53± 41	73± 11	26± 23	40± 34
Positive-3 (no sediment)	93 ± 11	93 ± 11	53± 11	53± 11	53 ± 30	40± 34	33± 11	60± 40	40± 52	40± 34

Figure 4.27 Sediment toxicity assay: 10 day sediment toxicity assay results using the amphipod (*Hyallela azteca).* Results represent mean percent survival of three replicates of each sediment per day on each day with standard deviation. Positive controls PC1 and PC2 contain the Water Accommodated Fraction (WAF) of 80,000 ppm of Mississippi Canyon 252 Weathered Crude Oil (Louisiana Light Sweet Crude) emulsified in distilled water and PC3 contains the Water Accommodated Fraction (WAF) of 133,000ppm of Louisiana Light Sweet Crude oil emulsified in distilled water. The sample names are defined by their location (see Figure 3.1).

Both Negative Controls throughout the 10 day period had a mean percent survival more than 86% (Figure 4.27) which is more than the minimum mean control survival (70%) required for test acceptability in the ASTM protocol (ASTM, 1993).

Table 4.5 Two-sample T-test in R comparing the toxicity of each sediment to the Negative Control 1 (NC1):

No.	Sediment	P-value (two sample t-test)
1	EIAC	0.3384
2	CP2	0.03329*
3	CP3	0.3797
4	CP4	0.2863
5	CP5	0.1342
6	CP6	0.1869
7	CP7	0.3797
8	EI6	0.7589
9	EIC	0.8829
10	GB1	0.5495
11	GB2	1
12	GB3	0.01165*
13	GB4	0.09415
14	GB5	1
15	GB6	0.7438
16	GB7	0.1209
17	GB8	0.1342
18	GIEP	0.4791
19	EIOC	0.4968
20	GIEP	0.04722*
21	RWR1	0.3426
22	RWR2	0.5418
23	RWR3	0.1342
24	RWR4	0.01165*
25	RWR5	NA

Data of the 10 day sediment toxicity assay was not normally distributed as indicated by the Shapiro Wilks test ($p < 2.2e-16$) and had unequal variances as demonstrated by Levene's test for

homogeneity of variances ($p < 2.2e-16$ ***). Kruskal Wallis test showed that there was significant effect of time on sediment toxicity ($p = 7.089e-07$). Further testing using the Post-hoc Kruskal-Nemenyi tests showed that there was a significant difference in survival of amphipods between Day 1 and Day 8 ($P= 0.01$) and Day 3 and Day 8 ($P= 0.02$). A significant difference in amphipod survival was seen between NC1 (Negative control with sediment) and PC1 (Positive control with sediment) using the Welch's two sample t-test that assumes non-normal data with unequal variances ($P = 0.0002365$). Similarly, NC2 (Negative control without sediment) displayed a significantly higher ($P = 1.746e-09$) amphipod survival than PC2 (Positive Control without sediment) and PC3 (Positive Control without sediment at a higher WAF, $P= 2.786e-07$). Amphipod survival was numerically but not significantly lower in the Negative Control without sediment (NC2) in comparison to the Negative Control with sediment(NC1) ($P = 1$). On the other hand, amphipod survival was significantly lower in both positive controls without sediment (PC2, $P= 0.03891$) and PC3, $P=0.05955$) in comparison to amphipod survival in PC1 (with sediment). Sediments CP2, GB3, GIEP and RWR4 had significantly lower amphipod survival in comparison to the Negative control NC1 (with sediment) whereas amphipod survival in the rest of the 21 sediments was not significantly different (Table 4.5). GB3 was minimally oiled and this could be a factor for toxicity in this case. However, GB3 had significantly higher amphipod survival than PC1 ($P= 2.528e-05$), PC2 ($P= 7.741e-10$) and PC3 ($P= 4.359e-08$). In addition, the PAH contents of GIEP in East Louisiana and CP2 and RWR4 in West Louisiana were below the established threshold of 700 ppm and thus, oiling cannot be the only explanation for toxicity. Toxic effects of unoiled sediments on *Hyallela azteca* mortality using similar experimental conditions such as in this study have been observed previously and can be caused by contaminants other than oil (see Chapter 5. Discussion).

Chapter 5. Discussion

Coastal marshlands including the salt marshes of Louisiana are threatened by a combination of natural and anthropogenic stressors (Barbier et al., 2011, Orth et al., 2006, Kirwan and Megonigal, 2013). Pollution caused by agricultural runoff, chemical and oil spills, land loss through sea level rise, subsidence, erosion and hurricanes, as well as physical destruction of marsh for oil and gas exploration projects are some of the known threats of coastal marshlands (Silliman et al., 2012, Kirwan and Megonigal, 2013, O'Riordan et al., 2000). When coastal marsh ecosystem health suffers, habitats of wildlife are converted, fragmented and/or destroyed thereby endangering the diverse and productive marshes (Reed and Wilson, 2004, DeLaune et al., 1983). Protective capacity of the coastal marshlands weakens, consequently making terrestrial ecosystems more vulnerable to ecological impacts (Silliman et al., 2012). Quick, accurate and reliable tools to assess marsh health are required not only after natural and anthropological impacts mentioned above but also to monitor recovery and positive impacts of coastal restoration projects (O'Riordan et al., 2000, Morris et al., 2015).

The green head horse fly *Tabanus nigrovittatus* is native to the coastal marshes from Texas to Nova Scotia (Hansens, 1979). More importantly, its larvae develop in the marshlands for long periods of time (Magnarelli and Stoffolano Jr, 1980). The functional role in the marsh ecosystem of the *T. nigrovittatus* larvae is that they are top invertebrate predators and their development is indicative of an undisturbed food web and, thus, a healthy marsh (Magnarelli and Stoffolano Jr, 1980). Hence, *T. nigrovittatus* has all the characteristics of a promising model for bioindication of a healthy marsh.

The Deepwater Horizon Oil Spill in 2010 provided an opportunity to test the *T. nigrovittatus* model for its capacity to indicate a disturbed marsh ecosystem. Population crashes of adults and larvae were observed in East Louisiana at or near sites where the oil made landfall. No such crashes were observed in West Louisiana, where the oil did not reach, despite similar collection efforts (Husseneder et al., 2016). This observed population crash of the larvae in the vicinity of oiled areas of East Louisiana led to the hypothesis that larval decline was a result of either direct or indirect effects of oil. Direct effects of oil could either be toxicity to adults, which would reduce their breeding population and/or toxicity to eggs and larvae in the sediment. Indirect effects could be caused by an alteration of the food web either due to oil itself or a change in sediment chemistry. The as yet undeciphered *T. nigrovittatus* larval food web would have to be studied to determine the key components that sustain the larvae in the *Spartina* salt marshes. Describing the taxa found in the larval gut and surrounding sediments not only showcases the constituents of the food web of the larvae but also answers questions about tabanid biology. Comparing food webs between sites where larvae were present and absent would indicate if certain meiofauna taxa can be used as predictors of larval presence. Measuring the effects of presence/ absence of oil and different parameters of sediment chemistry on the food webs was important and will help to assess whether absence of larvae was indirectly caused by alterations in food web composition. Lastly, comparing food webs among locations and based on geography (East and West) would aid in answering the question why larvae were absent in East Louisiana in comparison to the West.

5.1 Species ID of larvae

The first challenge was species ID of larvae collected in the study. There are morphological keys for adults and larvae (Merritt and Cummins, 1996, Sofield et al., 1984). However, morphological

keys might not be sufficient to identify cryptic species within the *T. nigrovittatus* species complex. Therefore, we employed DNA barcoding to identify larvae to the species level. The mitochondrial cytochrome oxidase subunit 1- 5' sequences of the adult reference specimen of *T. nigrovittatus* from Louisiana marshes identified by morphological measurements (Sofield et al., 1984) differed by 5% from a previously submitted *T. nigrovittatus* from Florida. While this variance might be population differentiation, it underscores the need for future study of the genetic variances to resolve the species complex. For the present study, larvae were DNA barcoded and compared to the reference specimen of *T. nigrovittatus*.

Barcoding of the tabanid larvae (see Results 4.1) indicated that most of them belonged to the species *T. nigrovittatus* (≥ 97% similarity at all three DNA loci) or at least were closely related species within the *T. nigrovittatus* complex. However, the ones that showed only a moderate percent identity match (92% to 95%) to the *T. nigrovittatus* reference specimen in the mitochondrial cytochrome oxidase 1 region likely belong to cryptic species within the *T. nigrovittatus* complex. There also is the possibility that some larvae with low similarity to the reference species (<95%) at three DNA loci were *Tabanus hinellus* as this species is known to be present in Louisiana marshes (Schutz et al., 1989, Mullen et al., 1988) and adult specimens have been collected at the sampling locations by our team.

Although more work is needed to resolve the species issue, for purpose of this study we considered all tabanid larvae equally as top predators in the marsh soil and therefore investigated their food web regardless of their exact species ID. Studies are in progress to DNA barcode additional tabanids from Louisiana marshes, which will help with species ID in the future.

5.2 Diets of tabanid larvae

Insects were the main component of tabanid larval diet (see Results 4.7). Insect families included Drosophilidae, Culicidae and Tabanidae in the larval guts (see Results 4.8). Invertebrate predators are known to forage specifically for proteins and lipids to redress specific nutrient imbalances (Mayntz et al., 2005) . Insect predators from the Belostomatidae and Nepidae families feed on snails, frogs, tadpoles and aquatic insects including mosquito larvae in the wetlands of Japan (Ohba and Nakasuji, 2006). Tabanid larval diets have been previously reported to consist of detritus, algae, bacteria and small invertebrates in the US (Frouz, 1999). Cannabilistic traits in tabanid larvae also have been reported (Axtell, 1976).

In our larval food web study (see Results 4.7), the most dominant "families" were hexapods and fungi and both were present in all larval guts. Moderate abundances of unknown metazoa followed by protozoa, algae and diatoms of the SAR superclass as well as low proportions of plant matter belonging to Liliopsida (*Spartina*) and Magniliophyta also were found in the guts. Insects were an important component of the tabanid larval diet in Louisiana marshes. Insect based diets have been reported in other tabanids. Insects have been previously reported as major components in tabanid diets in Poland (Frouz, 1999). Diet components are a subset of the major insect orders described in Louisiana marsh (Adams et al., 2017, Bam, 2015, Pennings et al., 2014, McCall and Pennings, 2012) and the sediments from this study.

Major insect orders found in the Louisiana marshes include Diptera, Coleoptera, Collembola, Hymenoptera, Hemiptera, Orthoptera, and Thysanoptera (Bam, 2015, Adams et al., 2017, Pennings et al., 2014). Of those, we found OTUs belonging to Diptera, Collembola, Hymenoptera and Hemiptera in the tabanid guts (see Results 4.2, 4.7). Insects also are known to parasitize tabanid larvae. Tachinidae (Diptera) parasitize the larvae whereas egg masses are

known to be parasitized by Hymenoptera (Scelionidae, Mymaridae and Trichogrammatidae) and pupae by *Trichopria tabanivora* (Axtell, 1976). Hence, the parasitic wasp found in the larval gut could be a larval parasite. As *T. nigrovittatus* larvae live in the sediment of *Spartina* marshes, insects associated with *Spartina* were a part of the larval food web (see Results 4.8). Hemipterans associated with the *Spartina* include *Prokelisia sp*., *Chaetopsis sp*. (stem-boring herbivore), grasshopper, *Orchelimum jidicin, Chlorochroa senilis* and *Ischnodemus badius* (Pennings et al., 2014, Pfeiffer and Wiegert, 1981).

Biodiversity of insects in salt marshes is not well described. Over 100 species of arthropods are found associated with *Spartina alterniflora* salt marshes (Pfeiffer and Wiegert, 1981, Wimp et al., 2010). However, few of these arthropods are described to the species level (Adams et al., 2017). Insects in salt marshes are vaguely described by the ecosystem they belong to (aquatic, terrestrial, benthic and wetland) or by their place in the environment (epiphytic and free-swimming) or their common names (mosquitoes, biting flies, and biting gnats) (Batzer and Wissinger, 1996, Pennings et al., 2014). Insects have been identified depending on their functional role such as detrivores, omnivores, herbivores, and predators (Pennings et al., 2014). These categories have been further divided into feeding guilds by other studies such as stem boring and sucking herbivores, parasitoids (McCall and Pennings, 2012).

Out of the 52 OTUs identified as Insecta in this study, only 23 were identified to the species level by the SILVA 119 database (see Results 4.8). These included *Aedes aegypti*, *Aedes albopictus*. *Drosophila sechellia, Haematopota pluvialis* and *Leptopilina heterotoma* (see Results 4.8). However, most of these insect species are not known to live in salt marshes. DNA metabarcoding indicated that dietary components of the tabanid larvae are related to species within the genera *Drosophila, Aedes* and the Family Tabanidae. *Drosophila sechellia* is not

known to occur in the marsh. The closest relative known to occur in LA marshes is a species from the genus *Scaptomyza* of the family Drosophilidae. *Scaptomyza pallida* (Zetterstedt) is a known parasitoid of a fungal gnat known to be associated with the marsh marigold (Eiseman et al., 2016) . *Aedes solicitans* and *Aedes taeniorhynchus* represent the Genus Aedes in the Louisiana marsh (Ailes, 1998), and ,thus, the *Aedes* sequences found in the larval guts originated most likely from those species. *Tabanus nigrovittatus, Tabanus hinellus* and *Chrysops spp.* are horse and deer fly species from the Family Tabanidae known to be present in the Louisiana marshes (Hansens, 1979, Mullen et al., 1988, Schutz et al., 1989). Hence, the sequences found in the larval guts likely belong to those species, indicating that some life stages of these species are the prey of the tabanid larvae. The presence of *T. nigrovittatus* DNA in larval guts both in the cloning (see Results 4.2) and 18S rRNA gene libraries (see Results 4.8) are likely a result of cannibalism, since cannibalistic traits have been reported in tabanid larvae (Axtell, 1976). However, contamination by host DNA cannot be excluded despite careful dissection.

Unclassified fungi was the other major component in the tabanid larval guts besides insects (see Results 4.7). The presence of fungi can be explained as fungal symbionts or parasites of tabanid larva itself, fungi associated with prey species, fungi as food source of tabanids or their prey, and/or fungi in general associated with the larval habitat. The family Saccharomycetaceae was found previously in the hindgut of mosquito larvae (Tajedin et al., 2009) suggesting that this fungus might be a part of the larval microflora. Capnodiales and Cyberlindnera have been isolated from beetle larval guts (Lou et al., 2014, Rojas-Jiménez and Hernández, 2015) whereas Kazachstania have been reportedly found in the gut of black soldier flies (Zheng et al., 2013). Sympodiomycopsis has been isolated from insect frass (Chen et al., 2013). Hence, Capnodiales,

Cyberlindnera Kazachstania and Sympodiomycopsis are fungi identified in the tabanid larval guts which have been associated with insect guts previously and might possibly be symbionts.

Symbiotic fungal associations of tabanid larvae have not yet been studied. However, fungi are known to be associated with the hindgut walls of other Diptera larvae such as Culicidae, Chironomidae, Simuliidae, Ceratopogonidae, Tipulidae and Thaumaleidae larvae (Pereira et al., 2005). Thus, there could be fungal symbionts in tabanid larva guts. Some tabanid larva fungal association could be parasitic as Coelomomyces fungus is known to parasitize tabanid larval haemocoel (Axtell, 1976).

Besides direct fungal association with tabanid larva, fungi also could be associated with the prey of tabanid larvae. For example, mosquitos (likely as eggs and larval stages) were a prevalent part of tabanid larval diet. The larval stages of mosquitoes harbor fungi as symbionts (Tajedin et al., 2009) and fungi (*Penicillium citrinum*) parasitize *Aedes* spp. eggs (Russell et al., 2001). Black fly larvae host at least 20 species of tricomycetes (Tajedin et al., 2009). Mycophagous Coleopteran larvae ingest Ganoderma as a food source (Kadowaki et al., 2011).

Besides fungi associated with the tabanid larva itself and/or its prey, the unclassified fungi could have originated from the environment, i.e. soil and plants. Fungi in the marshes are varied but the most abundant are Ascomycetes (Gessner and Kohlmeyer, 1976, Boland and Grund, 1979). The fungi isolated from *Spartina* included Ascomycetes, Zygomycetes and Coelomycetes where Ascomycetes (especially Genera Mycosphaerella and Phaeosphaeria) were abundant on the lower stems of *S. alterniflora* (Al-Nasrawi and Hughes, 2012, Boland and Grund, 1979). As fungi play such diverse roles in insect larval guts and were found in dominant proportions in the diet of tabanid larvae the unclassified fungal component of the tabanid guts should be studied further using fungal primers.

The fungi identified by the SILVA 119 database include Malassezia (most abundant), Saccharomycetaceae, Capnodiales, Cyberlindnera, Kazachstania and Sympodiomycopsis, Dothideomycetes. The genus Malassezia has been found in marine sediments previously and is reportedly ecologically hyper diverse. For example, Malassezia has been captured by metagenomics studies across a variety of habitats, including sediments from deep hypersaline anoxic basin redox clines and deep subsurfaces (Zhang et al., 2015, Rédou et al., 2014, Bernhard et al., 2014, Amend, 2014). Dothideomycetes are pathogenic microfungi of *Spartina* (Elmer, 2016) suggesting that they were acquired from the environment.

A significantly higher abundance of Agricomycetes/Agaricomycotina was observed in the diets of larvae found at Cypremort Point in comparison to the abundance of Agricomycetes/Agaricomycotina in the larvae found in Rockefeller Wildlife Refuge (see Results 4.9). However, the group of Agricomycetes/Agaricomycotina was not observed in the sediments of Cypremort Point (see Results 4.11) and was only present in the sediments from Rockefeller Wildlife Refuge. This suggests that this fungus is not derived from the surrounding sediment but could be a member of the larval gut community or associated with the prey of the larva.

Ciliated protozoa specifically *Tetrahymena* also were found in the larval gut (see Results 4.7) albeit with less than 200 reads in all larval guts except for one (RWR5.8.LG) which contained over 70,000 reads. Ciliated protists have been shown to be a part of mosquito diets (Merritt et al., 1992). Hence, the protists could be prey to the tabanid larvae, especially during early larval stages. Protozoa such as *Tetrahymena* are known to infect mosquito and black fly larvae (Hajek, 2000). *Tetrahymena chironomi* was shown to be a facultative parasite in chironomid larvae (Corliss, 1960). Another species of *Tetrahymena*, namely *Tetrahymena sialidos* found in the haemocoel of larval Megaloptera was proven to be a parasite involved in controlling invertebrate

populations using parasitism (Batson, 1985). Thus, the protozoa in the tabanid larva gut could originate from their prey species.

Non-prey dietary components in the larval guts include plant material and algae (see Results 4.7). Tabanid larvae were collected from *Spartina* marshes and their habitat contains plant roots and other below-ground biomass. Hence, it is possible that their prey is laced with some plant material and that DNA extracted from the gut contents of the larvae also contained DNA of the plants ingested by the larvae's prey. Plants/algae has been found in the guts of predatory larvae previously (Merritt et al., 1992) including tabanids (Axtell, 1976). Algae have been previously reported to be found in the diet of predaceous aquatic larvae (Merritt et al., 1992) including tabanids (Axtell, 1976) . Algae are part of aquatic environments and are likely associated with organisms that tabanid larvae prey on (Merritt et al., 1992). Chromulinales are photosynthetic single celled algae present abundantly in the marine environments (Reynolds, 2006). Presence of algae in the diets of insects have been previously correlated to their abundance in the surrounding environment suggesting that Chromulinales could be ingested during larval predation (Patterson et al., 1993).

Overall, the gut content of tabanid larva contains organisms that are likely to be the direct prey of larvae in their different development stages (ranging from ciliated protozoa to soil dwelling insects) but also include organisms that are likely to be associated with prey organisms, such as single celled algae and fungi.

5.3 Taxa present in sediments with and without larvae

A high proportion of Opisthokonta ("classes" Holozoa and Nucletmycea) in sediment samples was a good predictor for finding larvae in the West (see Results 4.10). This was confirmed by

LEfSe analysis showing a higher presence of Opisthokonta at the sites where larvae were found in comparison to the sites where larvae were absent. The Holozoa contain insects and the Nucletmycea contain fungi, both of which have been shown to be the major components in the larval gut and are, thus, likely associated with larval diet (see Results 4.7, 4.10). This suggests that a dominant presence of diet components in the sediment might be conducive to larval development.

While the dominance of Opisthokonta was associated with larval presence in the West, proportions of "families" and "family" diversity did not always predict larval presence (see Results 4.10). While most larvae were found at sites with predominant proportions of hexapods and fungi, high "family" diversity resulting in low relative proportions of hexapods and fungi did not preclude the presence of larva (RWR2).

In the East, however, larvae were absent even from sediments with high Holozoa and Nucletmycea ("class") or hexapods and fungi ("family") content. This suggests that there were other factors impacting larval numbers in the East besides food web composition (see last part of the discussion).

We initially hypothesized that sediments with high content of larval diet components would be better able to sustain larval development. However, it was not guaranteed that larvae can always be found even if they were present in those sediments. The distribution of larvae is patchy since females of *T. nigrovittatus* lay large numbers of eggs in batches and larvae cannibalize each other, lowering their density (Magnarelli and Stoffolano Jr, 1980, Axtell, 1976). Thus, larvae cannot always been found by our sampling method even if they are present in the general vicinity. Also, larvae are mobile and swim in pursuit of food making them less dependent on food web content of microhabitats (https://www.youtube.com/watch?v=GAdm_JqAuoM).

5.4 Effect of PAH on OTU composition

East Louisiana *Spartina* marshes took the brunt of oiling after the Deepwater Horizon Oil spill in 2010 (Silliman et al., 2012). *Spartina* marshes were a barrier between terrestrial and marine ecosystems preventing the oil from flowing inland (Silliman et al., 2012). However, due to the oil accumulated in the *Spartina* marshes heavy degradation of *Spartina* and associated organisms occurred (Silliman et al., 2012). *T. nigrovittatus* larvae live in the *Spartina* marshes where they develop for long periods of time (Magnarelli and Stoffolano Jr, 1980). Impact of oil in this region led us to the hypothesis that absence of larvae at most sites in the East (i.e. in the vicinity where oil made landfall) was due to oil contamination and changes in food web.

Surprisingly, we did not find a difference in the overall alpha- and beta diversity of taxa between Eastern sites where oil was present and absent. Even reducing overall variability by focusing on the same region (Grand Bayou) did not show significant differences in taxa composition between sites where oil detected a year after the DHOS and sites where oil was not detected a year after the DHOS (see Results 4.16). The lack of statistical significance was due to the limited number of oiled sites sampled, the low concentration of oil even at sites exceeding the background PAH level of 700 ppm, low sensitivity and/or fast recovery of the meiofauna community.

Our sites were determined before the oil made land fall. Although oil was reported along the Eastern coast of Louisiana, i.e. in the vicinity of our sample sites and our samples were collected at the high tide , within the oil penetration limit of <15 m in the marshes (Silliman et al., 2012), oiling was rather patchy (Boesch et al., 1994). Patchy distribution was due to wind and ocean currents driving the oil in some areas at a higher intensity than the others. Also, as observed in in Barataria Bay, intensity of oiling depended on the presence of heavily oiled above ground vegetation where oiled remained rooted in place even after the vegetation died and did not show

any signs of weathering. In addition, presence of wrack lines also contributed to overlaying emulsified oil on the marsh substrate. Therefore, only two of our sediment samples from Grand Bayou were directly contaminated with oil likely originating from the DWOH oil spill (see Results 4.15). Although oiled, these sites (GB2 and GB3) had only 0.00098 mg/g and 0.001219 mg/g of Total Petroleum Hydrocarbons (TPHs), which was lower than the lightly (6.2 mg/g - 13 months post DHOS) , moderately (70 mg/g - 9 months post DHOS) and heavily (510 mg/g - 9 months post DHOS) oiled sites that were the models for describing impact of the oil spill on soil fauna (McCann et al., 2017)

Evidence suggests that recovery at lightly oiled sites commenced as soon as within 17 months as the stem density of the *Spartina* marshes and associated benthic microalgae recovered (Fleeger et al., 2015). Most meiofauna, such as copepods which feed on the benthic microalgae recovered fast on pace with the *Spartina*. The arthropod population density also had rebounded by 2011 (McCall and Pennings 2012).

While the majority of meiofauna recovered fast (Fleeger, Carman et al. 2015), others such as polychaete *Manayunkia aestuarina*, ostracods and kinorhynchs, had not recovered even 4 years after the spill (Fleeger et al., 2015). Different sensitivity to oil impact and different rates of recovery might explain why we found differentially enriched taxa at our minimally oiled sites (see Results 4.16), such as Hymenostomatia (*Tetrahymena*), Bivalvia (mollusks), Maxillopoda (Crustacea) and Peronosporomycetes (fungus like eukaryotes).

Tetrahymena are ciliated protozoa. Ciliated protists have shown an increased abundance in the presence of oil in in vivo and in vitro studies because they feed on the oil degrading bacteria that increase in the presence of oil (Hazen et al., 2010). Bivalvia show depressed oxygen consumption via gills and decreased filtration rates in the presence of oil (Burger, 1994).

However, Bivalves were detected a year after the Exxon Valdez oil spill possibly due to their ability to close themselves immediately after an environmental stimulus (Burger, 1994). Physical barrier of the epicuticle and fast rebounding population capacity of Maxillopoda (Crustaceans) could be possible reasons why this group was detected in the minimally oiled sediments (Felder et al., 2014). Peronosporomycetes (fungus like eukaryotes) are plant pathogens. As such they might have infected weakened *Spartina* plants at the oiled sites (Pal and Gardener, 2006).

5.5 Effect of sediment chemistry on OTU composition

Similarity of the sediment chemistry parameter pH influenced the similarity of OTUs in sediments (see Results 4.17). In our study, pH had a positive correlation with the abundance of Liliopsida and Malasezzia in the sediments (see Results 4.17). In contrast, a previous study found a negative correlation of pH with *Spartina alterniflora* abundance under experimental conditions (Slocum and Mendelssohn, 2008). However, this discrepancy could also occur because salinity levels contribute to the effect of pH (Linthurst, 1979) . A negative correlation was observed between pH and the relative abundance of Gregarina, Aplanochytrium, Pseudocohnilembus, and Euplotes from the SAR supergroup, the fungus *Leptosphaeria maculans JN3*, the Nematode Nygolaimidae and the Annelid Enchytraeidae (see Results 4.17). However, a previous study did not detect any correlation of sediment pH and Annelids (Schlaghamerský and Tříska, 2009).

5.6 Sediment toxicity

Two sediments collected in 2011 from West Louisiana (RWR4 and CP2) and two sediments from the East (GB3 and GIEP) caused increased mortality in bioassays using amphipods (*Hyalella azteca*) as models (see Results 4.18). GB3 was the sediment sample with the highest

oil content (1,219 ppb) and thus oiling might have contributed to toxicity (see Results 4.15, 4.18). However, GIEP was not oiled above baseline (363 ppb), and the Western samples were collected outside of the range of the oil spill.

Since amphipods were chosen for their pronounced sensitivity to sediment toxicity (Landrum et al., 1991, Connolly et al., 2004, Emery et al., 1997), their survival shows that most sediments in the East were not toxic at the time of testing and, thus, tabanid larval decline in Eastern Louisiana might not have been due to sediment toxicity. However, the lab results have to be treated with caution since other studies reported variable results with similar bioassays.

Even in lab experiments using moderately oiled soil (0.6 $L/m^{2)}$ and similar experimental study design as our study a lack of toxicity on *H. azteca* has been observed and is attributed to time related adaptation decreasing species sensitivity and changes to bioavailability related to oil based contaminants (Blaise et al., 2004). In the same study, even reference samples showed toxicity (Blaise et al., 2004). Changes in hydrocarbon quantity and composition over time and modifications in oil chemistry profiles also could be other reasons for amphipod tolerance (Venosa et al., 2002, Blaise et al., 2004).

In addition, there is evidence of variability in the toxicity of sediments stored for long periods of time frozen at either - 4°C or -20°C (Geffard et al., 2004, Dillon et al., 1994, Becker and Ginn, 1995). Although many studies suggest increase in toxicity after long periods of storage, decrease in toxicity also has been observed. Overall, change in toxicity is unpredictable after long periods of storage (Schuytema et al., 1989) and references therein).The recommended ASTM conditions for a dependable toxicity test are storage of sediments at 4º C for not more than 8 weeks (ASTM, 1993) based on agreement that changes in the geochemical nature of sediments are minimized in these conditions (DeFoe and Ankley, 1998, Thomson et al., 1980). As toxicity assays in this

study were conducted after six years of storage, it is possible that the results of this study may not reflect toxicity of the sediment at the time of collection.

An increased survival of amphipods in the positive controls spiked with weathered crude oil with sediment in comparison to those without sediment was observed. The sediment likely offers a protective layer to the amphipods and reduces interaction with the overlaying WAF of oil thereby increasing their chances of survival.

5.7 Reasons for larval decline in the East

In 2011, tabanid larvae were found in higher numbers in West Louisiana in comparison to East Louisiana, where oil made landfall the year before. Although more sites were sampled in the East (14) compared to the West (11), only one larva was found in the East compared to 18 in the West.

We hypothesized that the lack of larvae in the east could be caused by (a) lack of specific food web components necessary for supporting larval development, which might be connected to (b) toxic effects of oil or (c) other parameters of soil biochemistry on larvae or their food web.

However, all these hypotheses were rejected. We found that (a) the group Opisthokonta, which encompasses the Holozoa including insects and Nucletmycea including fungi, was the main component of the larval diets (> 98%) as indicated by Result 4.10. A higher presence of Opisthokonta was a predictor of larval presence in the West. However, even with high abundances of Opisthokonta at sites in the East larvae were still absent. Overall, the was no significant difference in food web composition between sediments from East and West Louisiana and taxa enriched in the East or the West did not particularly reflect larval food components (see Results 4.13). This indicates that larval absence was not a result of a disturbed food web in the

East. (b) The presence of oil in sediments had no significant effect on food web composition (see Results 4.16) and was not a reliable predictor for the absence of larvae (see Results 4.10), since we failed to find larvae at a number of non-oiled sites in the East. Other studies that sampled moderately to heavily oiled sediments after the DWH oil spill found significant shifts in meiofauna composition (see literature review and above discussion). However, only two sites in our study were oiled and oil concentration was low (see Results 4.15).

Toxicity in bioassays occurred in equal numbers of samples in the East and in the West (see Results 4.18) and could not be reliably connected to oiling (see Results 4.15) nor other parameters of sediment chemistry (see Results 4.17). All of these results indicate that the larvae and their food web were not directly affected by the Deepwater Horizon Oil spill and/or sediment toxicity due to oil or biochemistry parameters in East Louisiana.

As mentioned above, one year after the DWH oil spill (2011) larvae were almost completely absent from collection sites in the East. This was not due to a permanent underlying difference in population numbers as 2016 the larval counts at Grand Bayou sites had risen to levels comparable to sample sites in the west (L. Foil, personal communication). This indicates that the oil spill did have an impact on larval populations, although the main cause was not direct toxicity on larvae and their food web. After eliminating direct effects of the oil spill on larvae and their food web, we conclude that the population crash of adult flies was the main cause of lack of larvae in the east. Immediately after the DWH oil spill the numbers of adult flies severely declined in the East, likely due to their being attracted to and landing on oil sheens because of reflected polarized light presumably mimicking fresh water (Horváth et al., 2008). The population crash led to pronounced genetic bottlenecks and a decrease of gene flow among populations in the east, and consequently, fewer breeders producing fewer larvae (Husseneder et

al. 2016). Secondly, the few remaining females of *T. nigrovittatus* probably did not get a blood meal to lay their second batch of eggs due to a decline in the terrestrial vertebrate population as result of the oil spill (Antonio et al., 2011). Since neither food web composition (see Results 4.10, 4.14) nor sediment toxicity (see Results 4.18) could sufficiently explain absence of larvae, the most likely explanation of the decline of larvae in the east remains the adult population crash in the vicinity of oil making landfall having an area-wide effect of larval population reduction even at sites that were not directly oiled.

5.8 Challenges in Taxonomic assignments

While 18S rRNA metagenomic sequencing is an invaluable tool for identifying food web dynamics (Xu, 2014, Mardis, 2008) this method has its limitations. Taxonomic assignments in DNA reference databases, such as SILVA, are not always in agreement with classical taxonomic assignments and the taxonomy files of the sequences in the database rarely allow identification to the species level. Hence, taxonomy of sequences already in the databases must be revised and reliable identification to the species level must be included. For example, in this study the main component of the tabanid larval diet belonged to "Insecta", which was identified at the "Genera" level based on the SILVA 119 taxonomy (see Results 4.8). Manual BLAST against the NCBI database confirmed that insects identified to the species level belonged to the families Drosophilidae, Culicidae and Tabanidae but the exact species could not be pinpointed (Supplementary Figure 2). There are closely related species known to occur in salt marsh, e.g. *Aedes solicitans, Aedes taeniorhynchus, Tabanus nigrovittatus, Tabanus hinellus* and *Chrysops spp.* However, their sequence information is not part of the reference databases. This points to the most important limitation of 18S sequencing: the lack of reference sequences in curated

databases. This is especially obvious when a study attempts to describe organisms and communities in less well researched environments, such as salt marshes.

Taxonomically, salt marsh insects have been lumped together at the level of orders, families and rarely identified to genera or species (Pennings et al., 2014, Batzer and Wissinger, 1996, Adams et al., 2017). Some species are identified by their functional role such as *Conocephalus aigialus* and *Orchelimum fidicinium* (omnivores), and *Hogna* sp. (predator) (Pennings et al., 2014). Even in studies where salt marsh insects have been identified to the species level DNA barcoding has not been done. Hence, 18SrRNA gene sequences of the salt marsh insect species in databases are scarce. Due to these gaps in the databases it is imperative to populate reference databases by accurately morphologically identifying and sequencing salt marsh organisms, specifically insects. Projects funded by Gulf of Mexico Research Initiative are under way to add sequences for these yet unrepresented salt marsh species. For example, Thomas et al.'s project "Genomic Responses to the Deepwater Horizon event and development of high-throughput biological assays for oil spills" is populating the databases by sequencing benthic organisms while Foil and Husseneder's project "A study of horse fly (Tabanidae) populations and their food web dynamics as indicators of the effects of environmental stress on coastal marsh health") is identifying and barcoding insect communities in the Louisiana salt marshes. Such studies will benefit future metagenomics studies of not only insect food webs but studies of all higher trophic levels.

In addition to gaps in reference databases, the low resolution power of 18SrRNA gene region targeted by the primer pair NF1 and 18Sr2b limits species assignment. This 400 bp region at the 3' end of the 18rRNA gene has been recommended to capture maximum eukaryotic diversity (see Chapter 2. Introduction) because of which it does not always have the ability to differentiate among closely related species. However, the taxonomic placements of species can still be

deciphered to a certain degree. Insects identified to the "species" level belong at least to the family classified in the database. For future studies focusing on insects, multiple regions that are better suited for insect species identification can be used (Valentini et al., 2009b). A combination of markers targeting the 18SrRNA gene and the mitochondrial CO1 gene can be used for barcoding or metabarcoding of insect species, because the 18SrRNA gene will capture overall insect diversity while the mitochondrial CO1 gene will differentiate between closely related species (Valentini et al., 2009b, Hajibabaei et al., 2007). Metabarcoding using insect specific primers 18S ai/bi used in this thesis for barcoding adults also could be a good alternative to be used in combination with mitochondrial CO1 gene or mitochondrial CO II gene (Whiting et al., 1997). ITS regions are widely used for barcoding, primarily to differentiate between closely related fungi (Schoch et al., 2012). However, with the high levels of fungi associated with the tabanid and other aquatic larvae (Tajedin et al., 2009) ITS might not be a good choice for insect larval barcoding.

Inaccurate species description of reference specimens in database also might lead to multiple or erroneous matches, but this should be the exception in curated databases. Morphological identification of adult insects is not always easy because taxonomic keys to differentiate between closely related species are not always available (Harvey et al., 2003). Insects in different stages of life cycle such as larvae or pupae usually require identification by an expert (Harvey et al., 2003). This causes insects to be inaccurately identified and consequently the sequences uploaded in the database might be from an incorrectly identified species.

Deficiency of sequences in databases identified to the species level, paucity of identified and sequenced insect species from the salt marshes, low resolution power of primers and inaccurately identified insect sequences were challenges that made taxonomic assignments difficult.

Chapter 6. Conclusions and Future

The food web of the larval stages of the green head horse fly primarily consist of the "Phylum" Opisthokonta further classified to insects and unclassified fungi. Larval diets are not particularly diverse with Opisthokonta dominating majority (15/16) of the larval guts followed by the SAR supergroup comprising algae and diatoms and minimal presence of plant materials from the "Phylum" Archeoplastida. In the future, DNA metabarcoding can be done using primers specifically targeting insects in the tabanid guts using a combination of genomic and mitochondrial regions (see Chapter 1 Introduction) to allow a more detailed classification of insect prey. The genomic DNA would cover the diversity of insects while the mitochondrial regions would be useful in discerning between closely related species. This is the first study showing that a wide array of fungi prevail in the tabanid larva guts. The Internal transcribed Spacer (ITS) region should be used to provide further details concerning fungal diversity as this region is widely used in fungal genomics especially to distinguish between closely related fungi (Schoch et al., 2012). A bacterial diversity study using the 16SrRNA gene region can be done to determine the lower trophic levels of the top invertebrate larval predator, *T. nigrovittatus*. 16S databases are comparatively more robust that 18S thereby reducing taxonomic challenges.

Bioindicators of larval presence in the East was dominance of the "Phylum" Opisthokonta supporting the hypothesis that presence of larval food web components is a predictor of larval presence. However, the reverse is not true. Absence of a high abundance of Opisthokonta does not indicate larval absence as we found larvae at sites with low Opisthokonta. In addition, dominance of Opisthokonta is not a predictor of larval presence in the East. Bioindicators that are enriched in minimally oiled sediments are Hymenostomatia (*Tetrahymena*), Bivalvia (mollusks), Maxillopoda (Crustacea) and Peronosporomycetes (fungus like eukaryotes), which

were significantly higher in minimally oiled sediments in comparison to unoiled sediments of Grand Bayou. In the future, primers used in this study can be used to determine the persistence of Opisthokonta in sediments to detect the dependability of this larval predictor in the West Louisiana. Primers also can be designed for bioindicator species prevalent in the minimally oiled areas and a spatial-temporal analysis can be done to determine efficacy of these species to detect low level of oiling. Sediment chemistry did not have an effect on OTU composition except pH which had a positive correlation with the abundance of Liliopsida and Malasezzia and a negative correlation with abundance of Gregarina, Aplanochytrium, Pseudocohnilembus, and Euplotes from the SAR supergroup, the fungus *Leptosphaeria maculans JN3*, the Nematode Nygolaimidae and the Annelid Enchytraeidae.

Neither sediment chemistry nor toxicity was different between sediments from the East and the West proving that sediment chemistry was not a contributor of the observed larval decline in the East. A wider array of sediment chemistry parameters can be used to determine the effect of direct/indirect toxicity on larvae. Toxicity assays should be conducted within eight weeks to eliminate variability due to change in sediment integrity (See Chapter 5. Discussion). In addition, the sensitivity of toxicity bioassays could be improved by observing growth rate, ability to swim, reproductive capacity and sediment avoidance besides mortality of the organism.

The lack of significant difference in food web composition and sediment toxicity between sediments from the East and the West indicates that larval decline in the East was not due to these factors. Most likely, the crashes in the adult population of T. *nigrovittatus* led to decline in breeding pairs and subsequently lower larval populations.

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Vita

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In January 2015, Devika started her MS program at the Husseneder Lab in the Department of Entomology at the Louisiana State University Agricultural center. Her research is focused on deciphering the food web of the green head horse fly using the state-of-the-art technique of DNA metabarcoding using Next Generation Sequencing.