Genomic and Metagenomic Characterization of Halophilic Microorganisms with Insights into The Bioremediation of Produced Water.

By

WILLIAM MARSH

Bachelor's of Science in Microbiology and Molecular Genetics

Oklahoma State University

Stillwater, OK

2018

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
MASTER OF SCIENCE
May, 2020

GENOMIC AND METAGENOMIC CHARACTERIZATION OF HALOPHILIC MICROORGANISMS WITH INSIGHTS INTO THE BIOREMDIATION OF PRODUCED WATER.

Thesis approved:

Dr. Babu Fathepure
Thesis Adviser

Dr. Noha Youssef
Committee Member

Dr. Mark Krzmarzick
Committee Member

ACKNOWLEDGEMENTS

I'd like to first thank Dr. Babu Fathepure for suppling me with the knowledge and the environment essential to my degree. The members of my committee, Dr. Noha Youssef and Dr. Mark Krzmarzick, were extremely helpful and I would have never picked anyone else. I'd like to thank other members of the scientific community, specifically Dr. Jason Belden, Dr. Robert Murdoch, Dr. Souk-Jhin and his Ph.D student Diako. It was always helpful having other hard working members of our lab namely, Christain Wilson and Teresa Mccarrell. I'd like to think Dr. Mostafa Elshahed and other members of the Graduate Student Acceptance committee for allowing me the chance to do such great work.

Of course, none of this would possible without the loving support of my friends and family. Firstly, words can not describe the loving support given to me by my mom, dad, and brother. From all of the care-packages, little cards in the mail, and simply having an ear to listen, made my hard days bubble away. I'd like to appreciate my friends for being there for me through every problem I had and being there to listen. My roommate, David supplied me with daily joy and weekly traditions we both grew to love. My friends Sarah, Nina, Chris, Somalisa, Alec, Joe, and Collin really made me open up and experience things I never would have had the confidence to explore. It is really difficult to describe the level of emotion I have for these people. They have grown to be the wind under my wings and I wouldn't have had it any other way. I'd like to send some love to all of my other colleagues trudging hard through their degrees, you are each incredibly driven and intelligent people and I wish everyone the absolute best.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: WILLIAM MARSH

Date of Degree: MAY, 2020

Title of Study: GENOMIC AND METAGENOMIC CHARACTERIZATION OF HALOPHILIC MICROORGANISMS WITH INSIGHTS INTO THE BIOREMDATION OF PRODUCED WATER.

Major Field: MICROBIOLOGY AND MOLECULAR GENETICS

Abstract: Produced water is an extremely toxic environmental wastewater source derived from oil-production. Produced water is typically a highly saline waste water containing a litany of hydrocarbon and heavy metal pollution. It is very cost ineffective to remediate and return this water to the natural sources it was obtained from prior to oil-production. We have metagenomically characterized a halophilic mixed culture (KWT) and identified the most abundant organism to be an *Arhodomonas* sp. Within the metagenome, we successfully identified a large assortment of genes theoretically responsible for BTEX degradation belonging to the *Arhodomonas* sp. Using this KWT culture (and another microbial enrichment Rozel), a complete removal of BTEX in raw produced water from Payne county was achieved. Degradation capability was rescued in extremely toxic produced water from Grant county via a wastewater dilution strategy. In addition to the above mixed culture characterization, we were able to successfully isolate an organism with a 99% 16S rRNA similarity to a novel organism, *Modicisalibacter tunisiensis*. *Modicisalibacter* sp. Wilcox genome encodes an assortment of genes responsible for a variety of different hydrocarbon degradation including aromatic ring compounds (BTEX and benzoate), aliphatic compounds (hexadecane, decane, eicosane) as well as biphenyls. We functionally confirmed the degradation of BTEX, benzoate, and hexadecane. We saw an optimum BTEX degradation rate in 1M NaCl concentrations but also noted complete BTEX degradation in 4M NaCl, granted over a 72-day period. *Modicisalibacter* sp. Wilcox was also capable of degrading BTEX natively in produced water from Payne county. *Modicisalibacter* sp. Wilcox genome encodes genes responsible for the first step of nitrate-reduction suggesting its ability to respire nitrate anaerobically. Interestingly, under nitrate-reducing conditions, *Modicisalibacter* sp. Wilcox seems to be able to oxidize and degrade only ethyl benzene in a mixture of BTEX. As the sole carbon source, *Modicisalibacter* sp. Wilcox was able to degrade ethyl benzene completely in 5 days. More work needs to be done to quantify the amounts of nitrate used in ethyl benzene oxidation under anaerobic conditions.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Produced water is a wastewater source derived from the hydraulic fracturing of oil-containing rock sediments. In short, hydraulic fracturing sites obtain water from local lakes, rivers and streams, and inject it into the subsurface under high pressure to perturb and displace the oil. The oil-water mixture is then pumped back to the surface for separation and crude oil-processing. At this stage, the water that is retrieved back to the surface is known as produced water. Historically, it has been extremely cost ineffective to completely remediate produced water before returning it back to local lakes and rivers. This is due, in part, to high salt concentrations and a myriad of other potent environmental pollutants that not only inhibit microbial life but are a concern to human health. In addition to the expected hydrocarbons, many of these polluted saline waters contain a collection of dissolved heavy metals (Pb, Mg, Mn, Co, and Cu for example) and small concentrations of naturally occurring radioactive materials (NORM's). All aforementioned pollutants can be removed through a variety of industrial and chemical means, which, as it stands, is extremely costly to the oil production industry. To avoid costly remediation, many oil production sites simply dispose of this water via deep well injection upon separating it from the produced oil.

This results in millions of gallons of previously surface water to be lost into the subsurface of the Earth. Produced water (PW) is the oil industries largest waste concern. On average, for every 1 barrel of oil, 3 barrels of PW are created. Reducing the remediation cost will not only lower the financial burden on the oil industry but may also result in clean water for irrigation or other industrial applications.

Halophilic microorganisms (salt-loving organisms) capable of degrading hydrocarbon pollution might stand as an excellent candidate to lower the cost of remediating this water. Several examples of halophilic organisms capable of degrading a litany of hydrocarbons derived from oil pollution (e.g. long chain n-alkanes, monoaromatic and polyaromatic ringed hydrocarbons, and branched hydrocarbons) have been documented in the literature. Halophilic microorganisms have evolved genetic and physical characteristics to survive in environments with copious amount of salt. These adaptations include the uptake and synthesis of compatible solutes that prevent excess Na^+ ions from entering the cell and disrupting the preferential osmotic pressure. In addition, most halophilic organisms have robust mechanisms to evade heavy metal intracellular accumulation usually in the form of ion pumps specific for heavy metals like Mg, Co, Cu and others specific for singular heavy metals like Pb or As. They also have enzymes designed to reduce said metal ions in order to precipitate and remove them from solution.

CHAPTER II

LITERATURE REVIEW

II.1 – Produced Water & Water Usage Overview

Produced water, water derived from oil and petroleum recovery operations; serves as one of the industry's largest waste components. Produced water is a very significant industry concern, it is estimated that for every barrel of oil recovered there are 3 barrels of produced water (Fakhru'l 209). Produced water contains an assortment of hazardous and toxic components. This water waste is highly saline, roughly 6-20% (Fidelis 2018). Toxic hydrocarbons exist in a variety of concentrations in the water. These hydrocarbons include simple aromatics like benzene, toluene, ethyl benzene, and xylene (BTEX). Polycyclic aromatic compounds like naphthalene and anthracene are also present in varying concentrations. Along with these compounds a variety of heavy metals can be found (aluminum, cadmium, chromium, cobalt, copper, iron, lead, manganese, nickel and zinc). There are also trace amounts of natural occurring radioactive materials (NORM's) present in produced water (Azetsu-Scott 2006).

There should be a distinction between fracking/flowback water and produced water. Fracking sites utilize high pressure surface waters to break apart rock formations. The water/oil mixture is pumped back to the surface, where the water is

separated from the oil fraction. The water first brought back to the surface (2-6 months when the oil-well was brought online) is known as flowback water. This is typically reused for further fracking operations. However, water being retrieved past the flowback window is described as produced water and is far more toxic (Dwyer, 2016).

The remediation and recycling of flowback or produced water can be facilitated in a variety of different ways depending on the shale formation, the state, and oil remediation plant. Produced water is typically handled in one or more of the following strategies.

1. Flowback water can be quickly reused in further oil recovery operations
2. Contaminated water can be processed through extensive biochemical engineering procedures to remove the aforementioned contaminants
3. Heavily contaminated produced water can be pumped into the deep subsurface.

An overview of modern (fracking-type) and conventional oil-drilling strategies are depicted in Figure 1.

Hydraulic fracturing in the United States used a cumulative 44 billion gallons of water in the period spanning from 2011-2012 (U.S EPA, 2015). This water is initially taken from local ground and surface waters and occasionally reused from previous fracking operations. It is important to note that reused water (flowback water), on average, only constitutes 5% of the total water used for fracking. Ground and surface water, on average, constitutes 95% of the water volume used for fracking operations (~ 41.8 billion gallons annually)(U.S EPA, 2015). A summary of modern fracking operations is depicted in Figure 2 below.

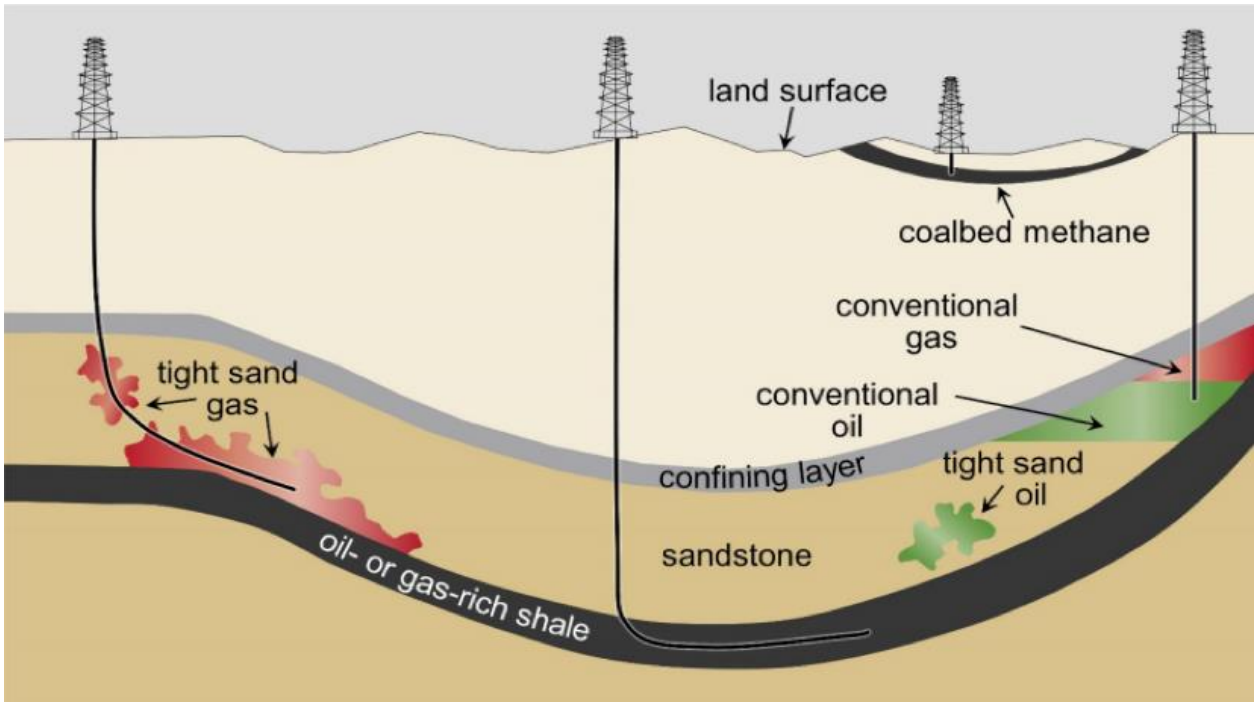

Figure 1. The different oil-drilling strategies most commonly used. The left two examples indicate a fracking-type oil and natural gas displacement strategy. The far right depicts a conventional oil-production site. (U.S EPA 2015)

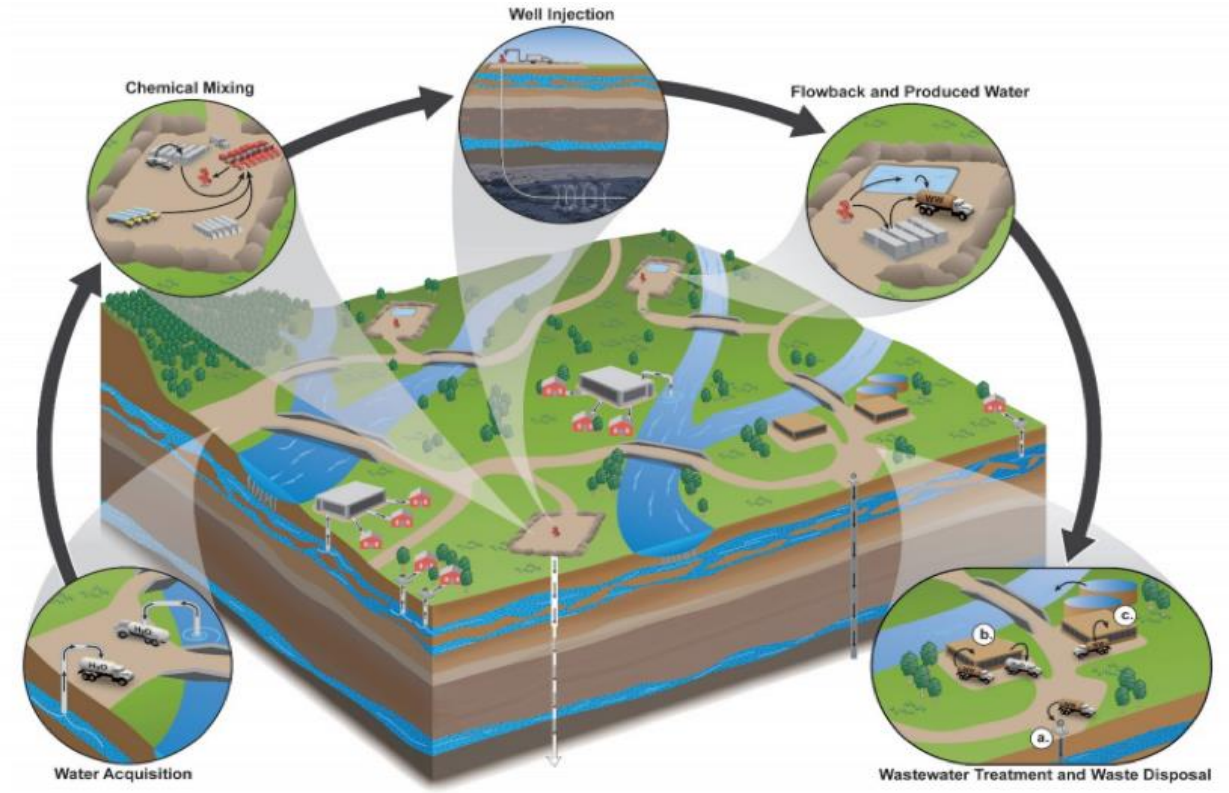

Figure 2. Modern fracking operations. Fracking operation starts with surface or ground water acquisition. Chemical mixing to reduce the surface tension of the water to assist in oil-displacement, well injection, flowback and produced water separation and partitioning, and finally disposal or treatment. (U.S EPA 2015)

The United States oil-production sites account for slightly less than 1% of the countries' total water usage. At some local levels, water usage per oil-production site is reported to be as high as 10% in some counties. In this small group of counties (2.2% of all counties containing at least 1 oil-fracking site) 10% of the counties' water was used for fracking. The majority (50% of all counties containing at least 1 oil-fracking site) account for ~1% of the counties' water usage (US.EPA, 2015).

Water consumption via hydraulic fracturing does not directly result in negative impacts to drinking water resources. There have been no documented instances where fracking alone has caused a surface or ground water supply to become completely depleted. However, the lack of documentation could possibly be the reason why no negative impacts are observed (U.S EPA, 2015). The most updated models have estimated a depletion volume of 25 km^3 per year from 2000-2008. This is a staggering 2.5 times more water usage compared to the previous 108 years (9.2 km^3 per year) (Konikow, 2013).

II.2 – Halophiles' Environmental Adaptations

Halophilic organisms thrive in high salt environments. They have accumulated specific adaptations to survive in such extreme environments. These organisms can regulate their internal osmotic pressure to prevent loss of water and desiccation. Halophilic microorganisms have single site and domain specific protein mutations to resist high NaCl concentrations.

II.2.1 - Osmoprotection

Disrupting osmotic pressure, especially in saline environments, leads to drastic cell deformation due to water leeching to the external environment. Water is not only quintessential for anabolic biochemical (condensation) reactions in the cell but helps the cell maintain its shape. These specific cell shapes have been classically related to

the diffusion equilibriums cells use to control uptake and excretion of nutrients and waste respectively. Cell deformity often collapses the cell and leads to cell lysis (Koslov 1984, Logisz 2005).

Organisms use two distinct mechanisms to withstand high salt concentrations 1. Production or uptake of compatible solutes whilst removing Na^+ and Cl^- ions (classically known as the "salt-out" strategy). 2. Maintaining a high intracellular level of K^+ to balance the high extracellular Na^+ concentrations (the salting-in strategy) (Shivanand, 2011).

The salt-out strategy mainly comprises of the uptake or synthesis of compatible solutes (e.g. proline, glycine betaine, glutamate, and/or ectoine) whilst removing specific Na^+ and K^+ ions (Oren 2008)(Kempf 2008). Halophiles typically prefer to uptake these small organic compounds rather than synthesize them due to their metabolic cost to the organism (Galanski 1994)(Vyrides, 2017). These organic solutes all have strong positive charges thereby preventing excess Na^+ ions from accumulating inside the cells.

The salt-in strategy utilizes a build-up of K^+ ions intracellularly to robustly counteract the invasion of exogenous Na^+ ions. Building up intracellular salt is more energetically favorable than synthesizing organic solutes and is typically the first response to immediate salt stress in low-moderate salt concentrations (1-5%) (Welsh 2000).

II.2.2 – Heavy metal tolerance

Halophilic microorganisms, among others, have an assortment of heavy metal ion tolerance mechanisms. These include heavy metal ABC transporters (Silver 1994). These proteins act as efflux pumps to remove Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+} Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, $TeO3^{2-}$, Ti^+ and Zn^{2+} and others (*Silver et al.)*

Microorganisms can also change the oxidation state of some metal ions to precipitate them out of solution (Ayangbenro 2017), thus reducing the heavy metal toxicity for the whole environment. Finally, while not explored in halophilic bacteria, many bacterial genera, e.g. *Pseuodomonas* and *Enterobacter*, have been shown to have modest biosorption capabilities (Ayangbenro 2017).

II.2.3 – Unique protein adaptations

Halophilic organisms have acidic proteins (proteins with abundance of dispersed aspartic acid (Asp, D) and glutamic acid (Glu, E) residues) that are more adapted to highly saline aqueous environments. Aspartic acid and glutamic acids have carboxylic side chains imparting negative charge upon deprotonation. Negative charges protect larger proteins from salting-out even in the presence of high salt concentrations (Fukuchi 2003).

II.3 – Aerobic degradation of BTEX

Benzene, toluene, ethyl benzene and xylene are predominant oil-derived aromatic hydrocarbon pollutants. These compounds are predominantly broken down aerobically via stepwise activation of their aromatic rings. This is accomplished via mono- or dioxygenases that introduce one or two hydroxyl groups (-OH), respectively. The aromatic ring activation results in two main intermediates, catechol and protocatechuate, the chemical structure is denoted below in Fig. 3 & 4 (Pérez 2013)

Catechol is the most common intermediate of aerobic BTEX degradation. Catechol-like intermediates with methyl and ethyl groups are also representative of this degradation pathway (Çınar 2004). Once catechol is formed, the ring is cleaved into a linear molecule in 2 distinct fashions, *ortho*- or *meta*- cleavage. *Ortho*-cleavage of catechol is done by the enzyme 1,2-dioxygenase and generates *cis,cis*-muconic

acid. *Cis,cis*-muconic acid can undergo further *beta*-oxidation with acetyl-CoA and succinyl-CoA for energy conversion in the citric acid cycle (Viswanathan 1998*).* *Meta*-cleavage follows a similar but characteristically different pathway resulting in different intermediates. *Meta*-cleavage is performed using the enzyme 2,3-dioxygenase and produces 2-hydroxymuconic semialdehyde. This molecule is eventually broken down to pyruvate and acetaldehyde. Pyruvate can then enter the citric cycle for energy extraction and oxidation (Kita 1999).

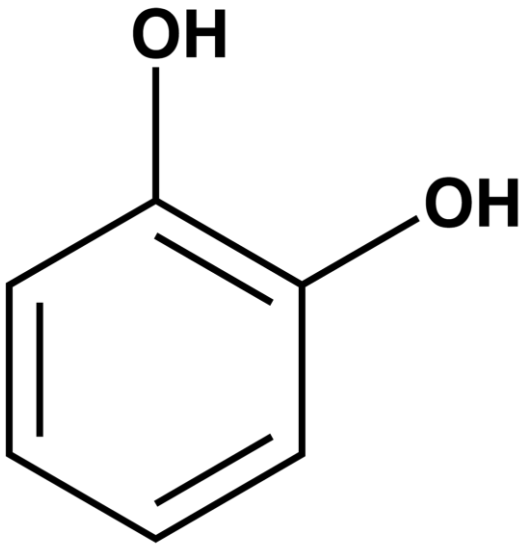

Figure 3 depicts Catechol, a central intermediate in upstream aromatic ring activation. The hydroxyl groups here are in the ortho- position.

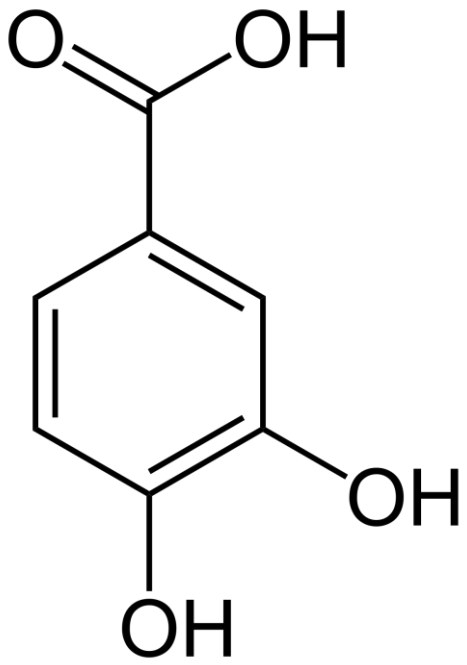

Figure 4 depicts protocatechuate, a central intermediate in upstream aromatic ring activation.

II-4 – Anaerobic degradation of BTEX

Anaerobic degradation of BTEX proceeds at a slower rate than aerobic degradation. Many microorganisms have been described to anaerobically degrade one or more of the compounds in BTEX. In many cases, the upstream pathways all converge on benzoate and its CoA derivative as central intermediates (Chakraborty 2004). Benzoyl-CoA ring cleavage proceed via benzoyl-CoA reductase coupled to ATP hydrolysis.

II-4.1 – Anaerobic benzene degradation

Anaerobic benzene degradation has only been described in mixed culture anaerobic sediments and enriched cultures in Fe (III)-reducing, nitrate-reducing, sulfate-reducing, and lastly methanogenic conditions (Lovely 1997),(Grbi'c-Gali'c 1987), (Vogel 1986),(Anderson 1998). Relatively recently, isolates from a newly described *Dechloromonas* genus in the beta subclass of the Proteobacteria were found that completely oxidize benzene with nitrate as its final electron acceptor (Coates 2001). The current upstream benzene activation pathway is unknown but there are some theoretical intermediates. Benzene could be acted upon by a carboxylase, methyl transferase, or a hydroxylase to create the intermediates benzoate, toluene, or phenol respectively (Chakraborty 2004).

II4.2 – Anaerobic toluene degradation

Anaerobic toluene degradation is probably the most understood component of BTEX degradation. Toluene can be degraded in the presence of Fe(III), nitrate, sulfate, Mn(IV), or CO_2(Coates 1996b, Dofling 1990, Edwards 1992, Evans 1991, Fries 1994) as electron acceptors. The first characterized step in toluene activation happens through a glycyl radical enzyme, benzylsuccinate synthase (BSS), which attaches a fumarate on the end of the methyl group on toluene. This forms

benzylsuccinate and is believed to be the genetic inducer of downstream ring cleavage (Leuthner 1998).

II 4.3 – Anaerobic ethyl benzene degradation

Anaerobic degradation of ethyl benzene in the presence of nitrate or sulfate has been relatively uncharacterized with only 4 total isolates described; strains EbN1, PbN1, a newly described *Dechloromonas* strain RCB, and EbS7 (Rabus 1995) (Kniemeyer 2003) (Chakraborty 2005). Generally, the complete oxidation of ethyl benzene to CO_2 is achieved with the reduction of nitrate completely to N_2. Ethyl benzene is initially activated by the dehydrogenation of the methylene group to form 1-phenylethanol. This is further oxidized to aromatic ketone acetophenone (Ball 1996). In contrast, strain RCB, under sulfate reducing conditions, attaches a fumarate to the terminal methyl group of the ethylene extension of the aromatic ring. This fumarate addition forms 1-phenylethyl-succinate. The main difference concerning nitrate and sulfate lies within sulfate's higher redox potential yielding more ATP overall (Kniemeyer 2003).

II 4.4 – Anaerobic xylene degradation

Anaerobic degradation of all the xylene isomers (*meta*-xylene, *para*-xylene, and *ortho*-xylene) has been mostly studied under nitrate and sulfate reducing conditions. The initial aromatic ring activation of *m*-xylene and *o*-xylene follows similar themes to anaerobic toluene degradation. Upstream activation of xylene involves an addition of a fumarate group to one of the methyl groups to form 3-methylbenzoate. This reaction is catalyzed by 3-methylbenzyl-succinate synthase (Krieger 1999).

Chapter III

BIODEGRADATION OF BTEX COMPOUNDS IN PRODUCED WATER BY HALOPHILIC MIXED CULTURES

BACKGROUND

The processing and disposal of large volumes of high salinity produced water (PW) pose a significant environmental and economic burden for the industry and communities. Clearly, there is a critical need to develop cost-effective approaches for the treatment and management of PW. Hydrocarbon-degrading halophiles can play important role in the remediation of PW. We have enriched a hydrocarbon-degrading mixed culture from crude oil contaminated saline soils from Kuwait (KWT enrichment). This culture is capable of degrading benzene, toluene, ethylbenzene, and xylenes (BTEX) as the sole sources of carbon at high salinity (optimum 2.5 M NaCl). Amplicon sequencing of the culture showed an abundance of *Arhodomonas* sp. (~99.3%), previous shown to degrade aromatic hydrocarbons optimally at 2.5 M salt. Metagenome analysis of the KWT enrichment showed the presence of several oxygenases, hydroxylases, and other genes involved in hydrocarbon degradation pathways. In this study, we have explored this culture's ability to directly remediate toxic PW from different locations.

MATERIALS AND METHODS:

Chemicals: Benzene, toluene, ethyl benzene, xylenes were purchased from Sigma-Aldrich Co. All the chemicals were of analytical grade and were used without further purification.

BTEX quantitation using Gas Chromatography (GC) - Biodegradation of BTEX compounds were assayed by using a Hewlett Packard 6890 GC equipped with a flame ionization detector and a DB-1 capillary column (30 m by 0.320 mm by 1 m; J&W Scientific, Inc.). Nitrogen served as both carrier and makeup gas at flow rates of 10 and 40 ml/min, respectively. The flow rates of hydrogen and air were set at 40 and 450 ml/min, respectively. The operating GC conditions were the following: oven temperature, 70°C for 7 min; inlet temperature, 150°C; and detector temperature, 220°C. Approximately, 100 µl of headspace gas from microcosms was injected into the GC for quantification. The GC response for each compound tested was calibrated to give the total mass in that bottle. Standards were prepared in 160-ml bottles filled with 50 ml of NaCl solution (0 to 4 M) and sealed with Teflon-faced septa and aluminum caps. The quantification of BTEX in enrichment bottles was accomplished as described above by using a calibration curve prepared with 1-liter bottles containing 500 ml of 2.5 M NaCl solution. The GC detection limit for benzene using our method was <1.0 umol/bottle.

Produced water composition for Grant and Payne county derived PW – Measurement of TDS and ion concentrations in the PW samples was carried out in Oklahoma State Univseristy's Soil and Water testing lab. Table 1 describes the composition for water derived from Grant county, OK. Table 2 describes the composition for water derived from Payne county, OK.

Test	Conc.	unit
Chloride	168000	mg/L
Total Nitrogen	104	mg/L
Nitrate	BPQL	mg/L
Total Dissolved Solids	394000	mg/L
Total Organic Carbon	0.293	mg/L
Total Kjeldahl Nitrogen	104	mg/L
Boron	5.09	mg/L
Calcium	7370	mg/L
Chromium	0.002	mg/L
Iron	<.01	mg/L
Lead	<.01	mg/L
Magnesium	1244	mg/L
Manganese	0.55	mg/L
Potassium	590	mg/L
Sodium	68900	mg/L
Phosphorus	BPQL	mg/L
Mercury	BPQL	ug/L
Salinity	237000	mg/L
Vanadium	BPQL	mg/L

Table 1 shows the analytical results for water derived from Grant county, OK.

Test	Conc.	unit
Alkalinity	146	mg/L
Bicarbonate	146	mg/L
Bromide	301	mg/L
Carbonate Alkalinity	BPQL	mg/L
Chloride	76700	mg/L
Cyanide	BPQL	mg/L
Fluoride	BPQL	mg/L
Ortho-Phosphate	0.26	mg/L
pH	7.41	pH Units
Total Nitrogen	74.2	mg/L
Total Suspended Solids	84.7	mg/L
Conductivity	250200	umhoc/cm
Nitrate	BPQL	mg/L
Sulfate	251	mg/L
Total Dissolved Solids	146600	mg/L
Total Organic Carbon	0.632	mg/L
Total Kjeldahl Nitrogen	74.2	mg/L
Oil & Grease	BPQL	mg/L
Aluminum	BPQL	mg/L
Arsenic	0.0492	mg/L
Barium	2.73	mg/L
Boron	6.79	mg/L
Calcium	7120	mg/L
Chromium	BPQL	mg/L
Copper	0.269	mg/L
Iron	0.317	mg/L
Lead	BPQL	mg/L
Lithium	4.48	mg/L
Magnesium	1240	mg/L
Manganese	0.303	mg/L
Nickel	BPQL	mg/L
Potassium	698	mg/L
Selenium	0.0006	mg/L
Silica	20.5	mg/L
Silver	BPQL	mg/L
Sodium	42600	mg/L
Sodium Adsorption Ratio	123	
Strontium	445	mg/L
Zinc	BPQL	mg/L

Table 2 shows the analytic results for water derived from Payne county, OK.

Wastewater collection - Wastewater was collected from the Stillwater wastewater treatment plant prior to any primary treatment. Water was vacuumed filtered to remove large sediments.

Sequencing and Bioinformatics – Total genomic DNA of the KWT enrichment was extracted using the Ultra Clean soil DNA kit (MO BIO Laboratories, Inc., CA). Extracted DNA was used for both amplicon as well as Shotgun metagenomic sequencing. Shotgun sequencing using MiSeq platform of the KWT enrichment yielded 6.7 and 4.7 Gbases of raw and trimmed sequence data, respectively. Reads were assembled using SPAdes v 3.12 to a minimum contig size of 500bp. The 16S rRNA-gene amplicon library of the KWT enrichment was processed using qiime2 pipeline with a pre-trained Silva v132 classifier to determine the community architecture of the enrichment.

The 5,010,546 raw 150bp read pairs were randomly subsampled using Seqtk 1 to 1.7 million read pairs to achieve an approximate genome coverage of 100X. Reads were then trimmed using Trimmomatic v0.39 2 with a 4:30 sliding window and minimum length of 36bp, yielding 1,315,515 read pairs and 155,408 and 64,471 forward and reverse unpaired reads, respectively. All trimmed reads were assembled using SPAdes v3.13 3 –careful with a minimum approximate coverage of 10X and minimum contig size of 500bp. The resulting assembly consisted of 1,169 scaffolds with an N50 15,766, L50 of 64, and total assembly length of 4,552,116 bp. A single 16S rRNA gene was detected using barrnap v0.9 4 (1 from each) and taxonomy was investigated by submission to Silva database v1325. Assemblies were submitted to the Integrated Microbial Genomes (IMG) database6 for gene-calling, functional annotation, and to provide public access under genome ID number 2844779970. Un-

annotated scaffolds were also deposited to GenBank under accession number WNXE00000000.1.

Enrichment culture maintenance - The KWT enrichment culture was enriched from a crude oil contaminated site in Kuwait. KWT culture was kept in 500 ml of mineral salt media (MSM) (Nicholson et al.) with 2.5M NaCl. Every 7-10 days, 5 µl of an equally mixed benzene, toluene, ethyl benzene, and xylene (BTEX) was spiked into the culture as the sole carbon source. Culture was maintained at these standards for the past 5 years. Approximately once a month, culture volume was halved and replaced with fresh MSM media to the same initial volume.

The Rozel enrichment was developed from an oil-brine soil obtained from an oil production facility in Seminole County, Oklahoma. The Rozel culture was held in similar constraints as the KWT culture. Rozel was kept in 600 ml of MSM with 2.5M NaCl. 8 µl of BTEX was spiked every 7-10 days as the sole carbon source. Subculturing in fresh media was performed monthly.

Biodegradation assays - Unless otherwise mentioned, all experiments involving the KWT or Rozel enrichment culture were performed in 160-ml-capacity serum bottles filled with a total volume of 50 ml. Produced water was filled to this volume unless diluted with waste water to achieve 50ml of total volume. Bottles were inoculated with 2 ml of KWT or Rozel (~1.6 x 10^6 cells) enrichment culture and were spiked with 2 µl (17 to 22 mol) of undiluted benzene, toluene, ethyl benzene, or xylenes. The bottles were then sealed with Teflon-coated septa and aluminum caps and were incubated under static conditions in the dark at 30°C. The headspace gas was

withdrawn periodically, and degradation of BTEX was monitored by gas chromatography (GC).

RESULTS AND DISCUSSION:

Metagenomic Analysis: 16S rRNA-amplicon sequencing of the KWT culture showed a community enriched in *Ahrodomonas* species (99.3% of the total abundance) (Table 3). Results of shotgun sequencing of the community are shown in Figure SL.5.

16S rRNA-amplicon sequencing of the KWT culture showed a community enriched in *Ahrodomonas* species (99.3% of the total abundance) (Table 3). Further metagenomic KEGG functionality shows the presence of key monocyclic aromatic hydrocarbon degradation genes, e.g. catechol-2,3-dioxygenase for catechol ring meta cleavage (Shu 1995), phenol hydroxylase subunits responsible for oxidizing the adjacent carbon from the primary hydroxyl group on phenol (Cafaro 2004), a crucial early step into aromatic hydrocarbon compound activation.

Bacterial Genus	Sequence counts	Relative abundance
Ahrodomonas sp.	453177	99.31%
Clostridium sp.	1276	0.27%
Bacillus sp.	591	0.12%
Candidatus Dichloromethanomonas sp.	201	0.04%
Desulfovibrio sp.	101	0.02%
Pseudomonas sp.	93	0.02%

Table 3: depicts the 16sRNA amplicon population results from metagenomic sequencing. Ahrodomonas sp. is the most prevelant bacterial species being 99.3% of the amplicon.

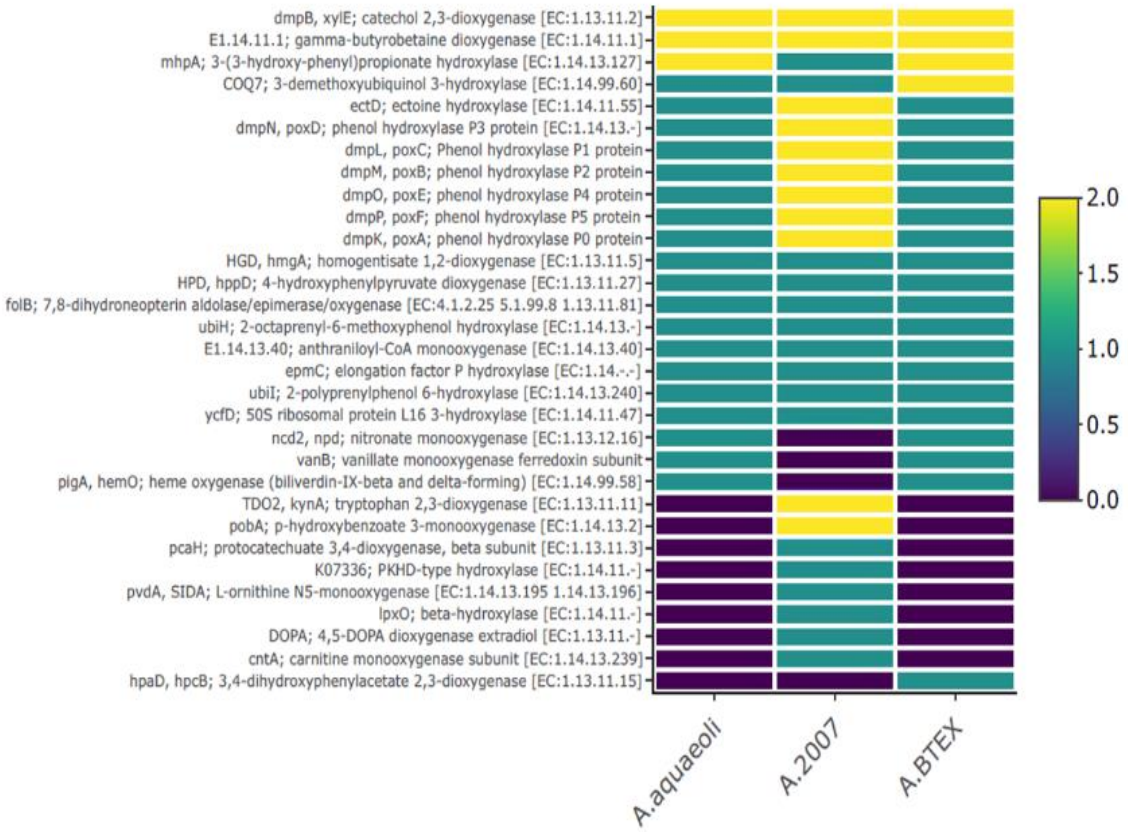

Figure 5 depicts a relative gene copy number heat map of the *Ahrodomonas* species found in the KWT culture (A.BTEX) compared with other closely associated species. A. aquaeoli is a marine species capable of degrading monocyclic hydrocarbons. A.2007 is an isolate from oil-contaminated soil. Some important genes responsible for aromatic ring oxidation are present in a list on the left. Relative gene copy number are illustrated for other species accordingly.

We compared gene copy of essential hydrocarbon degradation genes identified in *Ahrodomonas* species genome to other related species (Figure 5). The *Ahrodomonas* species in the KWT culture showed a higher relative gene copy number for genes encoding enzymes implicated in primary aromatic hydrocarbon activation.

Hydrocarbon degradation by the KWT culture: The KWT culture was able to degrade a mixture of benzene, toluene, ethyl benzene, and xylene (BTEX) natively in raw produced water from Payne county, OK in 9 days (Figure 6). Benzene, toluene, ethyl benzene, and xylene were, on average, degraded at a combined rate of 5.922 µmol/day. In an attempt to accelerate BTEX degradation, the produced water was 50:50 diluted with wastewater. Complete degradation of BTEX was accelerated to 7 days (Figure 7).

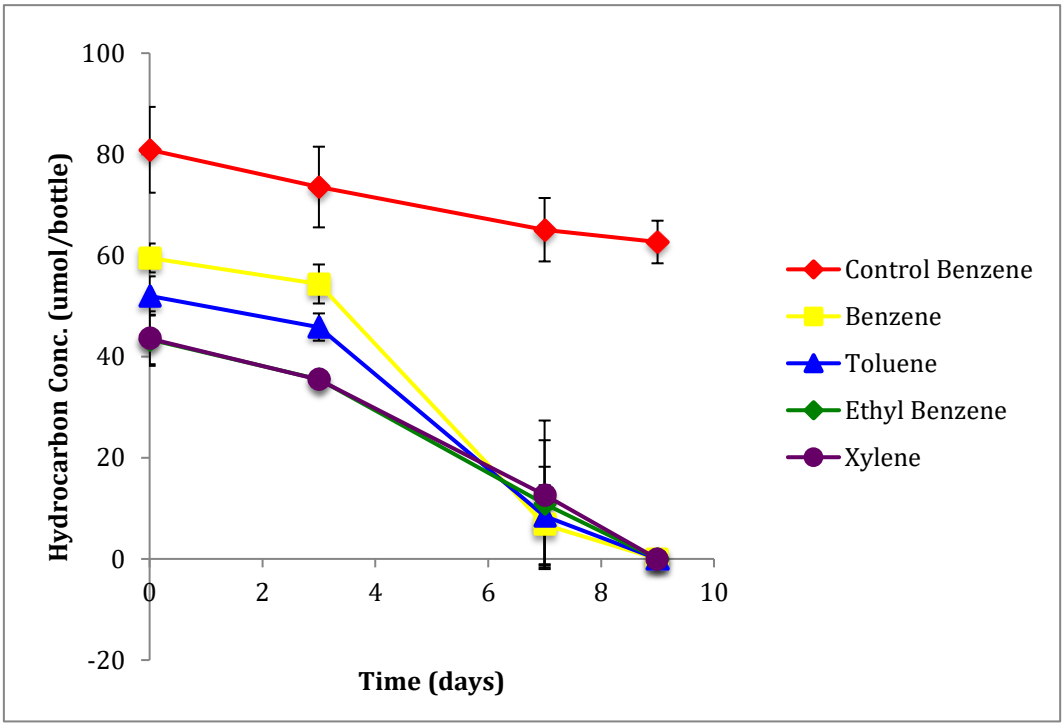

Figure 6 shows the BTEX degradation by the KWT mixed culture in 100% undiluted produced water from Payne county. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Benzene Control line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. The benezene, toluene, ethyl benzene, and xylene removal rates are 7.43, 6.3225, 5.006, 4.933 umol/day, respectively. Error bars represent standard deviation.

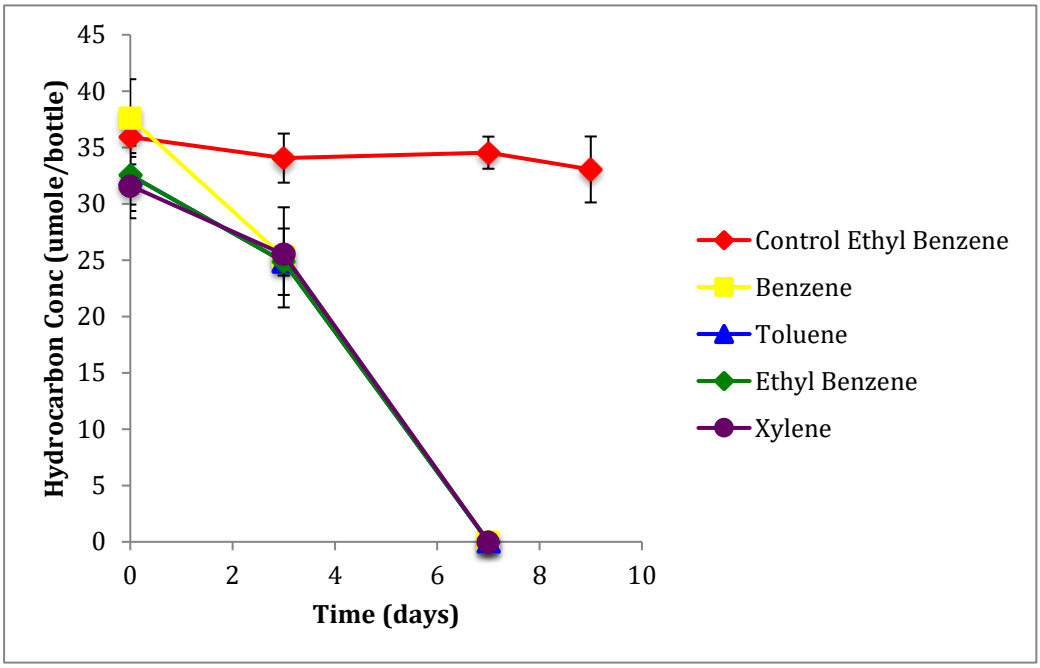

Figure 7 shows the BTEX degradation by the KWT mixed culture in a 50:50 dilution with autoclaved waste water and produced water from Payne county. The control represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Ethyl Benzene line (◆) are averages from 2 microcosm replicates. The benezene, toluene, ethyl benzene, and xylene removal rates are 5.423, 4.734, 4.034, 4.617 umol/day, respectively. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

The KWT culture was also inoculated into produced water from Grant county. We observed no BTEX degradation over the course of 28 days (Figure 8). Using the same dilution strategy of diluting PW 50:50 with wastewater, degradation was successfully recovered and complete degradation was seen in 5 days (Figure 9). The average combined hydrocarbon degradation rate can be noted as 9.128 µmol/day. Similar results were seen in the Rozel culture as well. The Rozel culture was able to completely degrade BTEX natively in 100% Payne county PW in 21 days (Figure 10). The average degradation rate was lower compared to the KWT culture in the same PW. The average hydrocarbon removal rate can be noted as 1.863 µmol/day. However, diluting with wastewater as mentioned before, accelerated BTEX degradation to 10 days (Figure 11). Diluting with wastewater, in this case, accelerated the BTEX degradation rate to 4.404 µmol/day. Similar to the KWT enrichment, BTEX degradation by the Rozel culture was never observed in undiluted Grant County produced water over 29 days (Figure 12). Again, using the same 50:50 produced water and waste water dilution, benzene and toluene were eliminated in 5 days while ethyl benzene and xylenes were completely degraded by 7 days (Figure 13). This likewise increased the rate of degradation to 5.747 µmol/day. A table summarizing this information is present in the figure appendix (SL.4).

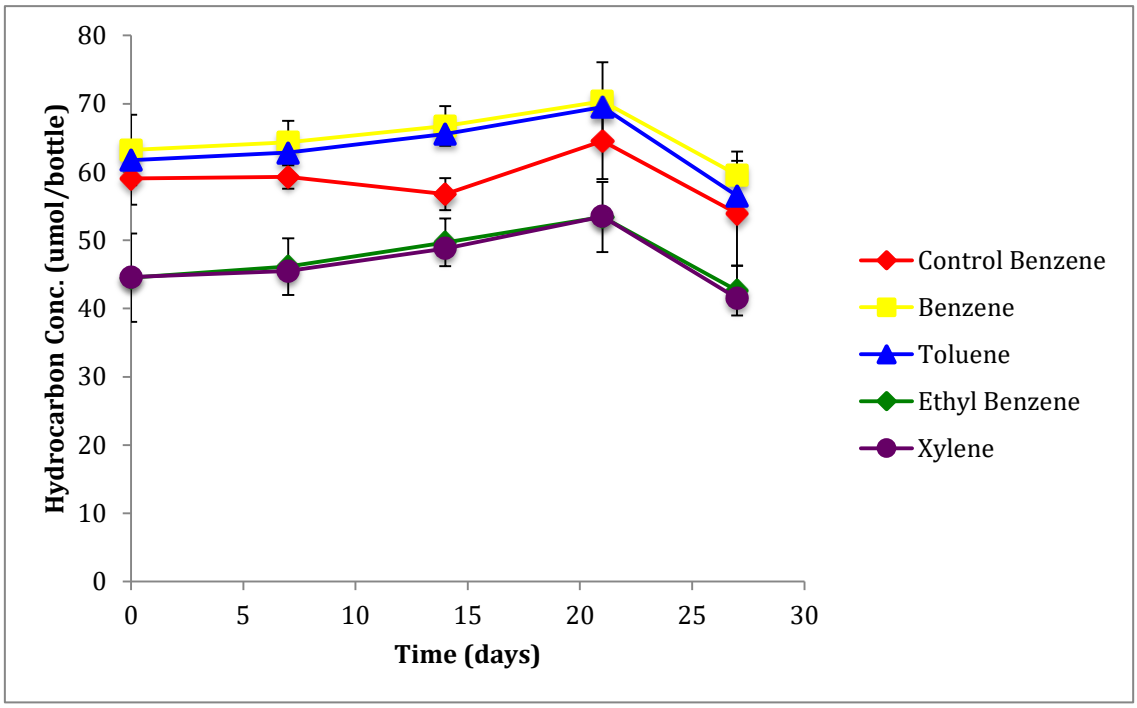

Figure 8. shows the BTEX degradation by the KWT mixed culture in 100% undiluted produced water from Grant county. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

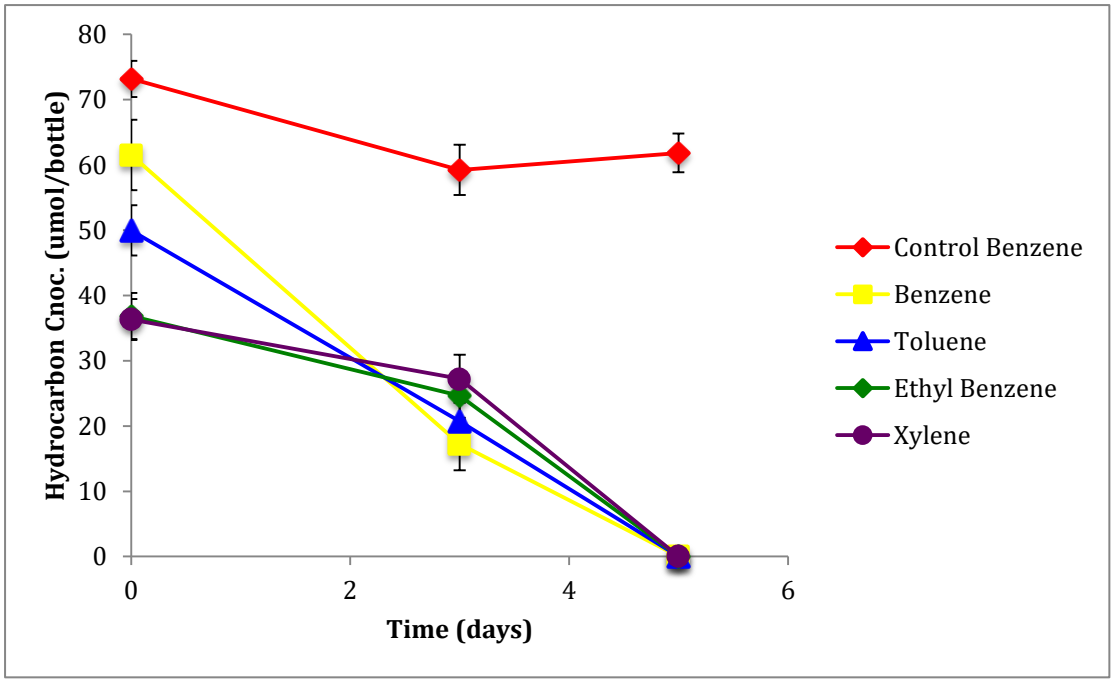

Figure 9 shows the BTEX degradation by the KWT mixed culture in a 50:50 dilution with autoclaved waste water and produced water from Grant county. The control represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Benzene line (◆) are averages from 2 microcosm replicates. The benzene, toluene, ethyl benzene, and xylene removal rates are 12.46, 9.97, 7.11, 6.933 umol/day, respectively. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

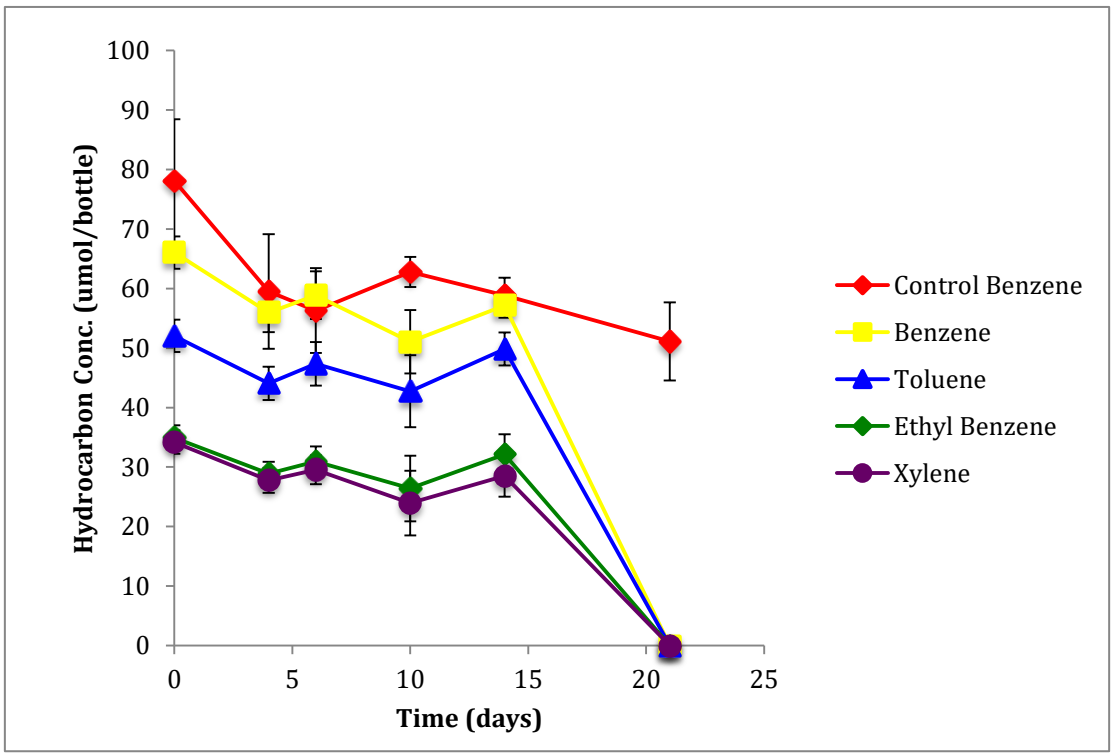

Figure 10. shows the BTEX degradation by the Rozel culture in 100% undiluted produced water from Payne county. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts per microcosm. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. The benzene, toluene, ethyl benzene, and xylene removal rates are 2.676, 2.03, 1.369, 1.378 umol/day, respectively. Error bars represent standard deviation.

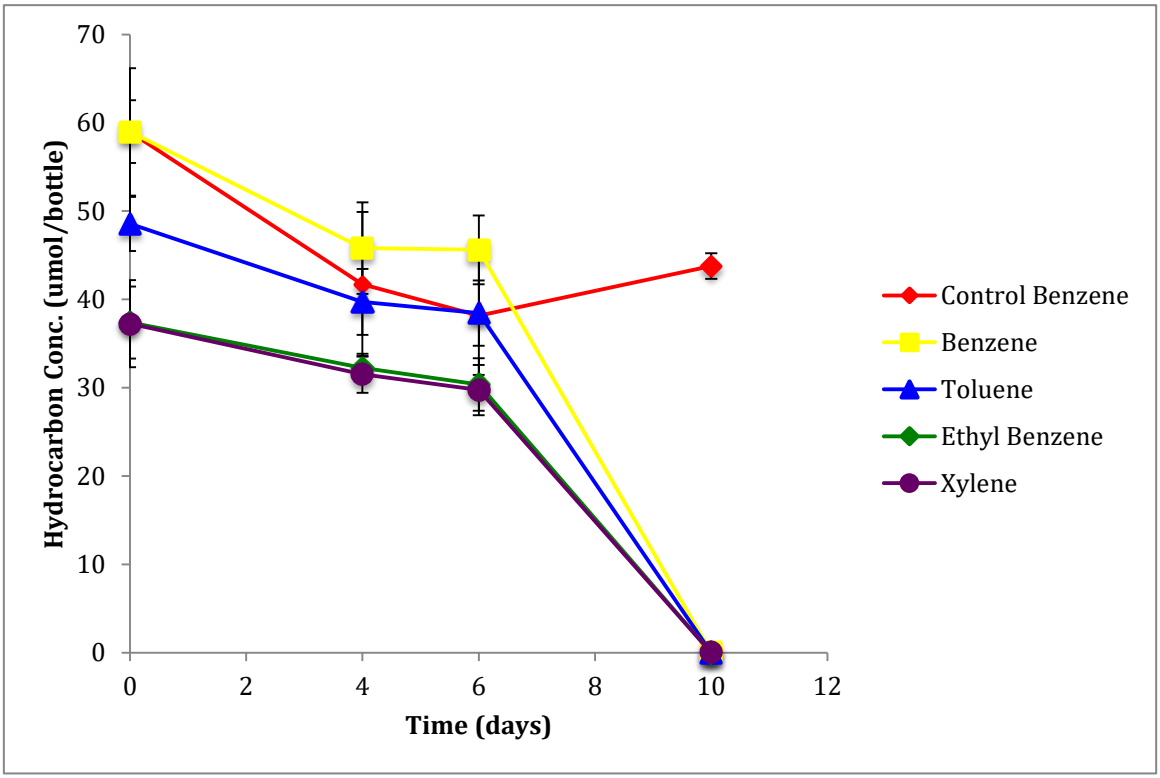

Figure 11. shows the BTEX degradation by the Rozel culture in 50:50 diluted produced water from Payne county with waste water. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. The benezene, toluene, ethyl benzene, and xylene removal rates are 5.676, 4.693, 3.632, 3.616 umol/day, respectively. Error bars represent standard deviation.

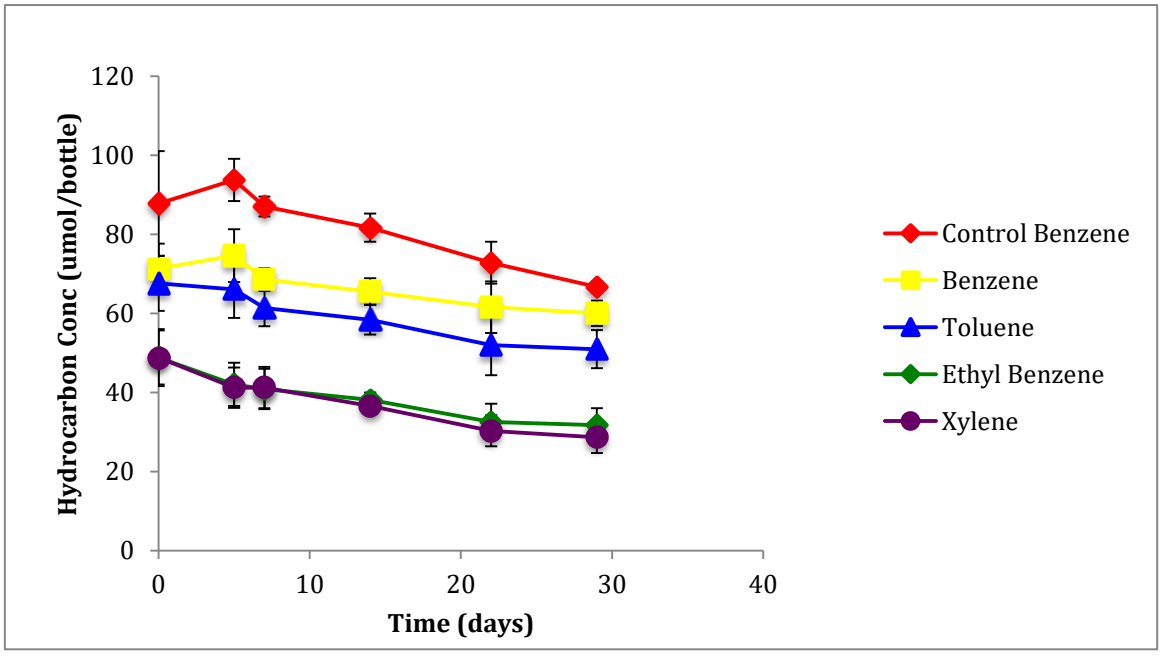

Figure 12. shows the BTEX degradation by the Rozel culture in 100% undiluted produced water from Grant county. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

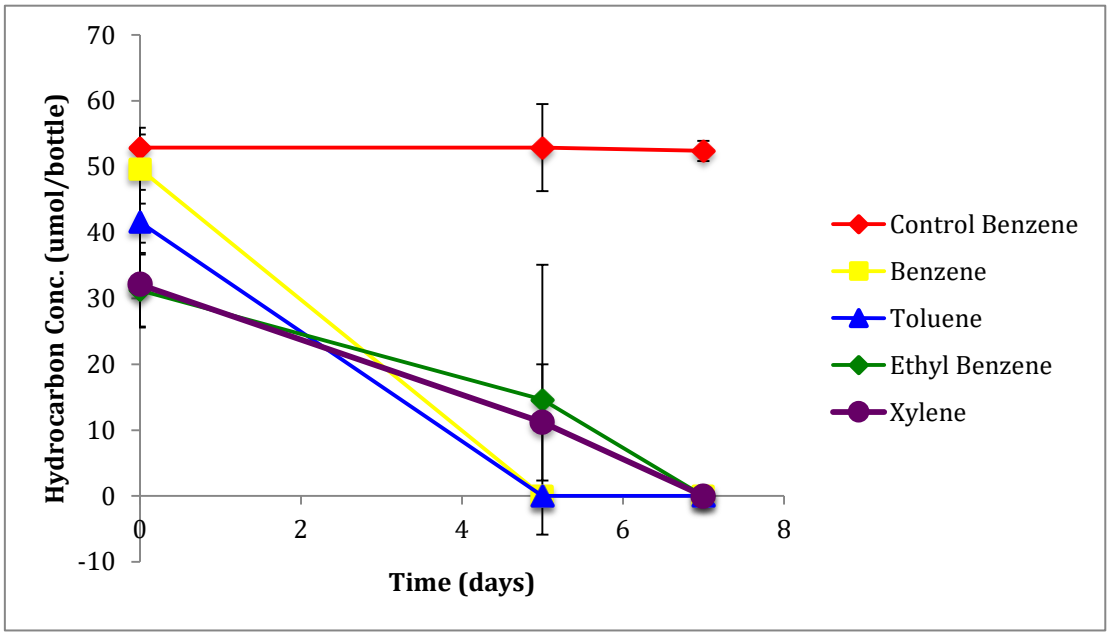

Figure 13. shows the BTEX degradation by the Rozel culture in 50:50 produced water from Grant county diluted with waste water. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. The benezene, toluene, ethyl benzene, and xylene removal rates are 7.638, 6.598, 4.234, 4.509 umol/day, respectively. Error bars represent standard deviation.

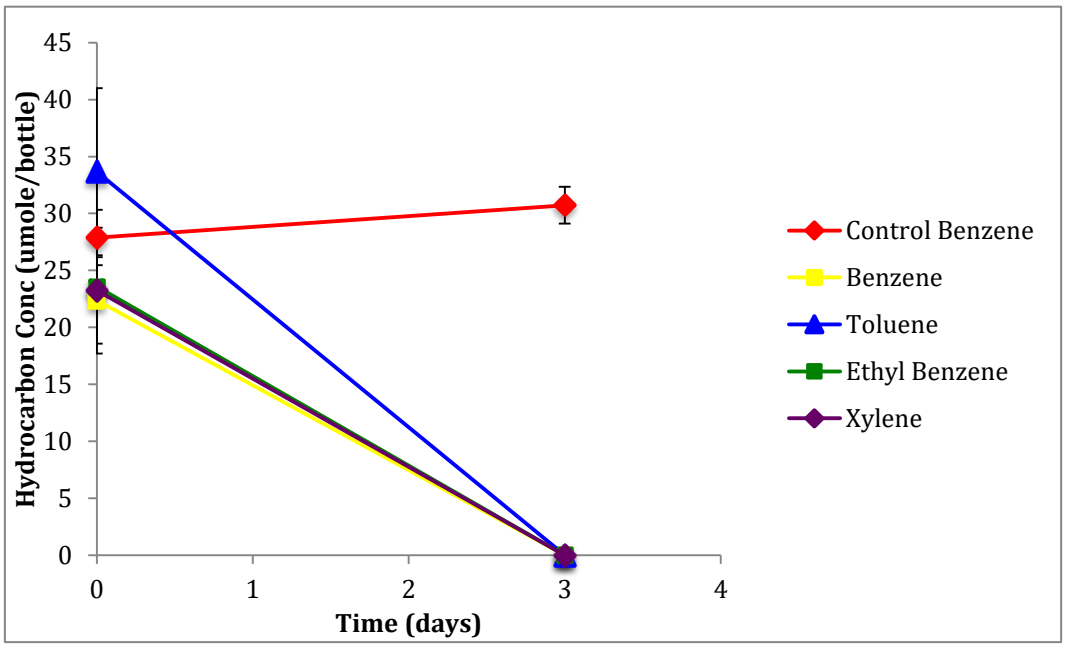

Figure 14 shows the BTEX degradation by the KWT culture in 50:50 produced water from Grant county and un-autoclaved waste water. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts per microcosm. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

CONCLUSIONS:

High salinity and heavy metal contamination of produced water from oil fields render bioremediation industrially difficult and costly. A large majority of this produced water from oil-production sites are typically disposed of via deep well injection (U.S. EPA 2016). Applying a halophilic culture or bacterial species with robust abilities to degrade hydrocarbons can help remove these toxic compounds, and render the process more cost effective.

Monocyclic hydrocarbon degradation capability of *Ahrodomonas* species in KWT and Rozel cultures was both genetically and functionally confirmed in toxic produced water. Key genes encoding enzymes responsible for cyclic ring activation and break down were detected in the *Ahrodomonas* species genomes. Both cultures were able to completely degrade BTEX mixtures in produced water from Payne county. Diluting the produced water with waste water both accelerated, and enabled BTEX degradation in the produced waters from Payne county, and Grant county, respectively.

Bacterial degradation of hydrocarbons in toxic produced water with relatively high levels of salinity could be directly applied to remediation efforts of the produced water itself, as well hydrocarbon-contaminated sites with high salinity. The produced waters were diluted with municipal waste water primarily to dilute with a readily available waste water stream. Similar results were observed in a microcosm with 50:50 produced water from Grant county and tap water (Sl.1). Waste water, not only dilutes down the salinity and other heavy metal concentrations, but might also provide essential vitamins and nutrients to the growing culture. All the previous experiments used autoclaved produced water from either source, Grant or Payne county. Using

autoclaved waste water would kill any potential nutrient competitors as well as destroy any possible bacteriophages that would otherwise decrease the hydrocarbon degrading capability of the KWT and Rozel cultures. However, a replication experiment using a 50:50 mixture of produced water from Grant county and unautoclaved waste water yielded an even faster degradation rate. In this experiment, the BTEX was completely degraded and removed in 3 days (Figure 14). This might be due to the presence of other natural BTEX degrading bacteria and archaea in waste water and other nontradional oil derived-water sources (Ojo 2006, Shokrollahzadeh 2008, Chavan 2008).

More work is needed on more diverse produced waters from other industrial produced water sites to further assess the holistic application of halophilic enrichment cultures to the wide spectrum of toxic produced water.

CHAPTER IV

CHARACTERIZATION OF NOVEL HALOPHILE *MODICISALIBACTER SP* WILCOX

BACKGROUND: PW has recently been an emerging environment for microbial studies and bioremediation applications. Isolation of individual microbial community members from PW could facilitate understanding the metabolic capabilities and PW resistance mechanisms in a single bacterial species. We have isolated a hydrocarbon-degrading organism from PW with a 99.3% 16S-RNA similarity to *Modicisalibacter* sp Wilcox. *Modicisalibacter* sp Wilcox is a halophilic rod-shaped Gram-negative bacterial species originally isolated from an oilfield water injection sample, a similar environment to PW. *Modicisalibacter* sp Wilcox, as reported in Gam *et al.* grows most optimally in 8-10% NaCl. It is capable of reducing nitrate, utilizing fructose and glucose. Little is known about *Modicisalibacter* sp Wilcox hydrocarbon degradation capability, or heavy metal ion resistance which might relate to the strain's ability to survive in PW, and it's PW bioremediation potential.

MATERIALS AND METHODS:

Chemicals**:** Benzene, toluene, ethyl benzene, xylenes, hexadecane, phenanthrene, and naphthalene were all purchased from Sigma-Aldrich Co. Hexane was purchased from Pharmco-Aaper. Sodium Benzoate was purchased from Fischer Scientific. Methane was purchased from Sigma-Aldrich Co. Dotriacontane (98%) was purchased from Alfa Aesar. All the chemicals were of analytical grade and were used without further

purification. PW used in this study was obtained from Wilcox oil production facility, Payne County, OK.

Isolation of a *Modicisalibacter* sp. WILCOX from PW: The enrichment was performed in 160-ml serum bottles (Wheaton) containing 50 ml of raw PW and sealed with 20-mm Teflon-lined septa and aluminum caps (The West Co). The bottles were injected with 2 uL of each benzene, toluene, ethylbenzene and xylenes (BTEX) and incubated in the dark in an inverted position. Air in the headspace (100 ml headspace) served as the source of oxygen. Biodegradation of added BTEX was monitored using a gas chromatograph (GC) as described before (Sei et al). The bottles were repeatedly spiked with BTEX for 3 -4 months at which time a stable enrichment culture was obtained that degraded BTEX as the sole carbon source within 7 days.

For the isolation of pure culture, a 10-fold serial dilution of enrichment was plated on to mineral salts medium (MSM) and agar plates supplemented with 1 M NaCl. The plates were incubated in a desiccator containing a vial with BTEX (1-2 ml) until colonies appeared (1 week). Well isolated colonies were transferred to serum bottles containing 50 ml of MSM (Nicholson et al 2004) supplemented with 1 M NaCl and 30-40 µmole of benzene, toluene, ethylbenzene, or xylenes (120-160 µmol of BTEX/bottle) as the sole source of carbon. Bottles were closed with Teflon-coated septa and aluminum caps. Head-space samples were withdrawn periodically and monitored for the added BTEX by GC using method described elsewhere. The culture that showed BTEX degradation was selected for further characterization.

16S rRNA gene analysis of the isolate: Total genomic DNA of the isolate was extracted using the Ultra Clean soil DNA kit (MO BIO Laboratories, Inc., CA). The isolate was identified by amplifying the 16S rRNA gene using the 27F and 1492R primers as described before [Spear et al. 2005]. Amplified PCR products was cleaned using ExoSAP-IT and sequenced at the DNA core facility, Oklahoma State University, Stillwater, OK. Amplified 16S rRNA gene sequences were analyzed using BLASTn against the nt database. The 16s RNA sequences of closely related members of the family *Halomonadaceae* and our isolate using the ClustalW algorithm. Using the generated alignment, A phylogenetic tree showing relationship between strain Wilcox and to other members in the family *Halomonadaceae* was constructed using the maximum likelihood method in MEGA 7 [Kumar et al 2016]. [NZ_WNXF00000000]. The strain, designated as Wilcox, was found to belong to the genus *Modicisalibacter* in the class *Gamma-Proteobacteria* and family *Halomonadaceae* on the basis of 16S rRNA gene sequence similarity.

Genome of strain Wilcox**:** The genome of strain Wilcox was sequenced using the Illumina HiSeq platform using 250 x 2 paired-end reads by Novogen Corporation. In brief, a total amount of 1.0 μg DNA was used as input material. A sequencing library was generated using the TruSeq Nano DNA HT Sample Preparation kit (Illumina) following the manufacturer's recommendations, and index codes were added to attribute sequences. The genomic DNA was randomly fragmented to a size of 150 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP system), and libraries were analyzed for size distribution by an Agilent2100 Bioanalyzer and quantified using real-time PCR. The

qualified libraries were sequenced by a HiSeq sequencer. The 5,747,630 raw 150-bp read pairs were randomly subsampled using Seqtk 1 to 1.7 million read pairs to achieve an approximate genome coverage of 100X. Reads were then trimmed using Trimmomatic v0.39 2 with a 4:30 sliding window and minimum length of 36bp, yielding 1,317,369 read pairs and 169,285 and 53,458 forward and reverse unpaired reads, respectively. All trimmed reads were assembled using SPAdes v3.13 3 –careful with a minimum approximate coverage of 10X and minimum contig size of 500 bp. The resulting assembly consisted of 43 scaffolds with an N50 of 180,819, L50 of 7, and total assembly length of 3,584,069 bp. A single 16S rRNA gene was detected using barrnap v0.9 4 (1 from each) and taxonomy was investigated by submission to Silva database v132. Assemblies were submitted to the Integrated Microbial Genomes (IMG) database6 for gene-calling, functional annotation, and to provide public access under genome ID number 2844586914. Un-annotated scaffolds were also deposited to GenBank under accession number WNXF00000000.1.

General features of strain Wilcox genome: The genome size is ~3,584,069 bp in length, with a ~67% GC content. *Modicisalibacter* sp. Wilcox has 3283 genes with 2850 being associated in COGs. There are 62 RNA genes with 53 genes belonging to tRNAs.

Mother Inoculum**:** The strain Wilcox was maintained in 1-liter bottles with 500 ml of MSM supplemented with 2.5 M NaCl and 30-40 µmole of benzene, toluene, ethylbenzene, or xylenes as the sole source of carbon. Bottles were closed with Teflon-coated septa and aluminum caps. Headspace was withdrawn periodically and monitored for consumption of added BTEX by GC (Nicholson et al 2004). Bottles

were repeatedly fed BTEX and consumption of the added BTEX monitored. At the end of 4-6 weeks, 50% of the culture was replaced with fresh MSM supplemented with 2.5 M NaCl and BTEX as the sole carbon source. This culture served as the source of inoculum for all the experiments presented in this article unless until stated otherwise.

Microcosm set up to study hydrocarbon degradation:

Long-Chain alkane degradation**:** Flasks (250 ml capacity) containing 100 ml of MSM supplemented with 2.5M NaCl with no yeast-extract were prepared. Long-chain n-alkanes such as decane, hexadecane, eicosane or dotricontain was added (2-5 mM) as the sole source of carbon. Flasks were inoculated with 2 ml ($1.2x10^6$ CFU) of strain Wilcox. Un-inoculated autoclaved flasks containing n-alkane served as the control. Flasks were closed with a cotton plug and incubated at 35^o C static in the dark. Liquid culture was withdrawn periodically and performed a 10x serial dilution and plated on MSM agar plates containing 1 M NaCl and 5 mM acetate as the sole carbon source. Plates were incubated at 35 ^{o}C for 3 – 5 days and colony forming unit was performed.

Short-Chain alkanes (methane and hexane): Degradation of methane was assessed in 160 ml-capacity serum bottles were filled with 50 ml of MSM containing 2.5 M NaCl. Bottles were inoculated with 1 ml of strain Wilcox (6×10^5 CFU). Uninoculated autoclaved bottles were set up similarly. All bottles were amended with 100 uM methane and air in the headspace served as the source of oxygen. Methane quantification was done as performed in Liu et al 2014.

Biodegradation of hexane was assessed in 160 ml-capacity serum bottles filled with 50 ml MSM containing 2.5 M NaCl. Bottles were amended with 5 mM hexane as the sole carbon source and sealed with Teflon-coated septa and aluminum caps. Bottles were inoculated with 1 ml of strain Wilcox (6 x 10^5 CFU). Uninoculated autoclaved bottles were set up similarly. Bottles were incubated upside-down at 35^{o}C static in the dark. Culture (1 ml) was withdrawn on day 0, 7, and 14 and plated on MSM agar plates containing 1 M NaCl and 5 mM acetate as the carbon source. Plate count (CFU) was performed after 3-5 days of incubation at 35^o C.

BTEX degradation: Unless otherwise mentioned, all experiments involving the BTEX were performed with 160-ml-capacity serum bottles filled with 50 ml MSM supplemented with 2.5 M NaCl. Bottles were amended with 2 µl of each BTEX compounds (17 – 22 µmol/bottle each) and inoculated with 2 ml (1.2x10^6 CFU) strain Wilcox. Uninoculated autoclaved control bottles were set up similarly. Bottles were then closed with Teflon-coated septa and aluminum caps and were incubated under static in the dark at 35°C. The headspace was withdrawn periodically, and degradation of BTEX was monitored using GC as mentioned before

Naphthalene and Phenanthrene degradation: Degradation of the PAHs was assessed in 160-ml-capacity serum bottles filled with 50 ml of MSM supplemented with 2.5M NaCl. Naphthalene (1 mM) or phenathrene (0.5 mM) was added as the sole source of carbon. Bottles were inoculated with 1ml (6 x 10^5 CFU) of strain Wilcox culture. Serum bottles were then sealed with Teflon-coated septa and aluminum caps. Bottles were incubated statically upside-down at 35^{o}C. Culture sampling was withdrawn from

each bottle and plated on to MSM agar with 1M NaCl and 5 mM acetate as the carbon source on day 0, 7, 14, 21, and 28.

Benzoate degradation**:** Biodegradation of benzoate in the presence or absence of benzene was performed as follows. Flasks (250 ml capacity) containing 100 ml MSM supplemented with 2.5 M NaCl and 2 mM benzoate as the sole source of carbon were set-up. Flasks were inoculated with 1.2 x 10^6 CFU of strain Wilcox and incubated at 35ºC. Autoclaved control flasks devoid of strain Wilcox were set up similarly. Culture samples were withdrawn periodically and MSM agar plates containing 1M NaCl and 5 mM acetate as the sole carbon source and CFU was determined after 3-4 days of incubation.

Degradation of benzoate was also assessed in the presence of benzene as the co-contaminant. Microcosms containing MSM containing 20 µmol/bottle of benzene and 2 mM benzoate as the carbon sources and inoculated with 6 x 10^6 CFU of strain Wilcox were set up. Headspace samples were monitored for the degradation of benzene using GC as described above. Also, culture sample (1 ml) was withdrawn on weekly basis and plated on to MSM agar containing 1 M NaCl and 5 mM acetate and CFU unit was determined after 3-4 days of incubation.

Benzoate levels were monitored at 223nm on a Ultraspec 200 UV/Visible spectrum spectrophotometer. Benzene was monitored with a Hewlett Packard 6890 GC as previously described. Plating on 1M NaCl MSM with 5mM acetate monitored population dynamics.

Salinity and BTEX degradation**:** The effect of salinity on BTEX degradation was determined in microcosms using MSM supplemented with 0, 1.0, 2.0, 3.0, or 4.0 M NaCl. Serum bottles (160 ml) filled with 50 ml of MSM and various concentration of NaCl salt were set up and inoculated with 6 x 10^5 CFU of strain Wilcox. Headspace (100 uL) was withdrawn periodically and degradation of the hydrocarbons was measured using GC.

BTEX degradation in produced water**:** PW samples were sent to Oklahoma State Univseristy's Soil and Water testing lab to determine TDS and ion concentrations. Table 4 (supplementary) describes the composition of produced water derived from Payne county, OK. Serum bottles (160ml) were filled with 50 ml of PW, sealed with Teflon-coated septa and closed with aluminum caps. Bottles were inoculated with 6x 10^5 CFU of strain Wilcox and spiked with 2ul of BTEX solution. Headspace (100ul) was withdrawn periodically and degradation of hydrocarbons was monitored using GC.

RESULTS AND DISCUSSION:

Gene annotation and hypothetical hydrocarbon-degradation capability A genomic analysis of strain Wilcox provided the theoretical basis for the mechanism of hydrocarbon degradation by the bacterium. The Integrated Microbial Genome (IMG) server (http://img.jgi.doe.gov) was the primary source used for genome predictions and comparisons. We also BLASTp searched Prokka-annotated amino acid sequences for homologs using NCBI non-redundant protein database. Using both KEGG results in IMG and NCBI BlastP search against NCBI n.r, we predicted complete and partial

metabolic pathways for several aromatic compounds in Silico in strain Wilcox genome. The genome analysis yielded the presence of several genes needed for the degradation of both aromatic and aliphatic compounds. Our analysis predicted complete or near complete set of genes needed for the metabolism of a variety of hydrocarbons including BTEX, phenol, and benzoate. We detected several oxygenases and hydroxylases (dioxygenases) needed for the initial oxidation of aromatic compounds such as phenol hydroxylase, benzene 1,2-dioxygenasse, benzoate 1,2-dioxygenase, salicylate hydroxylase, and 2-halobenzoate 1,2-dioxygenase (Gibson and Parales 2000; Reineke and Knackmuss 1978; Pérez-Pantoja 2016; Yamamoto 1965). Also, genome analysis predicted the presence of biphenyl dioxygenase subunit alpha and beta subunits. These genes code for enzymes needed for the initial oxygenation of mono and polyaromatic compounds. Oxygenases and hydroxylases participate in oxidation of reduced substrates such as hydrocarbons by transferring oxygen from molecular oxygen (O_2) utilizing various co-enzymes such as FAD, NADH and NADPH as a cosubstrate. Oxygenases are grouped into two categories; the monooxygenases and dioxygenases on the basis of number of oxygen atoms used for oxygenation. Monooxygenases are classified into two subclasses based on the presence cofactor: flavin-dependent monooxygenases and P450 monooxygenases. Flavin-dependent monooxygenases. contain flavin as prosthetic group and require NADP or NADPH as coenzyme. P450 monooxygenases are hemecontaining oxygenases and require NADPH as co-enzyme.

The monooxygenases comprise a versatile superfamily of enzymes that catalyzes oxidation of various substrates, both alkanes and aromatic compounds (Durairaj 2016). Dioxygenases are multicomponent enzyme systems that introduce molecular

oxygen into their substrate. Aromatic hydrocarbon dioxygenases, belong to a large family of Rieske nonheme iron oxygenases (Chandrakant 2011; Pérez-Pantoja 2016). Dioxygenases primarily oxidize aromatic compounds and, therefore, have applications in environmental remediation.

Aromatic hydrocarbon dioxygenases belong to a large family of Rieske non-heme iron oxygenases. The dioxygenases have abroad substrate specificity and catalyze enantiospecific reactions with a wide range of substrates. Interestingly, we did not find naphthalene-related genes, specifically genes that code for the upper pathway enzymes involved in the conversion of naphthalene to salicylate (Peng 2008). In addition, the genome analysis predicted genes for aromatic ring cleavage by ortho cleavage pathway such as catechol 1, 2-dioxygenase and protocatechuate 3,4-doixygenase (Macchi 2018; Seo 2009) to yield intermediates that are subject to oxygenolytic ring cleavage followed by channeling of ring-cleavage products into the central metabolism via beta-ketoadipate pathway (Harwood and Parales 1996; Pérez-Pantoja, et al. 2016). Table 4 below depicts certain monoxygenases, and dioxygenases responsible for both ring oxidation and ring cleavage of a variety of aromatic ringed structures.

Gene ORF	Substrate derivitive	Gene name	Putative function	Organism	% Identity	E-Value	NCBI accession no.
2806	Benzoate	benA-xylX	2-halobenzoate 1,2-dioxygenase small subunit	unclassified Halomonas	91.98	1.00E-109	WP_078089514.1
2805	Benzoate		2-halobenzoate 1,2-Fgenase large subunit	Halomonadaceae bacterium T82-2	100	1.00E-109	KXS37862.1
2807	Benzoate		Benzoate 1,2-dioxygenase electron transfer component	unclassified Halomonas	91.98	0	WP_078089514.1
1102	Benzoate	benD-xylL	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase	Halomonadaceae bacterium T82-2	95.83	0	KXS36894.1
2804	Catechol	catA	Catechol 1,2-dioxygenase	Halomonas nitroreducens	85.49	0	WP_126480973.1
2801	Catechol	catB	Muconate cycloisomerase	Halomonadaceae bacterium T82-2	94.09	2.00E-145	KXS37865.1
2802	Catechol		Muconate cycloisomerase	Halomonas heilongjiangensis	91.41	5.00E-75	WP_110286202.1
2803	Catechol	catC	Muconolactone Delta-isomerase	Halomonadaceae bacterium T82-2	97.92	3.00E-64	KXS37864.1
1734	Catechol	pcaD	3-oxoadipate CoA-transferase subunit B	Halomonadaceae bacterium T82-2	99.22	0.00E+00	KXS38043.1
1735	Catechol	pcaL	3-oxoadipate CoA-transferase subunit A	Halomonas sp. 362.1	94.85	0.00E+00	WP_129138993.1
44	Catechol	fadA	Acetyltransferase	Halomonas sp. 362.1	81.94	2.00E-78	WP_129138321.1
3015	Catechol	fadI	Acetyl-CoA acetyltransferase	Halomonadaceae bacterium T82-2	98.47	0.00E+00	KXS37177.1
1425	Catechol		3-ketoacyl-CoA thiolase	Halomonas xianhensis	84.65	0.00E+00	WP_134016041.1
26	Benzene		Benzene 1,2-dioxygenase system ferredoxin--NAD(+) reductase subunit	[Halomonas sp. A11-A	89	0.00E+00	WP_110068219.1
3230	Phenol	salA	Phenol hydroxylase P5 protein	Marinobacter pelagius	80.73	7.00E-47	WP_113861973.1
573	Salicylate	pcaH/pcaG	Salicylate hydroxylase	Halomonadaceae bacterium T82-2	88.25	0.00E+00	KXS36864.1
1724	Protochatuate	pcaA	Protocatechuate 3,4-dioxygenase alpha chain	Halomonadaceae bacterium T82-2	95.22	1.00E-145	KXS38033.1
1725	Protochatuate	pcaB	Protocatechuate 3,4-dioxygenase beta chain	Halomonadaceae bacterium T82-2	99.18	2.00E-180	KXS38034.1
1736	Protochatuate	pcaC	3-carboxy-cis,cis-muconate cycloisomerase	Halomonadaceae bacterium T82-2	86.91	0.00E+00	KXS38045.1
2803	Protochatuate		Muconolactone Delta-isomerase	Halomonas bacterium	95.83	5.00E-63	WP_035565030.1
2783	Phenylacetate	paaK	Phenylacetate-coenzyme A ligase	Halomonas pacifica	83.52	0.00E+00	WP_146804038.1
2778	Phenylacetate	paaE	1,2-phenylacetyl-CoA epoxidase, subunit E	Halomonas sp. HAL1	70.47	0.00E+00	WP_008957201.1
2780	Phenylacetate	paaC	1,2-phenylacetyl-CoA epoxidase, subunit C	Halomonas sp. JS92-SW72	79.92	1.00E-150	WP_119023124.1
2781	Phenylacetate	paaB	1,2-phenylacetyl-CoA epoxidase, subunit B	Halomonas bacterium	89.25	2.00E-57	WP_022521442.1
2782	Phenylacetate	paaA	1,2-phenylacetyl-CoA epoxidase, subunit A	unclassified Halomonas	90.99	0.00E+00	WP_027958342.1
2787	Phenylacetate	paaG	1,2-epoxyphenylacetyl-CoA isomerase	Halomonas sp. JS92-SW72	80.99	8.00E-156	WP_119023117.1
1733	Phenylacetate	paaZ	3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase	Halomonadaceae bacterium T82-2	97.51	0.00E+00	KXS38042.1
2788	Phenylacetate	paaF	2,3-dehydroadipyl-CoA hydratase	Halomonas sp. 3(2)	80.47	1.00E-144	WP_151442123.1
1742	Phenylacetate	paaH	3-hydroxyadipyl-CoA dehydrogenase	Halomonas sp. JS92-SW72	84.89	0.00E+00	WP_119023241.1
2784	Phenylacetate	paaJ	Beta-ketoadipyl-CoA thiolase	Halomonas sp. 3(2)	87.53	0.00E+00	WP_151442119.1
2723	Gentisate	nagL/sdgD	Homogentisate 1,2-dioxygenase	Halomonadaceae bacterium T82-2	95.86	0.00E+00	KXS37722.1
1776	Gentisate	nagL	Maleylpyruvate isomerase	Halomonadaceae bacterium T82-2	87.27	1E-133	KXS38997.1

Table 4 depicts the important upstream and downstream hydrocarbon degradation gene collections with putative functions found in *Modicisalibacter sp Wilcox*.. This includes complete or semi-completes genetic pathways for catechol, benzoate, procochatuate, salicylate, phenol, phenylacetate, and gentisate. All E-values are within signifigance and the majority of %identities are above 85% with the exception of genes belonging to phenylacetate degradation.

Gene annotation of hypothetical heavy metal resistance**:** *Modicisalibacter* sp Wilcox genome encodes a collection of p-type ATPases and efflux pumps responsible for removing and displacing heavy metal ions both specifically and non-specifically. Examples include *ars*C, and *arc*3 known to reduce arsenate arsenide and pump it outside the cell, as well as 3 potential copper exporting ATPases and a copper oxidase. The genome also encodes general metal ion exporters for cadmium, zinc, cobalt and lead. A concatenated list of these genes can be found in Table 5.

Gene ORF	Gene ı	Metal Ion	Putative function	Organism	% Identity	E-Value	NCBI accession no.
118	acr3	As	Arsenical-resistance protein	Halomonas xianhensis	100	0	WP_092850561.1
120		As	Arsenate reductase	Halomonas xianhensis	100	9.00E-95	WP_092850563.1
121	arsC	As	Protein ArsC	Halomonas xianhensis	100	8.00E-98	WP_092850567.1
818		As	Arsenical pump-driving ATPase	Halomonadaceae bacterium T82-2	100	0	KXS39846.1
2719		As	Arsenical pump membrane protein	Halomonas sp. SL1	87.82	0	WP_107181297.1
2720		As	Arsenate reductase	Halomonadaceae bacterium T82-2	91.18	8.00E-84	KXS37725.1
558		Cu	Copper-exporting P-type ATPase	Marinobacter salarius	94.67	0.00E+00	WP_085682201.1
859		Cu	Copper-exporting P-type ATPase	Halomonadaceae bacterium T82-2	93.61	0.00E+00	KXS39808.1
923		Cu	Copper-exporting P-type ATPase	Halomonadaceae bacterium T82-2	91.27	0.00E+00	KXS39748.1
2895	mco	Cu	Multicopper oxidase	Halomonadaceae bacterium T82-2	99.57	0.00E+00	KXS37229.1
411		Cd, Co, Zn	Cadmium, cobalt and zinc/H(+)-K(+) antiporter	Halomonadaceae bacterium T82-2	98.73	0.00E+00	KXS37941.1
1265		Zn	Zinc uptake regulation protein	Halomonadaceae bacterium T82-2	96	7.00E-99	KXS39292.1
1619	zntB	Zn	Zinc transport protein	Halomonadaceae bacterium T82-2	98.5	0.00E+00	KXS38423.1
1651		Cd, Zn, Pb	Zinc/cadmium/lead-transporting P-type ATPase	Halomonadaceae bacterium T82-2	97.4	0.00E+00	KXS38456.1
2740		Mn, Zn	putative manganese/zinc-exporting P-type ATPase	Halomonadaceae bacterium T82-2	94.96	0.00E+00	KXS37705.1
456	mntB	Mn	Manganese transport system membrane protein	Halomonadaceae bacterium T82-2	94.61	0.00E+00	KXS36360.1
945	mntP	Mn	putative manganese efflux pump	Halomonas sp. 362.1	83.07	2.00E-99	WP_129141166.1
2971	corC	Mg, Co	Magnesium and cobalt efflux protein	Halomonadaceae bacterium T82-2	98.97	0.00E+00	KXS39516.1
3129		Co	Sirohydrochlorin cobaltochelatase	Halomonadaceae bacterium T82-2	91.27	1.00E-74	KXS38081.1

Table 5 depicts heavy metal ion pumps and toxicity regulatory genes responsible for evading metal ion toxicity. *Modicisalibacter sp Wilcox* has specific and general ion pumps for As, Cu, Cd, Co, Zn, Pb, Mn, and Mg. Most of these transporters are efflux pumps coupling ion transport with ATP hydrolysis, but one general transporter for Cd, Co, and Zn is coupling transport via the cross transport of K+ ions. *Modicisalibacter sp Wilcox* also has an interesting arsenate reductase to produce arsenite. E values are all within significance cut-off and %identities are all above 85%.

Functional hydrocarbon degradation**:** Numerous studies have shown that petroleum-degrading microorganisms are widely distributed in diverse environments and have the potential to degrade petroleum compounds various degradation pathways under aerobic and anaerobic conditions. However, such information for high salinity environments is limited and only recently some studies have shown the capacity of halophiles and halotolerant to degrade petroleum compounds at high salinity (see Fathepure 2014; Le Borgne 2008; Martins and Peixoto 2012). Based on the genome sequence of strain Wilcox and its annotation, we bioinformatically predicted the degradation pathways of a few monoaromatic aromatic and aliphatic compounds. We have set up laboratory microcosms to experimentally validate the genome predicted degradation information for some important hydrocarbons. Table 6 shows the strains ability to degrade several monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, xylenes, benzoate as well as aliphatic compounds such as hexadecane and n-decane at 2.5 M NaCl as the sole source of carbon.

Class	Substrate	(+/-) Degradation	(+/-) Dergradation genes
MAH	BTEX (Combined)	(+)	(+)
MAH	Benzene	(+)	(+)
MAH	Toluene	(+)	(+)
MAH	Ethyl Benzene	(+)	(+)
MAH	Xylene	(+)	(+)
MAH	Benzoate	(+)	(+)
PAH	Naphthalene	(-)	(-)
PAH	Phenanthrene	(-)	(-)
n-alkane C-16	Hexadecane	(+)	(+)
n-alkane C10	Decane	(+)	(+)
n-alkane C-32	Dotriacontaone	(-)	(-)
n-alkane C-20	Eicosane	(-)	(-)
n-alkane C-6	Hexane	(-)	(-)
C-1	Methane	(-)	(-)

Table 6 depicts a concatenated list of functional hydrocarbons degradation. This was done via CFU growth quantitation or detection of the hydrocarbon being reduced overtime.. (+/-) indicate the positive or negative observation of hydrocarbon degradation. The presence or absence of genes primarily responsible for breaking down these compounds are also denoted here.

BTEX degradation in the presence of different NaCl concentrations**:** Graphs depicting BTEX removal in different NaCl concentrations can be found below in Figure 15 A-D. *Modicisalibacter* sp Wilcox appears to have an optimal BTEX degradation in 1M NaCl but can still degrade BTEX completely in all other tested NaCl concentrations. A BTEX removal rate in 1M NaCl can be reported as 6.32 umole of BTEX/day. The other NaCl concentrations are 3.96 umole of BTEX/day, 2.67 umole/day, .728 umole/day in 2M, 3M, 4M NaCl respectively. A chart depicting each individual rate can be found in the appendix in Figure SL.6.

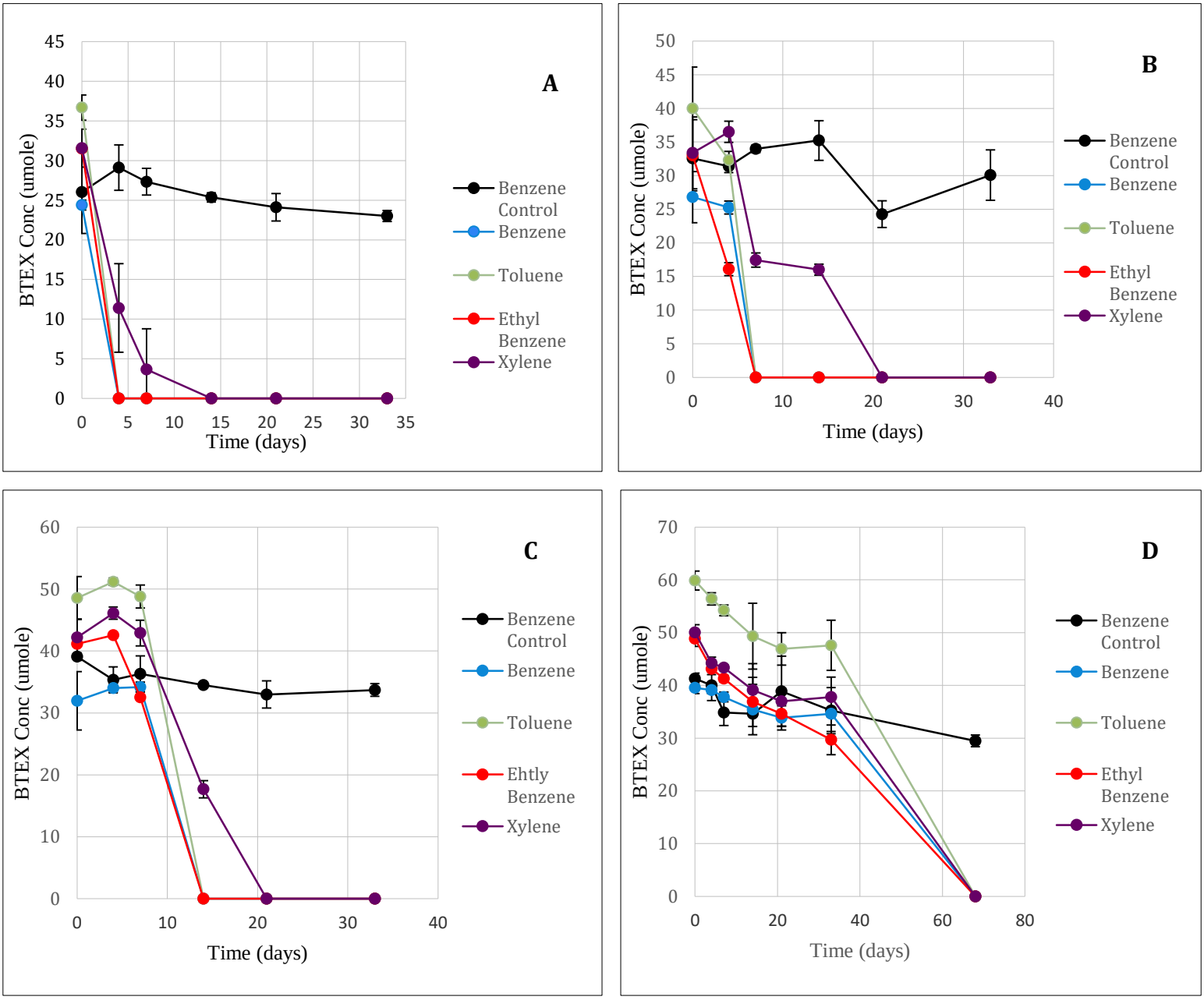

Figure 15 depicts BTEX degradation of *Modicisalibacter* sp Wilcox in different NaCl concentrations. Panel A-D represents 1M, 2M, 3M, 4M NaCl MSM media respectively. In panel A, benzene, toluene, and ethyl benzene were removed in 4 days, while xylene was completely removed later at 14 days. The concatenated rate of complete BTEX removal at 1M NaCl is 9.14 umole BTEX/day. Panels B-D all have slower rates of BTEX removal (6.33, 7.91, and 2.94 umole BTEX/day respectively). Even in all cases, BTEX was completely removed.

Benzoate degradation in the presence of benzene**:** Benzoate is successfully removed after complete BTEX degradation. In the presence of benzoate, benzene was completely removed after 10 days, having an average removal rate of 33 umole/day (Fig 16). This was much slower than benzene removal rate in the absence of benzoate. (167 umole/day of benzene). After the complete removal of benzene (10 days), the beginning of benzoate removal was noted. From this point on, benzoate was removed in 18 days (28 days from the start of the experiment). Benzoate was removed at a rate of 55 umole per day. (Fig 17)

Degradation of BTEX by *Modicisalibacter* sp Wilcox in PW**:** BTEX was successfully removed by *Modicisalibacter* sp Wilcox in produced water from Payne County. All BTEX constituents were degraded by 35 days but it can be observed that benzene, toluene and ethyl benzene were all removed in 21 days. Results are depicted in Figure 18.

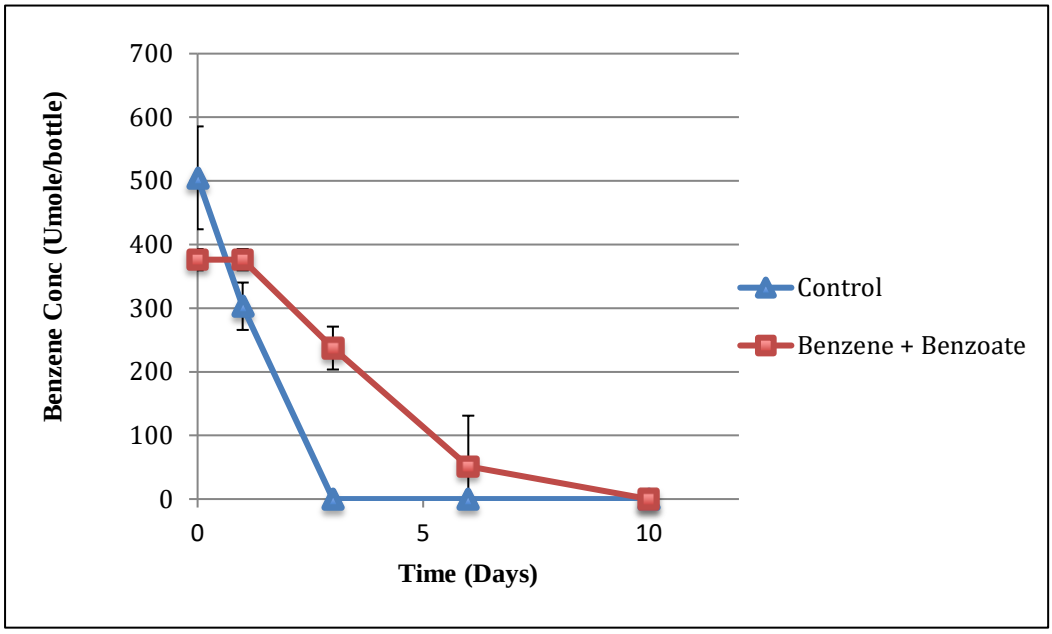

Figure 16 shows the isolate *Modicisalibacter* sp Wilcox degrading benzene in the presence (Benzene + Benzoate) or absence of benzoate (Control) over 10 days. Benzene was removed at an average rate of 167.33 umole/day and 38 umole/day for the control and benzene + benzoate microcosms respectively.

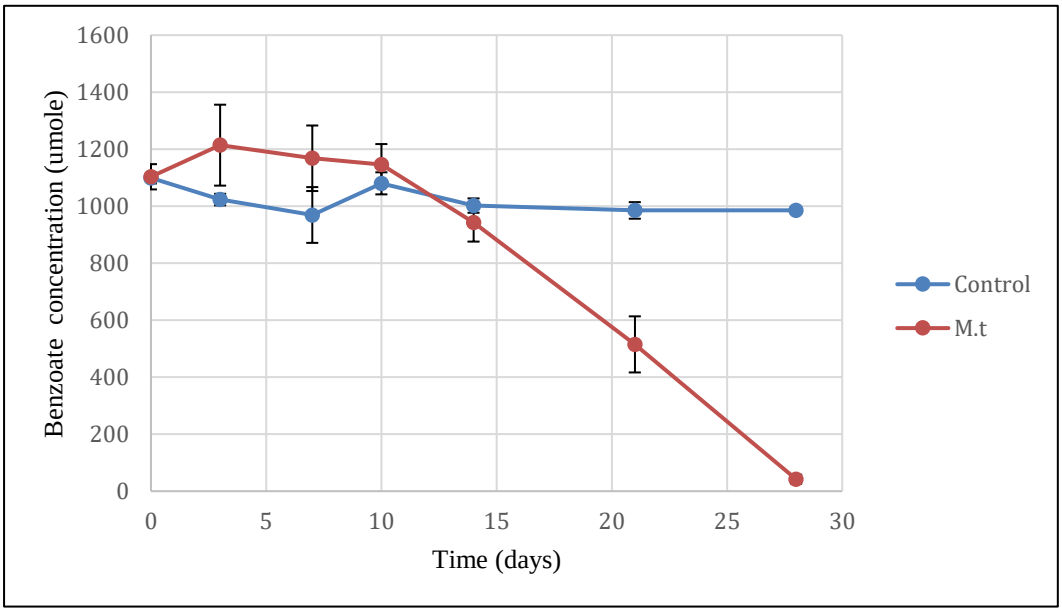

Figure 17 shows the isolate *Modicisalibacter* sp Wilcox degrading benzoate over the course of 28 days. Benzoate was removed at a rate of 55 umole/day. The control here represents microcosms without the presence of bacteria.

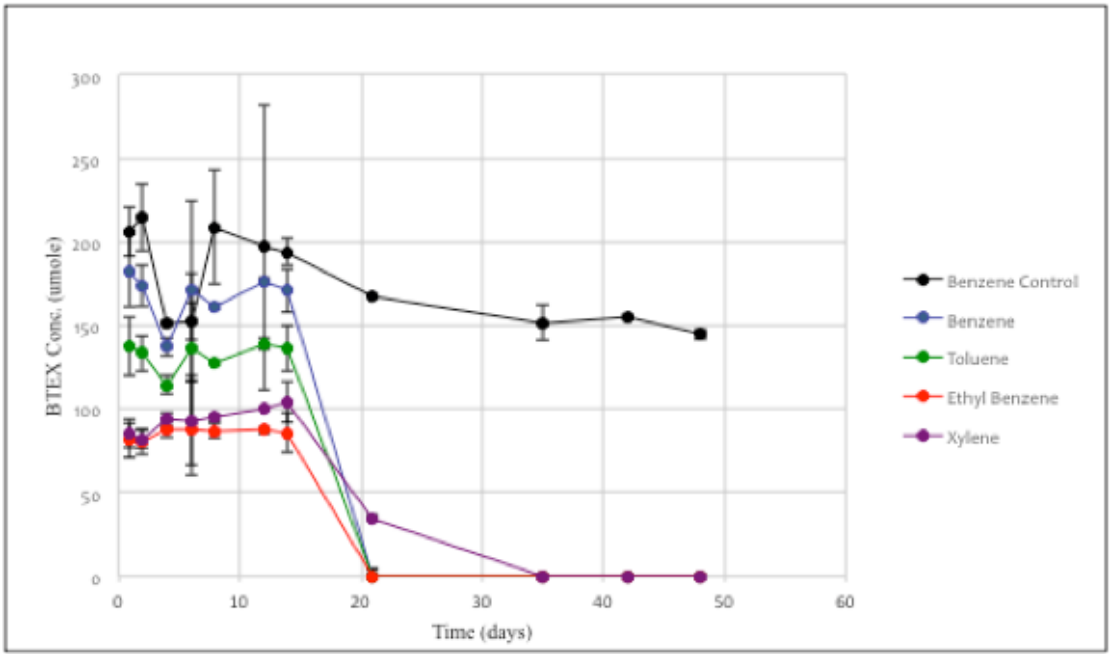

Figure 18 shows the isolate *Modicisalibacter* sp Wilcox degrading BTEX completely in 35 days in produced water from Payne county. Benzene, toluene, and ethyl benzene were all removed in 21 days while xylene was not detected to be absent until 35 days.

CONCLUSIONS:

Modicisalibacter sp Wilcox could be a strong candidate for hydrocarbon remediation in environments containing moderate-high levels of salt and an assortment of heavy metals (Cd, Cu, Co, Pb, As, Mn, Mg). *Modicisalibacter* sp Wilcox genome has an assortment of general and specific metal ion efflux pumps and reductases that lower the metal ion's toxicity. As shown, *Modicisalibacter* sp Wilcox is capable of degrading and utilizing an assortment of MAH and n-alkanes in 2.5 M NaCl. Based on genomic analysis, *Modicisalibacter* sp Wilcox can theoretically degrade phenol, salicylate, and phenyl acetate, although functional analysis of these compounds was not performed. The metal ion efflux pumps and reductases outline an interesting environmental regulation dynamic and resistance. The genome encodes multiple arsenic reductases and no arsenite reductases. As(III) is much more systemically toxic than As(V). The As(III) produced from the action of arsenic reductases is theoretically exported outside the cell where other microorganisms will likely further reduce and precipitate it out of solution (Jonnalagadda 1993, Zobrist 2000, Radabaugh 2000).

The genomically encoded hydrocarbon-degrading potential was functionally characterized and confirmed for BTEX, hexadecane, and benzoate. These are all hydrocarbons derived from oil production mediated contamination and other industrial processes. BTEX was removed, on average, to below detection limits in 5-7 days when all BTEX constituents were present in one experimental microcosm. These compounds are of greater municipal concern due to their relatively high solubility making it difficult to remove industrially during water remediation (Njobuenwu 2005, Štandeker 2009).

The diverse presence of metal ion exporters could potentially belay *Modicisalibacter* sp Wilcox' ability to withstand environments containing a diverse set of heavy metals at potentially high concentrations. *Modicisalibacter* sp Wilcox could stand as an excellent candidate to assist in remediation of oil-production derived produced water, one such heavy metal and highly saline contaminated water source that typically contains hydrocarbon pollution.

CHAPTER V

ANAEROBIC ETHYL BENZENE DEGRADATION BY *MODICISALIBACTER SP* WILCOX

BACKGROUND: Hydrocarbon pollution has been described in both aerobic and anaerobic environments. Hydrocarbon degradation under anaerobic conditions is less characterized than its aerobic counterpart. Anaerobic ethyl benzene degradation primarily occurs through $-NO_3$ and $-SO_4$ reduction with the addition of fumarate or dehydrogenation of the terminal methyl group extending from the aromatic ring. Anaerobic ethyl benzene degradation has only been reported in a few particular organisms (EbN1, PbN1, a newly described *Dechloromonas* strain RCB, and EbS7 respectively (Rabus 1995) (Kniemeyer 2003) (Chakraborty 2005)).

MATERIALS AND METHODS:

Anaerobic microcosm preparation**:** 50 ml of anaerobic 2.5M NaCl MSM media were prepared in 160 ml glass serum bottles (anaerobic MSM media contains 5 mM $NaNO_3$, 0.4g/l L-Cysteine, 500 mg/L resazurin with N_2 gas flushing for ~15-20 min prior to autoclaving). Microcosms were aliquoted with 1 µl of BTEX-mixture or ethyl benzene alone to reach final concentrations of 35-65 µmole of each BTEX constituent or 90-100 µmole of ethyl benzene alone. Microcosms were inoculated with 1 ml of *Modicisalibacter* sp Wilcox culture. Uninoculated control microcosms were also prepared.

BTEX quantitation using Gas Chromatography(GC) - Biodegradation of BTEX compounds were assayed by using a Hewlett Packard 6890 GC equipped with a flame ionization detector and a DB-1 capillary column (30 m by 0.320 mm by 1 m; J&W Scientific, Inc.). Nitrogen served as both carrier and makeup gas at flow rates of 10 and 40 ml/min, respectively. The flow rates of hydrogen and air were set at 40 and 450 ml/min, respectively. The operating GC conditions were the following: oven temperature, 70°C for 7 min; inlet temperature, 150°C; and detector temperature, 220°C. Approximately 100 µl of headspace gas from microcosms was injected into the GC for quantification. The GC response for each compound tested was calibrated to give the total mass in that bottle. Standards were prepared in 160-ml bottles filled with 50 ml of NaCl solution (0 to 4 M) and were closed with Teflon-faced septa and aluminum caps. After equilibration at room temperature the GC response for a range of mass (in micromoles/bottle) of each compound tested was plotted, and the slopes were used to quantify the unknown. The quantification of BTEX in enrichment bottles was accomplished as described above by using a calibration curve prepared with 1-liter bottles containing 500 ml of 2.5 M NaCl solution. The GC detection limit for benzene using our method was <1.0 umol/bottle.

RESULTS AND DISCUSSION:

Selective anaerobic degradation of ethyl benzene in BTEX-mixture by *Modicisalibacter* sp Wilcox: As shown in Fig 21, ethyl benzene concentrations were below detectable levels after 7 days. Other BTEX constituents remained at a concentration similar to the control benzene concentration and did not decrease for the duration of experiment. On day 21, ethyl benzene was re-spiked back to an

increased concentration (~100 µmole), and within 5 days the levels of ethyl benzene dropped to 9 µmole. In this case, the effective rate of ethyl benzene removal was 5.7 µmole/day in the first instance and > 20 µmole/day following re-spiking.

Anaerobic removal of ethyl benzene by *Modicisalibacter* sp Wilcox in the absence of other BTX-constituents**:** In the absence of BTX, *Modicisalibacter* sp Wilcox was able to remove ethyl benzene in 4 days at an average rate of 22.5 µmole/day, the fastest anaerobic ethyl benzene removal rate for *Modicisalibacter* sp Wilcox. Results can be noted in Figure 22.

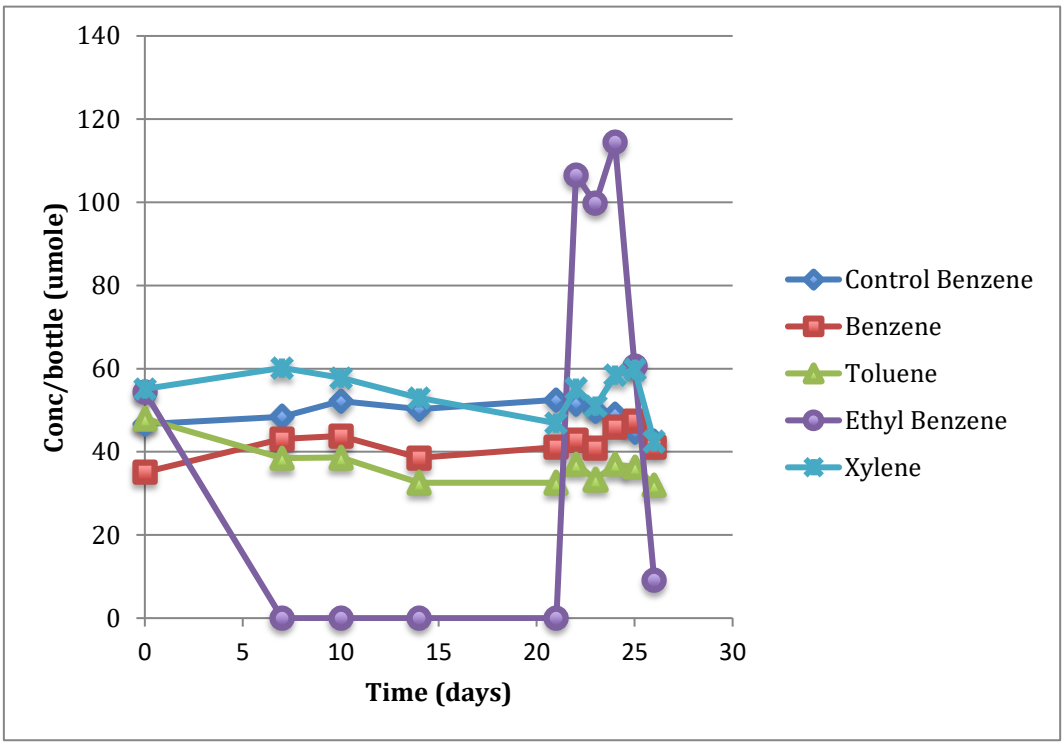

Figure 21 shows the anaerobic degradation of ethyl benzene in 7 days with a re-spike of ethyl benzene being likewise removed in 5 days following. The effective rate for the instance of ethyl benzene removal stands at 5.7 umole/day and at the second instance, 20 umole/day.

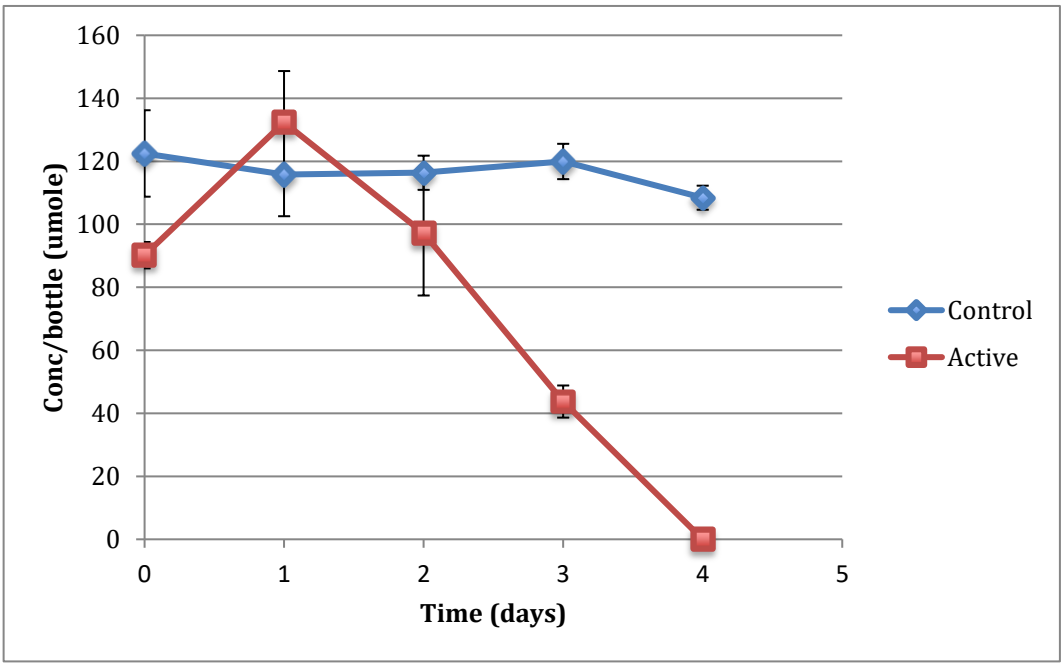

Figure 22 shows the anaerobic degradation of ethyl benzene in 4 days. The effective rate of ethyl benzene removal stands at 22.5 umole/day.

CONCLUSIONS:

These preliminary observations show *Modicisalibacter* sp Wilcox's ability to degrade ethyl benzene in nitrate-reducing anaerobic conditions. Interestingly, ethyl benzene was the only aromatic hydrocarbon to be removed over the duration of the experiment. As previous literature has shown, toluene is more readily degraded (Coates 1996b, Dofling 1990). More work needs to be done to truly confirm the nitrate reducing-ethyl benzene oxidation coupling through stoichiometric comparisons of nitrate and ethyl benzene.

CHAPTER VI

OVERALL CONCLUSIONS

Oil-polluted hypersaline environments are ubiquitous. These environments may be man-made or naturally occurring, but in either case, pollution could seep into and contaminate important municipal infrastructure. Organisms found in saline and hypersaline environments could have the potential to degrade certain oil-derived pollution. Enrichment and characterization of halophilic bacteria could help, in part, reduce remediation costs of these environments. Hydrocarbon degrading halophilic organisms could be potentially enriched and applied to heavily polluted saline environments. One of these environments include oil-production derived produced water; a heavy metal, saline, and hydrocarbon contaminated waste water stream being produced by the billions of gallons a year.

Microbial enrichments from Rozel point (Rozel) and Kuwait (KWT) were enriched, characterized, and applied to different produced waters. The KWT culture was enriched to 99% with one organism, an *Arhodomonas sp.* Metagenomic sequencing and KEGG pathway construction revealed the presence of certain genes encoding enzymes responsible for BTEX degradation.

These enrichment cultures were both able to degrade aromatic hydrocarbons (BTEX) in raw produced water from Payne county (TDS= ~146,000). Each culture, KWT and Rozel, achieved complete degradation in 9 or 21 days respectively.

However, when the cultures were applied to produced water from Grant County (TDS ~394,000), both cultures were unable to degrade any of the BTEX constituents. Diluting this Grant county produced water with municipal waste water not only rescued BTEX degradation by both the Rozel and KWT microbial culture, but it also accelerated the BTEX degradation rate in wastewater diluted Payne county produced water.

To switch gears, our group was interested in isolating novel organisms from produced water itself. An organism was isolated from this produced water having a 99% 16s rRNA (SL2) sequence similarity to a novel halophilic organism, *Modicisalibacter* sp Wilcox. Our isolate branches closely to other known *Modicisalibacter* sp and away from closely related halophilic organisms from *Chromohalobacter* and *Halomonas* (SL3). *M. tunisiensis* was initially isolated from fracking flow-back water living optimally at 1M NaCl. *Modicisalibacter* sp Wilcox genome was sequenced and genes encoding enzymes for BTEX and benzoate degradation were identified. These enzymes funnel aromatic intermediates through catechol via catechol-1,2-dioxygenase for downstream ring cleavage. *Modicisalibacter* sp Wilcox also have the genetic potential to withstand a variety of heavy metals with its assortment genes responsible for metal ion ATP-ases and reductases. In an attempt to functionally confirm both benzoate and BTEX degradation, we developed microcosms containing these hydrocarbons. Interestingly, benzoate was never removed when used as the lone carbon source (Data not shown). However, in the presence of benzene, benzoate was detected to decrease over a 5 day period following the complete removal of benzene. BTEX was also degraded at a rate of 9.7 umole of BTEX/day in an optimal NaCl of 1M. In order to look at BTEX degradation rates in higher salt concentrations, we performed experiments in MSM

media microcosms with increasing levels of NaCl (1-4M). We saw an optimal BTEX degradation rate at 1M NaCl, marginally slower rates in the 2-3M NaCl range, but surprisingly, we saw complete BTEX degradation even at 4 M, albeit with a much slower average rate, at about 2.94 umole BTEX/day. Lastly, in attempt to apply this organism to produced water, we saw complete BTEX degradation in Payne county produced water in 21 days.

During KEGG pathway metabolic reconstruction, our group noticed the presence of Nrt and nitrate reductase, and sought to explore the potential anaerobic BTEX degradation. Although results are preliminary, *Modicisalibacter* sp Wilcox is capable of degrading ethyl benzene alone and in the presence of other BTX constituents under nitrate-reducing conditions. More work needs to be done to further confirm actual nitrate reduction coupled to ethyl benzene oxidation.

References

Anderson RT, Rooney-Varga J, Gaw CV, Lovley DR (1998) Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. Environ Sci Technol 32:1222–1229

Ayangbenro, Ayansina Segun, and Olubukola Oluranti Babalola. "A new strategy for heavy metal polluted environments: a review of microbial biosorbents." International journal of environmental research and public health 14.1 (2017): 94.

Azetsu-Scott, Kumiko, et al. "Precipitation of Heavy Metals in Produced Water: Influence on Contaminant Transport and Toxicity." Marine Environmental Research, vol. 63, no. 2, 2007, pp. 146–167., doi:10.1016/j.marenvres.2006.08.001.

Ball HA, Johnson HA, Reinhard M, Spormann AM (1996) Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol 178:5755–5761

Baquiran, J. P., Thater, B., Songco, K., & Crowley, D. E. (2012). Characterization of Culturable PAH and BTEX Degrading Bacteria from Heavy Oil of the Rancho La Brea Tarpits. *Polycyclic Aromatic Compounds,32*(5), 600-614. doi:10.1080/10406638.2011.651678

Cafaro, V., et al. "Phenol Hydroxylase and Toluene/o-Xylene Monooxygenase from Pseudomonas Stutzeri OX1: Interplay between Two Enzymes." Applied and Environmental Microbiology, vol. 70, no. 4, 2004, pp. 2211–2219., doi:10.1128/aem.70.4.2211-2219.2004.

Chakraborty, R., and J. D. Coates. "Anaerobic degradation of monoaromatic hydrocarbons." *Applied Microbiology and Biotechnology* 64.4 (2004): 437-446.

Chakraborty, Romy, et al. "Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by Dechloromonas strain RCB." *Appl. Environ. Microbiol.* 71.12 (2005): 8649-8655.

Chandrakant S, Karigar S, Rao S (2011). Role of microbial enzymes in the bioremediation of pollutants: A review. Enzyme Res. Article ID 805187. https://doi.org/10.4061/2011/805187.

Chavan, A., & Mukherji, S. (2008). Treatment of hydrocarbon-rich wastewater using oil degrading bacteria and phototrophic microorganisms in rotating biological contactor: effect of N: P ratio. *Journal of hazardous materials*, *154*(1-3), 63-72.

Çınar, Özer. "Biodegradation of central intermediate compounds produced from biodegradation of aromatic compounds." Bioprocess and biosystems engineering 26.5 (2004): 341-345.

Coates JD, Chakraborty R, Lack JG, O'Connor SM, Cole KA,

Bender KS, Achenbach LA (2001) Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas. Nature 411:1039–1043

Coates JD, Phillips EJP, Lonergan DJ, Jenter H, Lovley DR (1996b) Isolation of Geobacter species from a variety of sedimentary environments. Appl Environ Microbiol 62:1531–1536

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol 134:336–341

Durairaj P, Hur J, Yun H (2016). Versatile biocatalysis of fungal cytochrome P450 monooxygenases. Microb Cell Fact 15, 125

Dwyer, B. P. (2016). Treatment of Oil & Gas Produced Water. doi: 10.2172/1238102

Edwards EA, Wills LE, Reinhard M, Grbi'c-Gali'c D (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl Environ Microbiol 58:794–800

Evans PJ, Mang DT, Young LY (1991) Degradation of toluene and m-xylene and transformation of o-xylene by denitrifying enrichment cultures. Appl Environ Microbiol 57:450–454

Fakhru'l-Razi A1., Pendashteh A., Abdullah LC., Biak DR., Madaeni SS., Abidin ZZ. (2009). Review of technologies for oil and gas produced water treatment. *Journal of Hazardous Materials*,170(2-3):530-51.doi: 10.1016/j.jhazmat.2009.05.044.

Farkas, M., Szoboszlay, S., Benedek, T., Révész, F., Veres, P. G., Kriszt, B., & Táncsics, A. (2016). Enrichment of dissimilatory Fe(III)-reducing bacteria from groundwater of the Siklós BTEX-contaminated site (Hungary). *Folia Microbiologica,62*(1), 63-71. doi:10.1007/s12223-016-0473-8

Fathepure B.Z (2014). Recent Studies in Microbial Degradation of Petroleum Hydrocarbons in Hypersaline Environments. Frontiers in Microbiology 5, doi:10.3389/fmicb.2014.00173.

Fidelis Silva, L. C., Lima, H. S., Sartoratto, A., de Sousa, M. P., Rodrigues Torres, A. P., de Souza, R. S., & ... da Silva, C. C. (2018). Effect of salinity in heterotrophic nitrification/aerobic denitrification performed by acclimated microbiota from oil-produced water biological treatment system. International Biodeterioration & Biodegradation, 1301-7.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl Environ Microbiol 60:2802–2810

Fukuchi, Satoshi, et al. "Unique amino acid composition of proteins in halophilic bacteria." Journal of molecular biology 327.2 (2003): 347-357.

Galinski, Erwin A., and Hans G. Trüper. "Microbial behaviour in salt-stressed ecosystems." *FEMS Microbiology Reviews* 15.2-3 (1994): 95-108.

Gam, Zouhaier Ben Ali, et al. "Modicisalibacter tunisiensis gen. nov., sp. nov., an aerobic, moderately halophilic bacterium isolated from an oilfield-water injection sample, and emended description of the family Halomonadaceae Franzmann et al. 1989 emend Dobson and Franzmann 1996 emend. Ntougias et al. 2007." *International journal of systematic and evolutionary microbiology* 57.10 (2007): 2307-2313.

Gibson DT, Parales RE (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. Current opinion in biotechnology. 3:236-43.

Grbi'c-Gali'c D, Vogel T (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol 53:254–260

Harwood CS, Parales RE (1996). The beta-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50: 553–590.

Jonnalagadda, S. B., & Rao, P. P. (1993). Toxicity, bioavailability and metal speciation. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *106*(3), 585-595.

Kempf, Bettina, and Erhard Bremer. "Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments." *Archives of microbiology* 170.5 (1998): 319-330.

Kita, Akiko, et al. "An archetypical extradiol-cleaving catecholic dioxygenase: the crystal structure of catechol 2, 3-dioxygenase (metapyrocatechase) from Pseudomonas putida mt-2." Structure 7.1 (1999): 25-34.

Kniemeyer O, Fischer T, Wilkes H, Glockner F, Widdel F (2003) Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol 69:760–768

Konikow, L.F., 2013, Groundwater depletion in the United States (1900−2008): U.S. Geological Survey Scientific Investigations Report 2013−5079, 63 p., http://pubs.usgs.gov/sir/2013/5079.

Koslov, M. M., and V. S. Markin. "A theory of osmotic lysis of lipid vesicles." *Journal of theoretical biology* 109.1 (1984): 17-39.

Krieger CJ (1999) Initial reactions in anaerobic oxidation of mxylene by the denitrifying bacterium Azoarcus sp. strain T. J Bacteriol 181:6403–6410

Le Borgne S, Paniagua D, Vazquez-Duhalt R (2008). Biodegradation of organic pollutants by halophilic bacteria and archaea. J. Mol. Microbiol. Biotechnol. 15, 74-92.

Leuthner B, Leutwein C, Schulz H, Horth P, Haehnel W, Schiltz E, Schagger H, Heider J (1998) Biochemical and genetic characterization of benzylsuccinate synthase from Thauera aromatica: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 28:615–628

Logisz, Catherine C., and Jennifer S. Hovis. "Effect of salt concentration on membrane lysis pressure." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1717.2 (2005): 104-108.

Lovley DR (1997) Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. J Ind Microbiol 18:75–81

Liu, K., Atiyeh, H. K., Stevenson, B. S., Tanner, R. S., Wilkins, M. R., & Huhnke, R. L. (2014). Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols. Bioresource technology, 152, 337-346.

Macchi M, Martinez M, Tauil RN, Valacco M, Morelli I, Coppotelli B. (2018) Insights into the genome and proteome of *Sphingomonas paucimobilis* strain 20006FA involved in the regulation of polycyclic aromatic hydrocarbon degradation. World Journal of Microbiology and Biotechnology 34: 7. https://doi.org/10.1007/s11274-017-2391-6 PMID: 29214360

Nicholson CA, Fathepure BZ. 2004. Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. Appl. Environ. Microbiol. 70:1222–1225

Njobuenwu, D. O., Amadi, S. A., & Ukpaka, P. C. (2005). Dissolution rate of BTEX contaminants in water. *The Canadian Journal of Chemical Engineering*, *83*(6), 985-989.

Martins LF, Peixoto RS (2012). Biodegradation of petroleum hydro- carbons in hypersaline environments. Braz. J. Microbiol. 43, 865–872

Palmer, L. (1978). Brine Shrimp Bioassay Procedure For Determining Produced Water Toxicity. *SPE California Regional Meeting*. doi:10.2118/7129-ms

Pérez-Pantoja, D., González, B., & Pieper, D. H. (2010). Aerobic degradation of aromatic hydrocarbons. In *Handbook of hydrocarbon and lipid microbiology*.
.

Ohlendorf, D. H., Lipscomb, J. D., & Weber, P. C. (1988). Structure and assembly of protocatechuate 3, 4-dioxygenase. *Nature*, *336*(6197), 403.

Ojo, O. A. (2006). Petroleum-hydrocarbon utilization by native bacterial population from a wastewater canal Southwest Nigeria. *African Journal of biotechnology*, *5*(4), 333-337.

Oren, Aharon. "Microbial life at high salt concentrations: phylogenetic and metabolic diversity." *Saline systems* 4.1 (2008): 2.

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, et al (2008). Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol. Rev. 32: 927-955.

Pérez-Pantoja, D., B. González, and D. H. Pieper. "Aerobic degradation of aromatic hydrocarbons." Handbook of hydrocarbon and lipid microbiology. 2010.

Rabus R, Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 163:96–103

Radabaugh, T. R., & Aposhian, H. V. (2000). Enzymatic reduction of arsenic compounds in mammalian systems: reduction of arsenate to arsenite by human liver arsenate reductase. *Chemical research in toxicology*, *13*(1), 26-30.

Ranck, J. M., Bowman, R. S., Weeber, J. L., Katz, L. E., & Sullivan, E. J. (2005). BTEX Removal from Produced Water Using Surfactant-Modified Zeolite. *Journal of Environmental Engineering,131*(3), 434-442. doi:10.1061/(asce)0733-9372(2005)131:3(434)

Reineke W, Knackmuss H-J (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1, 2-dioxygena-tion of benzoic acid. Biochim Biophys Acta 532: 412–423.

Seo J-S, Keum Y-S, Li QX. (2009). Bacterial degradation of aromatic compounds. Inter. journal of environmental research and public health. 2009; 6:278–309. https://doi.org/10.3390/ijerph6010278 PMID: 19440284

Shergill, J. K., Joannou, C. L., Mason, J. R., & Cammack, R. (1995). Coordination of the Rieske-type [2Fe-2S] cluster of the terminal iron-sulfur protein of Pseudomonas putida benzene 1, 2-dioxygenase, studied by one-and two-dimensional electron spin-echo envelope modulation spectroscopy. *Biochemistry*, *34*(51), 16533-16542.

Shivanand, Pooja, and Gopal Mugeraya. "Halophilic bacteria and their compatible solutes–osmoregulation and potential applications." Current science (2011): 1516-1521.

Shokrollahzadeh, S., Azizmohseni, F., Golmohammad, F., Shokouhi, H., & Khademhaghighat, F. (2008). Biodegradation potential and bacterial diversity of a petrochemical wastewater treatment plant in Iran. *Bioresource technology*, *99*(14), 6127-6133.

Shores, A. R., Hethcock, B., & Laituri, M. (2018). Phytoremediation of BTEX and Naphthalene from produced-water spill sites using Poaceae. *International Journal of Phytoremediation,20*(8), 823-830. doi:10.1080/15226514.2018.1438352

Shu, Lijin, et al. "X-Ray Absorption Spectroscopic Studies of the Fe(II) Active Site of Catechol 2,3-Dioxygenase. Implications for the Extradiol Cleavage Mechanism." Biochemistry, vol. 34, no. 20, 1995, pp. 6649–6659., doi:10.1021/bi00020a010.

Silver, Simon. "Bacterial resistances to toxic metal ions-a review." Gene 179.1 (1996): 9-19.

Silver, Simon, and Guangyong Ji. "Newer systems for bacterial resistances to toxic heavy metals." Environmental health perspectives 102.suppl 3 (1994): 107-113.

Stainer, R. Y., & Ornston, L. N. (1973). The β-ketoadipate pathway. In *Advances in microbial physiology* (Vol. 9, pp. 89-151). Academic Press

Štandeker, S., Novak, Z., & Knez, Ž. (2009). Removal of BTEX vapours from waste gas streams using silica aerogels of different hydrophobicity. *Journal of hazardous materials*, *165*(1-3), 1114-1118.

U.S. EPA. Assessment of the Potential Impacts of Hydraulic Fracturing for Oil and Gas on Drinking Water Resources (External Review Draft). U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-15/047, 2015.

U.S. EPA. Hydraulic Fracturing for Oil and Gas: Impacts from the Hydraulic Fracturing Water Cycle on Drinking Water Resources in the United States (Final Report). U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-16/236F, 2016.

Wargo, Matthew J. "Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa." *Appl. Environ. Microbiol.* 79.7 (2013): 2112-2120.

Welsh, David T. "Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate." *FEMS microbiology reviews* 24.3 (2000): 263-290.

Viswanathan, Rathinam, et al. "Functional models for catechol 1, 2-dioxygenase. Synthesis, structure, spectra, and catalytic activity of certain tripodal iron (III) complexes." Inorganic Chemistry 37.12 (1998): 2943-2951.

Vogel TM, Grbi'c-Gali'c D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl Environ Microbiol 52:200–202

Vyrides, I., & Stuckey, D. C. (2017). Compatible solute addition to biological systems treating waste/wastewater to counteract osmotic and other environmental stresses: a review. *Critical reviews in biotechnology*, *37*(7), 865-879.

Yamamoto S, Katagiri M, Maeno H, Hayaishi O (1965). Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and gen-eral properties. J Biol Chem 240: 3408–3413.

Zobrist, J., Dowdle, P. R., Davis, J. A., & Oremland, R. S. (2000). Mobilization of arsenite by dissimilatory reduction of adsorbed arsenate. *Environmental Science & Technology*, *34*(22), 4747-4753

FIGURE APPENDIX

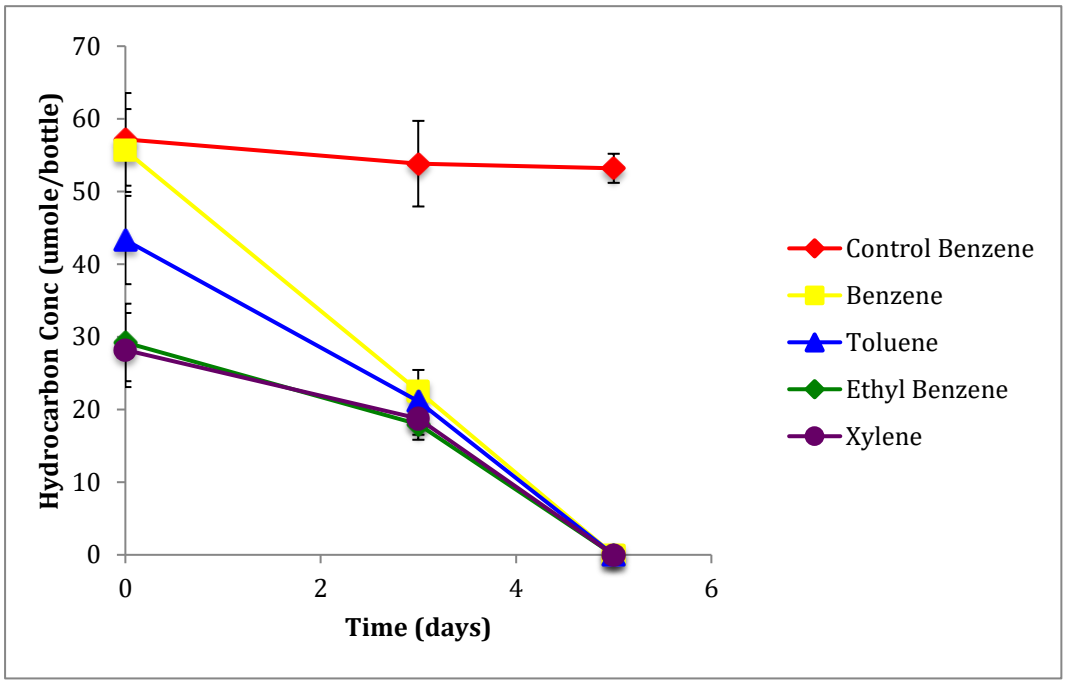

Figure SL.1 shows the BTEX degradation by the KWT culture in 50:50 produced water from Grant county and tap water. The Control Benzene represents an un-inoculated microcosm replicate. All lines represent specific monocyclic hydrocarbon amounts per microcosm. Control Benzene line (◆) are averages from 2 microcosm replicates. Active lines (■◆●▲) are averages from 3 biological replicates. Error bars represent standard deviation.

Modicisalibacter strain Wilcox 16s rRNA sequence –

GGCCTGGGGCAGCTTACCATGCAGTCGAGCGGAAACGATCCCAGCTTGCT
GGGAGGCGTCGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTGCCCG
GTAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTATCGGATGAGCCTATG
TCGGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCAACGATCCGTA
GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCC
TGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCAC
TTTCAGCGGGGAAGAAAGCGTGCCGGTTAATACCCGGCACGGACGACATC
ACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG
CGGCCTGTTAAGTCAGATGTGAAAGCCCCGGGCTCAACCTGGGAATGGCA
TTTGAAACTGGCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGT
GTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCG
GCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGT
TGGGTCCCTTGAGGACTTAGTGGCGCAGTTAACGCGATAAGTCGACCGCC
TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAGAACCTTA
CCTACCCTTGACATCCTCGGAACTTGGCAGAGATGCCTTGGTGCCTTCGGG
ACCGAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAATGTG
GGTAGTCCCGTACGAGCGCACCCTATCCTATTTGCAGCGATCGTCGGGAC
TCTAGGGAGACTGCGTGACAACGGAGAGGTGGGATGACGTCAGTCATCAT
GCCTACGTAGGCTACCACGTGCTACATGACGTACAAGGGTGCATCCTCGC
GGAGAGGGGAGCGAATCCAGAAG

Figure SL2 denotes the 16s rRNA sequence produced from the 27F PCR primer.

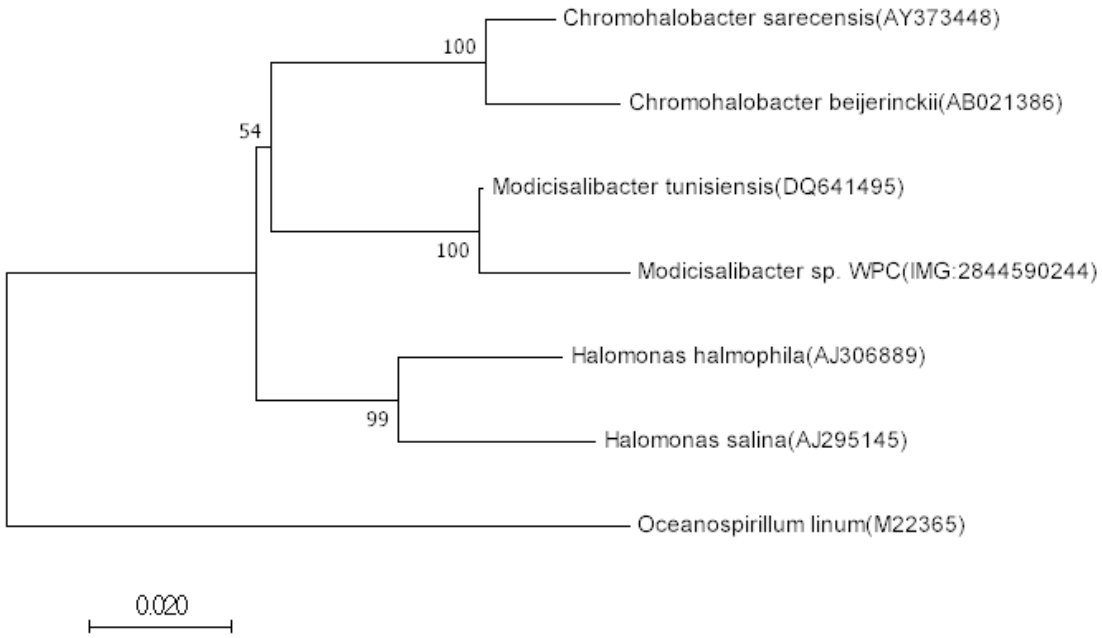

Figure SL3 depicts a 16s rRNA phylogenetic tree run with 100 bootstraps. Modicisalibacter sp WPC always associated itself next to the known *Modicisalibacter tunisiensis* while branching away from other known halophiles like *Chromohalobacter* and *Halomonas.*

Microcosm component	Culture name	Rate of BTEX removal (µmol/day)
Payne county PW	KWT	5.92
Payne county PW	Rozel	1.86
Grant county PW	KWT	0
Grant county PW	Rozel	0
Grant county PW/WW	KWT	9.13
Grant county PW/WW	Rozel	5.744

Figure SL.4 shows the average BTEX removal rates shown in figures 8-13 above. Rate of BTEX removal rate is shown as the average rate for each BTEX constituent averaged together per day.

Metagenomic Sequencing Results of KWT Enrichment	Value/Count
Raw reads (<150bp)	5,010,546
After Trimming	
Paired reads	1,315,515
Forward unpaired	155,408
Reverse unpaired	64,471
After Assembly	
N50	15,766
L50	64
Total Assembly Length	4,555,116

Figure SL.5 depicts the KWT enrichment's metagenomics sequencing data and statistics.

BTEX constituent rates from figure 15A-D	Rate (umole/day)
1M	[redacted]
Benzene	6
Toluene	9.25
Ethyl Benzene	8
Xylene	2.28
Avg	6.3825
2M	[redacted]
Benzene	3.71
Toluene	5.71
Ethyl Benzene	4.71
Xylene	1.6
Avg	3.9325
3M	[redacted]
Benzene	2.29
Toluene	3.43
Ethyl Benzene	3
Xylene	2.04
Avg	2.69
4M	[redacted]
Benzene	0.588
Toluene	0.882
Ethyl Benzene	0.72
Xylene	0.735
Avg	0.73125

Figure SL.6 depicts the individual BTEX removal rates in 1-4M by *Modicisalibacter* sp. Wilcox.

VITA

William Marsh

Candidate for the Degree of

Master of Science

Thesis: GENOMIC AND METAGENOMIC CHARACTERIZATION OF HALOPHILIC MICROORGANISMS WITH INSIGHTS INTO THE BIOREMDIATION OF PRODUCED WATER.

Major Field: Microbiology and Molecular Genetics

Biographical:

Education:

Completed the requirements for the Master of Science in Microbiology and Molecular Genetics at Oklahoma State University, Stillwater, Oklahoma in May, 2020.

Completed the requirements for the Bachelor of Science in Microbiology and Molecular Genetics at Oklahoma State University, Stillwater, Oklahoma in May, 2018.

Presentations at Academic and Professional Meetings:
William Marsh and Babu Fathepure. Hydrocarbon Degradation in the Remediation of Contaminated Produced Water. Annual ASM Missouri and Missouri Valley Branch meeting, University of Nebraska, Omaha, Nebraska, 16 March 2019.

William Marsh and Babu Fathepure. Bioremediation of Hydrocarbon polluted Produced Water using salt-loving Microbial Consortia. Oklahoma Governor's Water Conference. Midwest City, Oklahoma, 4 December 2019

Academic Leadership:
President, Microbiology and Molecular Genetics' Graduate Student Association, Oklahoma State University, August 1st 2019 – June 1st 2020.