7-2014

Isolation and characterization of polychlorinated biphenyl (PCB) degrading bacteria isolated from a PCB contaminated reservoir in Donna, TX

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ISOLATION AND CHARACTERIZATION OF POLYCHLORINATED BIPHENYL (PCB) DEGRADING BACTERIA ISOLATED FROM A PCB CONTAMINATED RESERVOIR IN DONNA, TX

A Thesis

by

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Submitted to the Graduate School of
The University of Texas-Pan American
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

July 2014

Major Subject: Biology

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ABSTRACT

Cepeda, Sergio., Isolation and Characterization of Polychlorinated Biphenyl (PCB) Degrading Bacteria Isolated from a PCB Contaminated Reservoir in Donna, TX. Master of Science (MS), July, 2014, 87 pp., 9 tables, 15 figures, references, 68 titles.

The Donna Irrigation System (DIS) located in Donna, Hidalgo County, TX, was confirmed to be contaminated with polychlorinated biphenyls (PCBs) in 1993 and remediation of the site will be attempted in the future. The need to isolate novel PCB degrading bacteria for bioremediation has been an active area of research in recent decades; thus, it was hypothesized that a variety of microorganisms able to degrade PCBs could be isolated from sediments taken from the DIS. Two genes of the PCB degradation pathway, *bphA1* and *bphC*, were detected in 43 bacterial isolates grown from DIS sediments using molecular and culture-based methodology, confirming the PCB degradation potential of these bacteria. Biochemical testing indicated differences in the enzymatic activity, carbohydrate fermentation ability, and carbon source utilization patterns of these isolates and strongly suggests that a variety of species, and possibly genera, of PCB degrading bacteria are present in the sediments of the DIS.

DEDICATION

I dedicate the completion of my Master's degree to all the members of my loving family. My parents and brothers have always been a source of encouragement and have provided me with the common sense I lack despite my advanced studies.

I also want to thank the many friends I have made during my studies. All of you will never be forgotten. Many of you helped in the completion of this work, whether you realize it or not, and I am eternally thankful for that.

Lastly, I would like to thank my advisors for all their support and patience in this long process. Without their expertise I could not have accomplished this task. I hope I can influence the younger generation in the same way you influenced my studies.

ACKNOWLEDGMENTS

I will be forever indebted to my committee for helping me throughout this project. I am deeply thankful to Dr. Luis Materon for helping start this project, his patience throughout its development and his editing of this manuscript. I cannot give enough thanks to Dr. Kristine Lowe for her assistance in the technical aspects of this project and her advice on many other matters. This manuscript would surely be of lesser quality without her help. I also greatly thank Dr. Michael Persans for his help in mediating during the toughest times of this project. His advice to take the project one small step at a time instead of focusing on a huge goal was a much needed turning point.

I also wish to thank the Biology department of the University of Texas-Pan American for their help in the many aspects relating to this thesis. The incredible generosity Dr. Erin Schuenzel showed in helping with my project, despite not being part of my committee, will never be forgotten. Dr. Anxiu Kuang was instrumental in acquiring the electron micrographs used in this manuscript and I cannot thank her enough for teaching me how to use the electron microscope. I also have a huge amount of gratitude for Mrs. Bonnie Gunn. She made me a far better scientific writer and was always there when I needed help, criticism, and a dose of realism.

I am eternally grateful to all the friends that volunteered their time to help me finish this project. Most of you will never realize how much you helped me get through this. Last, but certainly not least, I thank Ms. Jacqueline Valencia, who motivated me to get this degree, and Ms. Monika Martinez for her great help in sample collection.

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CHAPTER I

INTRODUCTION

Statement of Problem

In 1991, three babies with neural tube defects were born within a 36-hour period in the same hospital in Brownsville, Cameron County, TX. Neural tube defects are amongst the most severe birth defects and all three newborns died shortly after their birth (New York Times, 1992; Centers for Disease Control, 1997; Texas Department of State Health Services, 2010). This cluster of a rare birth defect was part of a larger problem in the Rio Grande Valley of South Texas. Between 1981 and 1986, the national average for neural tube defects was 6 per 10,000 births but was only 4.9 per 10,000 births for newborns in South Texas. The lower birth defect rate of South Texas increased from 1982-1992, however, when an estimated 42 babies in Cameron County alone were born with neural tube defects (New York times, 1992). Studies show that from 1987-1989, neural tube defect rates in South Texas increased to 14.7 per 10,000 births and further increased to 27.1 per 10,000 births from 1990-1991, a rate over 6 times higher than it had been just 10 years before (Reviewed in Centers for Disease Control, 1997).

While a clear, scientific explanation for the increased rate of birth defects in the region has yet to be established, locals hold the opinion that birth defects, along with other illnesses, are due to environmental contamination. The Rio Grande Valley is made up of four counties, Cameron, Hidalgo, Starr and Willacy, which all border Mexico directly. Across the border from

Hidalgo County, the city of Reynosa, Mexico, a highly industrious city home to many factories locally referred to as "maquiladoras", manufactures a wide variety of goods. Many local residents in both countries believe industrial run-off from Reynosa ends up in the Rio Grande, contaminates drinking water supplies, and exposes the population to dangerous chemicals.

Due in part to the 1991 neural tube defect cluster, the Environmental Protection Agency (EPA) launched a pilot investigation in 1993 which examined potential human contact with environmental pollutants in the Lower Rio Grande Valley. The study sampled indoor and outdoor air, household water, food, house dust, soil, blood and urine of nine families, all of which resided in Hidalgo and Cameron Counties, for a variety of organic and inorganic pollutants (EPA, 1994). The study found the families were not significantly exposed to most of the contaminants measured but one sample, taken from the freezer of a family residing in Brownsville, Cameron County, TX, stood out. This sample, a frozen fillet from a common carp (*Cyprinus carpio)*, contained 399 parts per million (ppm) of polychlorinated biphenyls (PCBs), an amount approximately 200 times higher than the established FDA limit (2 ppm) considered to pose a health risk for an adult (D'Itri and Kamrin, 1983; EPA 1994; ATSDR, 2000). Blood and urine samples from the family which consumed the contaminated carp confirmed two family members had been exposed to PCBs. Upon further investigation, the exposed family informed the EPA the carp had been caught from a Donna irrigation canal in Donna, Hidalgo County, TX (EPA, 1994).

In response to the results of the 1993 EPA investigation, the Texas Department of Health (TDH) and the Texas Natural Resource Conservation Commission (TNRCC) conducted a series of fish samplings between 1993-1994 in the Donna Reservoir and Canal System, also known as the Donna Irrigation System (DIS). The DIS is located southwest of Donna, Hidalgo County,

TX, and is composed of a 400-acre reservoir, which is divided into eastern and western sections, an 11.3 kilometer-long main irrigation canal and 168 miles of lateral canals and pipelines (EPA, 2012a). A pumping station, located on the Rio Grande, pumps water into the main irrigation canal at an average rate of 3.4 cubic meters per second. The main irrigation canal then flows northward via gravity flow into an inlet siphon, travels underneath the Arroyo Colorado stream, and resurfaces into an outlet siphon near FM1423 (Valley View Road). The main irrigation canal ultimately empties into the western-most reservoir of the DIS and water is carried into the eastern reservoir via two conduits located under FM1423 (ATSDR, 2010). Water from the reservoir is used to irrigate surrounding agricultural fields and provides drinking water for the cities of Alamo and Donna, TX.

The results of the 1993-1994 fish samplings showed elevated levels of PCBs in the fish caught from the DIS. The TDH deemed the presence of PCBs in the fish a threat to human health and issued Aquatic Life Order-9 on February 4, 1994. The order bans possession of fish taken from the reservoir, or any location of the main irrigation canal, and imposes a fine of up to $500 for possession of these fish (ATSDR, 2010). Despite the risk of a fine and multiple signs on the reservoir and canal banks warning that fish from the DIS are contaminated with PCBs, locals continue using the DIS as a popular fishing destination. The signs are often vandalized, used as shooting targets by local hunters and/or have graffiti on them. Many locals ignore the warning signs, believing they were placed to scare away local fishermen so only city workers could fish from the reservoir (ATSDR, 2010). During visitations to the site, multiple people were observed fishing from the DIS reservoirs and main irrigation canal. Signs of fish, which had been caught and fillet on the spot, then discarded on the ground, were also present at multiple locations around the site.

Between 1994 and 1997, the TNRCC and TDH expanded their studies beyond fish and tried localizing the source of the contamination. They gathered more than 75 samples of water and bed sediment from several locations of the DIS but only detected PCBs in a drainage ditch close to the canal (Mahler *et al*., 2002). In July 1997, the Texas commission on Environmental Quality (TCEQ) tested 10 additional fish samples and confirmed fish from the DIS still contained high levels of PCBs. An additional 13 samples, taken from the Rio Grande just below and above the pumping station, did not contain PCBs, indicating the source of contamination was somewhere in the main irrigation canal and not the Rio Grande (ATSDR, 2010). This also suggested PCB contamination could not be attributed to the maquiladoras of Reynosa, Mexico.

Due to difficulty in localizing the source of the PCB contamination, the TDH and TNRCC asked the United States Geological Survey (USGS) for assistance. Between February 1999 and July 2000, the USGS obtained suspended-sediment samples from twenty-one (21) locations in the main irrigation canal in an attempt to localize the source of contamination. Their findings narrowed the source of contamination to a 35 meter long area north of the outlet siphon, on the eastern bank of the main irrigation canal. PCBs were also detected in sediments taken from the 90° bend of the canal. These findings suggested the source of the contamination must be located between the outlet siphon and the 90° bend of the canal (Mahler *et al*., 2002; ATSDR, 2010).

In April 2001, the TCEQ conducted further sampling on soil, surface water, bottom sediments and suspended sediments in the DIS. Their results confirmed the presence of PCBs in the suspended sediment samples but failed to find PCBs in bottom sediments (ATSDR, 2010). Further studies were carried out on 30 fish samples between December 2005 and January 2006 by the DSHS and all samples were tested for inorganic and organic compounds, pesticides and

PCBs. Results concluded inorganic compound, organic compound and pesticide levels did not pose a public health hazard but PCB concentrations in fish from the DIS continued to be a risk for public health (ATSDR, 2010).

The preponderance of evidence for PCB contamination of the Donna Irrigation System led to the site being included in the National Priorities List (NPL) on March 19, 2008. The NPL is associated with the Superfund program, a federal program created to clean up uncontrolled waste sites, and intended to guide the EPA in their investigations into these sites (Federal Register, 2008; EPA, 2012b). Inclusion of the Donna Irrigation System to the NPL allows access to federal funds and personnel to assist in further investigation for the source of the PCBs contaminating the DIS. The most recent action on the DIS by the federal government was in August 2008-2009. During this time, an estimated 38,940 fish were removed from different portions of the DIS using electro-shocking and gill nets (EPA, 2012b).

The EPA held community meetings to discuss the fish depopulation in 2009 and met with Donna and Alamo city officials in 2011 and 2012 to discuss the status of the investigation of the site (EPA, 2012b). The EPA will consider dredging of contaminated sediments as a possible remediation strategy but alternative strategies, such as bioremediation using microorganisms, will also be considered. A final remediation plan has not been issued for the DIS, however, and development of a final strategy will not be considered until all data has been gathered and analyzed (Rafael Casanova, EPA, written communication, January 9, 2014). With field work scheduled to conclude by late May 2014, 21 years after the DIS was first identified to be contaminated with PCBs, it could be years before remediation of the DIS begins.

Statement of Purpose

Although EPA policy states there is no presumptive remedy for contaminated sediments, the historical method of remediation for PCB contaminated sites has been the dredging of contaminated sediments or a combination of dredging and other remediation strategies, such as capping of sediments or monitored natural recovery. The preference for dredging is due to the perception of permanence, since the contaminated sediment is permanently removed from the site, and well-established technology for dredging (NRC, 2007). Dredging is not an end-all solution, however, as it is the most complex and expensive remediation technique available. Dredging also has the drawback of potentially resuspending buried contaminants, leading to increased contaminant concentration in water and tissues of biota in the short-term. These drawbacks, along with findings that dredging alone achieved desired contaminant cleanup levels in only a few dredging projects, have caused the decision to dredge contaminated sites to become very controversial (NRC, 2007).

The National Research Council (2007) has suggested that dredging be considered along with other options. A potential remediation option that has gained popularity in the literature in recent decades is bioremediation using microorganisms to degrade contaminants into less toxic and/or less bioactive compounds. Bioremediation is most effective when a wide variety of organisms are available to degrade compounds and is of particular importance for PCBs, which are actually a group of 209 different congeners. Microorganisms are typically able to degrade only a few congeners and the congeners that are degraded vary between species. For this reason, this study aimed to isolate potential PCB-degrading bacteria from sediment samples taken from the PCB contaminated reservoir in Donna, TX. By isolating, characterizing and later identifying PCB degrading bacteria, it is expected that this work will increase the body of knowledge of

microorganisms able to degrade PCBs. Furthermore, since these microorganisms are a natural part of the reservoir, and able to be grown in laboratory conditions, it is hoped the EPA will place greater consideration into bioremediation as part of the final remediation strategy for the Donna Irrigation System.

It was hypothesized the sediments of the Donna Irrigation System would contain a variety of bacterial species which have evolved the ability to degrade PCBs. In order to test this hypothesis, a variety of microbiological and molecular techniques were employed. The amount of bacteria in the DIS able to grow on biphenyl as a sole carbon source was enumerated using media-based methodology. These bacteria were then screened for their potential to degrade PCBs by exposing them to a PCB analog, dibenzofuran, which is also cleaved by *bphC*, the third gene in the biphenyl/PCB degradation metabolic pathway. Bacterial isolates able to cleave dibenzofuran were subjected to a battery of characterization tests. Simple characterization tests included negative staining and scanning electron microscopy to analyze bacterial morphology and Gram staining to determine the cell wall structure of isolates. More complex characterization tests included API® 20E Strips to test the ability of isolates to perform various enzymatic and carbohydrate fermentation reactions and BIOLOG EcoPlates™ to examine carbon source utilization differences between isolates. Polymerase chain reaction (PCR) was utilized to determine if bacterial isolates contained the *bphA1* gene, the first gene of the biphenyl/PCB degradation pathway. Future work will identify the genus and species of isolates by sequencing their 16S rDNA gene and submitting the sequence to the NCBI *Blastn* database find the most similar 16S rDNA sequence in public databases.

CHAPTER II

REVIEW OF LITERATURE

Description of chemicals contaminating the Donna Irrigation System

Polychlorinated biphenyls (PCBs)

PCBs are a group of related, nonpolar, chlorinated hydrocarbons with a chemical formula of $C_{12}H_{10-n}Cl_n$, were n = 1-10 chlorine atoms (Borja *et al.*, 2005; Erickson, 1997). The chlorine atoms are attached to the biphenyl nucleus via catalysis with $FeCl_3$, producing a mixture of biphenyls with varying amounts of chlorine atoms (Grabowska, 2010). In total, 209 different combinations of chlorination, called congeners, exist. The least substituted PCB congener is monochlorobiphenyl and substitution increases by one chlorine until it reaches the most substituted congener, decachlorobiphenyl (Field and Sierra-Alvarez, 2008; Grabowska, 2010).

Aroclor

The contamination in the Donna Irrigation System is, in actuality, a mixture of PCB congeners which was marketed by the Monsanto Chemical Company (St. Louis, MO) under the trademark Aroclor 1254 (EPA, 2007). The first two numbers of the Aroclor represent the parent compound, biphenyl, and the last two numbers refer to the weight percentage of the chlorine in the mixture. The degree of chlorination in the Aroclor is controlled by the contact time with the $FeCl_3$ catalyst, which allows for a variety of Aroclors to be created.

Thus, Aroclor 1254 has an average weight percentage of chlorine of 54% (Field and Sierra-Alvarez, 2008). Although all 209 congeners could be theoretically present in Aroclors, they typically contained 20-60 congeners which were mostly tri-, tetra-, penta- and hexa-chlorinated congeners. Estimates of the main congeners in Aroclor are: 1.8% tri-chlorinated, 17.1% tetra-chlorinated, 49.3% penta-chlorinated and 27.8% hexa-chlorinated biphenyls (Faroon, 2003; Pieper, 2005).

PCB manufacturing

PCBs were first synthesized and described by Schmidt and Schultz (1881) but were not commercially manufactured until the 1920s, when economical synthesis of biphenyl from benzene became available (EPA, 1979). During their 48 years of manufacture, from 1929-1977, approximately 1.4 billion lbs. (635 million kg) of PCBs were produced in the United States and 44 billion lbs. (2.0×10^9 kg) worldwide (ATSDR, 2000; Faroon, 2003).

PCB chemical properties

The huge scale of PCB production was due to their chemical properties, which made them highly desirable to a variety of industries. The consistency of PCBs varies from congener to congener. Lower chlorinated congeners, from mono- to tetra-chlorinated biphenyls, are colorless or lightly colored oils. Pentachlorinated biphenyls are heavy, dark oil and the most highly chlorinated PCBs are greasy to waxy in consistency (Borja *et al.*, 2005). Despite differences in consistency, all congeners have a low flash point, thermal stability, chemical inertness, non-flammability, high electrical resistance, and resistance to acidic and alkaline substances (Boyle *et al*., 1992; Borja *et al*., 2005).

PCB usage in industry

PCBs were mainly used as heat-transfer agents in transformers and dielectrics (insulating material) in capacitors but their properties also made them useful for a wide variety of applications (Faroon, 2003; Borja *et al.*, 2005; EPA, 2013). Everyday items that contained PCBs included oils for hydraulic systems, lubricants for pumps, electrical devices and appliances, cable insulation, ballasts in fluorescent bulbs, adhesives, tapes, plasticizers, inks, oils, carbonless paper, and caulking (EPA, 2013; ATSDR, 2000). PCBs performed incredibly well as heat-transfer agents in electronics and replaced older, combustible insulating fluids. This property reduced risk of fire to such an extent that some city codes would only allow capacitors and transformers which were filled with PCBs (Ross, 2004).

PCBs in the environment

PCBs in wildlife samples

Despite being manufactured since the 1920s, concern about PCBs in the environment did not become widespread until the 1960s, long after concern for other chlorinated compounds, like DDT, had been established. DDT had received much attention in the 1950s due to its accumulation in the environment leading to relatively clear detrimental effects on biota. For example, when DDT was sprayed on plants as a pesticide and birds ate the seeds of these plants, the bird population would decrease or disappear. PCBs do not have this readily visible effect on the environment, however. Unlike DDT, which was deliberately sprayed out into the environment, PCBs inconspicuously leak into the environment through industrial waste, accidental spills and leaky electrical components. This sort of contamination made it difficult to

attribute environmental problems to a chemical which was not even known to be there in the first place (Jensen, 1972).

The first indications of a PCB problem were discovered in 1964, when Soren Jensen from the University of Stockholm began analyzing the levels of chlorinated pesticides, especially DDT and its metabolites, on human fat and wild life samples from Sweden. As Jensen studied the samples using gas chromatography he encountered chromatographic peaks which could be attributed to DDT but also found peaks which did not match any chlorinated pesticide. At first, it was assumed these unknown peaks must be an unknown substance that is found naturally in the samples. To test this hypothesis, samples of pike from relatively uncontaminated areas were gathered. Gas chromatography showed the further the samples got from known contaminated areas the lower the levels of the unknown compound in their tissue. This decrease of the unknown chemical strongly suggested the compound was not natural in origin (Jensen, 1972).

The second hypothesis was the unknown compound was a metabolite of chlorinated pesticides. To test this, a feather from each white-tailed eagle specimen in the Swedish Museum of Natural History in Stockholm was sampled using gas chromatography. The feather samples ranged in age and were gathered from 1888-1966. Results indicated the unknown compound was present in the feathers in 1942, but chlorinated pesticides were not used until 1945. This clearly indicated the compound could not be a metabolite of chlorinated pesticides. Further investigation in 1966 eventually concluded the unknown compound had to be polychlorinated biphenyls (Jensen, 1972).

Further findings of PCBs in animal samples

Several studies which investigated PCBs in the environment were published shortly after Jensen's work. Widmark (1967) published a discussion of the presence of PCBs in pesticides and mass spectrometry analyses of unknown compounds in fish and bird samples which resembled and behaved like highly chlorinated PCB congeners. This study led to further investigations to determine the extent of PCB environmental contamination (Reviewed in Veith and Lee, 1971 and EPA, 1979). Risebrough *et al*. (1968) studied declining populations of raptorial birds and their reproductive success. Sampling of these birds revealed high levels of PCBs in their system, even when the birds were only a few months old. One population of peregrines, with especially highly levels of DDT and PCBs, hatched or fledged only one young when two to four was the norm for healthy populations. Another peregrine population in Great Britain, which preyed on birds with low concentrations of chlorinated hydrocarbons, had a healthy breeding population compared to peregrines in California, which had their breeding population reduced by 80% or more. The finding that PCBs were sufficiently high in the environment to negatively influence the reproductive success of birds was picked up by the press and increased concern about PCBs in the environment and the possibility that they might also negatively affect human health (EPA, 1979).

Potential causes of environmental releases of PCBs

The huge amount of PCB production and usage inevitably led to releases into the environment. Potential sources of these releases include: legal and illegal releases, use of PCBs in open systems and unintentional releases. From 1929-1977, the Monsanto Chemical Company had many intentional, legal releases into the environment. Most of these releases involved PCB-

contaminated waste water which released an estimated amount of <1 pound of PCB/day (0.45 kg/day). Other legal releases by Monsanto involved an unknown amount of PCB-containing waste into landfills during the same 1929-1977 period (ATSDR, 2000). Illegal releases by both the private and public sector undoubtedly occurred. Due to the nature of such releases, however, it is impossible to quantify the full extent of PCBs released in such a manner (Erickson, 1997).

Unintentional releases of PCBs are the greatest source of the chemical in the environment. As previously mentioned, PCBs were ubiquitously used in many household products, such as inks, caulking, lubricants, etc. These open systems have resulted in unintentional, widespread, low-level contamination of the environment. Unintentional release of PCBs from closed systems, such as ballasts in fluorescent lights, transformers and capacitors, have greatly contributed to the contamination problem as well. Approximately 1 billion ballasts, each containing approximately 30 grams of PCBs, were installed in the U.S. and millions of these ended up in landfills. Similarly, an unknown number of PCB-filled capacitors and transformers have ended up in landfills. Larger capacitors could be filled with 2-3 pounds (~1 kg) of Aroclors while transformers, depending on their size, could require much more Aroclor to fill. As the enclosures of these ballasts, capacitors and transformers deteriorate, they slowly release their PCBs into the environment (ATSDR, 2000; Erickson, 1997). PCB leakage is not restricted to old, discarded transformers and capacitors, however. In 1987, The EPA estimated that 3.3% of PCB filled transformers would leak every year and each could spill upwards of 66 lbs. (30 kg) of PCBs. It was also estimated that 12,000 PCB-filled capacitors in use in electrical substations would leak each year. Many of these capacitors would rupture in a violent fashion, spraying PCBs in an 11-foot radius around them (As cited in Robinson and Lenn, 1994).

Extent of PCBs in the environment

The amount of PCBs released into the environment is impossible to calculate but is so widespread in the U.S. that 500 of 1,598 Superfund sites are confirmed to be contaminated with the chemical (Reviewed in ATSDR, 2000). Despite this shortcoming, different authors and organizations have provided estimates on the amounts of PCBs released into the environment. Borja *et al*. (2005) estimate 90 million lbs. (40.9 million kg) used in North America entered the environment. Meanwhile, Leisinger *et al*. (1981) estimate that 210 million lbs. (95 million kg) produced in the United States have entered the environment. The WHO and CDC provide a worldwide estimate on the amount of PCBs that have entered the environment and estimate that approximately 440 million lbs. (200 million kg) of PCBs remain in mobile environmental reservoirs (Faroon, 2003; ATSDR, 1990).

More than 99% of PCBs released into the environment are found in soil, causing long-term immobilization and slow release of the chemical into the environment (Travis and Hester, 1991; Faroon, 2003; Ross, 2004; Borja *et al*., 2005). PCBs are still mobile through the worldwide ecosystem via a complex cycle involving redistribution from one environmental compartment to another, however. PCBs can be transferred from soil to water and air, water to sediments and air, air to water, and move around bodies of water via sediment transport (Faroon, 2003). Despite this cycling accounting for 1% or less of PCBs in the environment, the amount of PCBs transported throughout the environment can be quite large. Volatilization into the air is now considered the most important mechanism of global dispersion of PCBs (Erickson *et al*., 1997; ATSDR, 2000) and in the U.S. alone, an estimated 2 million lbs. (900,000 kg) of PCBs cycle through the atmosphere every year (Murphy *et al.,* 1985). This has led to PCB contamination of even remote regions, such as Antarctica and the Arctic, despite a lack of

anthropogenic PCB sources in these regions (Risebrough *et al*., 1968; Risebrough *et al*., 1976; Montone *et al*., 2003).

Concern of PCBs in the environment becomes widespread

Public concern about PCBs in the environment and their potential toxicity reached a boiling point in October of 1968, when a major accident involving human exposure to high doses of PCBs occurred in Kyushu, Japan. The first indication that something was wrong was an epidemic of a skin condition called chloracne, swelling of the upper eyelids, increased eye discharge and pigmentation of the skin (Kuratsune *et al*., 1972). After an intense epidemiological study, the accident, now known as the "Yusho" or oil disease incident, was attributed to consumption of PCB contaminated rice oil. The source of the contamination was determined to be a leaky heating tube which used PCBs as heat transfer agents (Kuratsune *et al*., 1972 and Aoki, 2001). This incident, along with a similar incident in Thailand, raised further concerns in an already worried public, setting the stage for legislation against PCBs.

Regulatory action against PCBs

Actions against PCBs moved rapidly following the Risebrough *et al*. (1968) study and Yusho incident. Public outcry was so great it eventually lead to regulation of PCBs by the Toxic Substance Control Act (TSCA) in 1976, which directed the EPA to promulgate regulations concerning PCBs and required PCBs to be labeled with adequate warnings. It is important to put public concern about PCBs during this period into perspective. The only other toxins that were specifically targeted by the 1976 TSCA were asbestos and lead-based paint, both of which have clear and highly detrimental effects on human health (U.S. Senate, 1976; Erickson, 1997). The

TSCA set the date of January 1, 1978 for a ban on manufacturing, processing and distribution of PCBs, unless these processes could be done in a totally enclosed manner. Lastly, a complete ban of PCB processing and distribution would take effect on January 1, 1979 unless the EPA granted a 1 year exemption (U.S. Senate, 1976).

The Monsanto Chemical Company cooperated surprisingly well with the new regulations. The company had already voluntarily limited their sales of PCBs to closed applications and recommended that existing PCB-filled heat transfer systems be drained and refilled with a replacement fluid since 1970 (EPA, 1979). In 1971, the company limited its production of Aroclor to formulations containing less than 60% chlorine content and completely stopped manufacturing PCBs in 1977, two years before the required TSCA date (Ross, 2004).

Health effects of PCBs

After the Yusho incident and TSCA regulations, research into PCBs increased exponentially. Since the 1970s, more than 10,000 publications on the subject have been published, many of which investigated the health effect of PCBs on animals and humans (Ross, 2004). Detrimental health effects from PCB exposure were known long before Yusho and the TSCA, however, and were first published in the 1930's by Schwartz (1936) and Jones and Alden (1936). Schwartz examined workers involved in the chlorination of biphenyl which had developed an acne-like condition on the skin. The fumes of the PCBs caused acne in the face and neck but also penetrated clothes and caused acne to erupt on the shoulders, belt-line and genitals. Workers also complained of digestive disturbances, burning of the eyes, impotence and hematuria. Schwartz hypothesized PCB fumes clogged the follicles of the skin when they solidified and the chlorine had an irritating effect on the clogged follicles (Schwartz, 1936).

Jones and Alden reported similar symptoms in workers which were exposed to PCBs and determined their condition was indicative of "chloracne", a term first used in 1899 to describe acne like eruptions in the arms and faces of workers who manufactured chlorine gas (Jones and Alden, 1936).

Many recent publications have studied the health effects of PCBs on animals and a wide variety of symptoms and health effects have been attributed to PCB exposure. These symptoms include: neurobehavioral deficits in monkeys, neurotoxicity in several animal species, reduced thymus weights in rats, decreased immunoglobulin levels in monkeys, alterations of menstrual cycles and decreased fertility in rhesus monkeys. Of the many animal studies, a subset has investigated the carcinogenic potential of PCBs. Early studies suggested female rats exposed to four commercial mixtures of Aroclor developed liver tumors but these findings were initially met with skepticism (reviewed in ATSDR and EPA, 1998). Further experimentation by Mayes *et al.* (1998) provided strong evidence that all PCB mixtures are able to induce cancer in rats.

Data on the health effects of PCBs on humans has been gathered largely through examining occupational and environmental exposures or has been extrapolated from animal studies. For example, the EPA believes the abundance of animal data strongly suggests PCBs are probable human carcinogens and has labeled them as such due to standing policies and safety precautions (Ross, 2004). Besides the previously mentioned symptoms of the Yusho victims, a large number of other health effects on humans have been suggested and include: decreased cognitive and motor performance (Aoki, 2001; Park *et al*., 2010), decreased menstrual cycle length, miscarriage (Reviewed in Nicolopoulou-Stamati and Pitsos, 2001), decreased birth weight (ATSDR, 2010), decreased serum IgA and IgM, and impaired thyroid function (Aoki, 2001).

Despite the almost overwhelming amount of data and apparently conclusive effects of PCBs on animal and human health, the degree of PCB toxicity and the nature of their effects on humans and other organisms remains a controversial and highly debated topic (Erickson, 1997). Ross (2004) is particularly critical of the human health effects of PCBs. He, and other reviewers, argue the only consistent and verifiable health effects of PCBs are skin and eye irritations, such as chloracne (Erickson, 1997; Ross, 2004). These criticisms are not unfounded since it is now known that Yusho victims were not only exposed to PCBs but to polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) as well. Both of these chemicals are considered to be highly toxic and today it is accepted the symptoms of Yusho were due to these two compounds (Aoki, 2001; Ross, 2004). Another important criticism from Ross (2004) is the unrealistic PCB dosage levels used in animal studies. The estimated daily dietary intake of PCBs for an adult human was 0.016 µg/kg/day (0.016 ppb) in 1977 and 0.0005 µg/kg/day (0.0005 ppb) from 1982-84 (ATSDR, 2000). Animal studies, however, have fed adult rats upwards of 64 mg/kg/day of PCB congeners, an amount 4,000,000 times higher than normal adult humans would be exposed to in their diets (Schantz *et al*., 1995). Other studies have exposed animals to lower amounts of PCBs but these quantities are still much higher than what the average human would be exposed to. For instance, Tryphonas *et al*. (1989) exposed rhesus monkeys to a minimum of 5 µg/kg/day of Aroclor 1254, an amount that is approximately 300 times higher than the average human dietary exposure.

Despite controversy of whether or not PCBs have harmful effects on humans, they are treated as highly dangerous chemicals and are given high priority for cleanup. The chemicals consistently rank within the top 10 in the ATSDR Substance Priority List and are currently #5 on

the list. It should be noted that ranking is not determined based on the toxicity of the chemical alone but a combination of frequency, toxicity and potential for human exposure.

Removal of PCBs from the environment

Natural degradation and half-lives of PCBs in environmental compartments

Given the perceived danger of PCBs, much research into the chemicals has focused on the half-lives of the chemicals and on their removal from the environment. The half-life of PCBs in the environment has been particularly difficult to measure for two reasons: 1) half-life is different for each congener and 2) Half-life of PCBs changes depending on the media they are located in. In general, PCB half-life increases with chlorination and several studies have examined the length of time they remain in different environments.

When present in the atmosphere, PCBs are subject to photolysis and chemical reactions with hydroxyl (OH) and nitrate (NO_3) radicals and with ozone (O_3). Of these three chemical reactions, the hydroxyl radical reaction is most important for PCBs (Atkinson, 1987). Atmospheric half-lives have been calculated to range from 3 days for biphenyl to over 83 days for pentachlorobiphenyl (Atkinson, 1987; Faroon, 2003). For more highly chlorinated congeners, such as heptachlorobiphenyl, half-lives of 1.31 years have been reported (Sinkkonen and Paasivirta, 2000; EPA, 2013).

In water, photolysis appears to be the only chemical degradation process and mono- to tetrachlorobiphenyls have half-lives between 17 to 210 days, respectively. These lengths are based on summer daylight and shallow water with a depth of less than 0.5 meters. Thus, PCBs are expected to have longer half-lives during winter and when located in greater water depths. More highly chlorinated biphenyls do not significantly absorb sunlight and are expected to have

longer half-lives than less chlorinated congeners (Faroon, 2003). Sikkonen and Paasivirta (2000) estimate heptachlorobiphenyl could have a half-life upwards of 27.39 years in water.

Measuring half-life of PCBs in soils and sediments has been the most problematic since no natural abiotic process is known to significantly degrade PCBs in this medium (Faroon, 2003). Despite this difficulty, several studies have given estimates and one derived a half-life of 940 days for Aroclor 1254 (Hsieh *et al*.,1994) while another study estimated the half-life of PCBs in river sediments to be 9.5 (±2.2) years (ATSDR, 2000). A third study by Sinkkonen and Paasivirta (2000) estimated the sediment and soil half-life to be 2.97 years for trichlorobiphenyl and 38 years for heptachlorobiphenyl.

As old PCB-filled transformers, capacitors and other containers breakdown in dumps, a steady amount of PCBs leaks into the environment every year. Given the long half-life of these chemicals, it is clear PCBs will continue being an environmental problem well into the future.

Artificial degradation of PCBs

Many artificial processes have been utilized to either degrade PCBs or remove them from the environment. Of these, dredging has been particularly popular but runs the risk of resuspending and redistributing PCB-contaminated sediments. The EPA required dredged sediments with concentrations >50 ppm of PCB to be destroyed in an approved incinerator with 99.9% efficiency. Despite this high efficiency, incineration has slowly fallen out of favor due to concerns about highly toxic dioxins and dibenzofurans being formed during the incineration process (ATSDR, 2000). Other degradation technologies include wet air oxidation, electrochemical catalytic dechlorination, polyethylene glycol/potassium hydroxide chemical destruction, irradiation using gamma rays, and treatment of soils and sediments with titanium

dioxide-catalyzed photodecomposition with sunlight. These treatments vary in their efficiency and have not been widely adopted (ATSDR, 2000).

Biodegradation of PCBs

The last potential method of destroying PCBs in the environment involves biodegradation using microorganisms, which is the only known process to degrade PCBs in soil systems or aquatic environments (Borja *et al*., 2005). Complete biodegradation of PCBs in the environment requires three different processes. The first is reductive dechlorination by anaerobic microorganisms, which reduces the number of chlorines on the PCB molecule. The second reaction, oxidative degradation by aerobic bacteria, transforms PCBs into chlorobenzoic acid and reduces their toxicity. The last reaction involves mineralization of chlorobenzoic acid to CO_2, H_2O and chlorine (Abramowicz, 1995; Davison and Veal, 1997). Most bacterial species can only catalyze one of these reactions; therefore, complete biodegradation PCBs requires the use of mixed bacterial cultures (Komancova *et* al., 2003; Faroon, 2003). Due to the need for cultures containing a variety of PCB degrading bacteria, isolation of novel PCB degrading bacteria has been an active area of research and interest since the 1970s (Asturias *et al*., 1995).

As PCBs congeners increase in chlorination, their biodegradability decreases and their toxicity increases (Furukawa and Fujihara, 2008). Thus, efficient biodegradation requires PCBs to have the least number of chlorines as possible attached to the biphenyl nucleus. Anaerobic dechlorination of PCBs was first observed in sediments from the Hudson River by Brown *et al*. (1988) who reported increased number of lower chlorinated congeners and loss of non-*ortho* substituted congeners relative to the Aroclor known to contaminate the river. Research suggests the specificity of dechlorination varies between microorganisms, making isolation of multiple

species important for bioremediation via reductive dechlorination (Abraham *et al*., 2002; Furukawa and Fujihara, 2008). Identification of dechlorinating microorganisms has had limited success and no pure cultures of PCB dechlorinating bacteria have been obtained (Abraham *et al.*, 2002). Despite this shortcoming, 16s rDNA analysis has revealed sequences related to *Dehalococcoides* in microbial communities associated with reductive dechlorination of PCBs (Furukawa and Fujihara, 2008).

After anaerobic reductive dechlorination has lowered the number of chlorines in PCBs, aerobic oxidation of PCBs can occur. Bacteria which oxidize PCBs into chlorobenzoic acid were first isolated and characterized as Gram-negative rods by Lunt and Evans (1970) and Catelani *et al.* (1970). Since then, a variety of Gram-negative and Gram-positive PCB oxidizing bacteria have been isolated and identified. Gram-negative PCB oxidizing genera include *Pseudomonas*, *Achromobacter, Alcaligenes, Acinetobacter, Sphingomonas, Comamonas,*and *Vibrio* while Gram-positive PCB oxidizing genera include *Rhodococcus, Bacillus, Corynebacterium* and *Nocardia* (Reviewed in Abraham *et al.*, 2002; Borja *et al.*, 2005; Pieper, 2005; and Furukawa and Fujihara, 2008). Although a large number of known aerobic PCB degraders exist, isolation of novel PCB degrading genera, species and strains continues to be an active area of research due to each bacterium displaying activity towards specific PCB congeners (Abraham *et al*., 2002).

Oxidative degradation of PCBs involves the "upper" biphenyl metabolic pathway which consists of several genes termed *bph* genes (Pieper, 2005). The first gene, *bphA,* consists of four subunits, *A1,A2,A3,A4,* and encodes for the enzyme biphenyl 2,3-dioxygenase which catalyzes the formation of a chlorinated biphenyl-2,3-dihydrodiol. The second gene, *bphB*, encodes the enzyme biphenyl-2,3-dihydrodiol-2,3-dehydrogenase which yields chlorinated 2,3-dihydroxybiphenyl. The third gene, *bphC*, encodes the enzyme 2,3-dihydroxybiphenyl-1,2-

dioxygenase which yields a yellow *meta*-cleavage product called chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). Lastly, *bphD* encodes a hydrolase which converts HOPDA to chlorinated 2-hydroxy-penta-2,4-dienoate and chlorobenzoic acid (Fedi *et al*., 2001). Of these genes, the *bphA1* and *bphC* genes are of most importance to this study. The *bphA1* gene encodes for the large subunit of the enzyme and is responsible for substrate specificity. Major amino acid differences are found in this subunit in different species of PCB-degrading bacteria, accounting for the preference to certain PCB congeners different bacteria display (Brühlmann and Chen, 1998; Furukawa and Fujihara, 2008). Given the importance of this gene, previous studies have used the presence of the *bphA*1 gene in bacterial isolates as a measure of their genetic potential for PCB biodegradation (Dercova *et al*., 2008b). The *BphC* yields HOPDA, a yellow *meta*-product which has been useful in visually identifying potential PCB degraders. Bacteria possessing this gene are able to turn their growth media yellow, a property which has been used in several studies as a quick screening method for potential PCB degradation potential (Becher *et al*., 2000; Stope *et al*., 2002; Wesche *et al*., 2005; and Dercova *et al.*, 2008a).

Mineralization of chlorobenzoic acid to CO_2, H_2O and chlorine requires the "lower" biphenyl pathway and involves a separate set of four genes, *bphX0, bphX1, bphH* and *bphX3.* Although these are also *bph* genes, very few bacteria that are able to aerobically oxidize PCBs are able to mineralize them as well (Abraham *et al*., 2002). The limited ability of any bacterial species to degrade multiple PCB congeners necessitates the need for mixed bacterial cultures in order to fully biodegrade the chemicals and isolation of novel strains of PCB degraders continues to be an active area of research.

CHAPTER III

MATERIALS AND METHODS

Sediment samples

Sampling methodology

Samples of the upper sediment layer (0-10 cm) were collected from the western reservoir, eastern reservoir, main irrigation canal and outlet siphon of the main irrigation canal by plunging a copper tube into the sediment. Collected samples were pushed into sterile, 50 mL conical centrifuge tubes. After every sample, the copper collection tube was washed with deionized water to remove any sediment stuck to the inside. To minimize cross contamination between samples, the tube was sterilized by pouring isopropyl alcohol down the tube and incinerating the alcohol inside it. All sediment samples were stored in their conical tubes at 4°C until further use.

Western reservoir

Four locations in the westernmost reservoir were sampled. Sample sites included: the southeast shore (26° 8'37.81"N, 98° 4'52.49"W), the south-central shore (26° 8'38.56"N, 98° 4'57.57"W), the southwestern shore (26° 8'39.48"N, 98° 5'3.23"W) and a small canal running alongside the western reservoir (26° 8'38.70"N, 98° 5'4.06"W).

Eastern reservoir

Four locations on the easternmost reservoir were also chosen for sampling. Sample sites included: The southeast shore (26° 8'59.53"N, 98° 4'45.71"W), south-central shore (26° 8'53.67"N, 98° 4'27.08"W), southwestern shore (26° 8'51.44"N, 98° 4'12.44"W) and a small canal running alongside the eastern reservoir (26° 8'58.54"N, 98° 4'45.75"W).

Main Irrigation canal

The main irrigation canal, which feeds the reservoirs, was sampled at three locations: a bridge that traverses it (26° 6'44.94"N, 98° 5'10.94"W), the canal shore southeast of the bridge (26° 6'44.61"N, 98° 5'10.69"W), and the canal shore northeast of the bridge (26° 6'45.61"N, 98° 5'10.88"W).

Outlet siphon

The outlet siphon of the main irrigation canal was sampled at three separate locations: the outlet siphon exit (26° 5'45.27"N, 98° 4'22.61"W), the eastern shore north of the siphon (26° 5'46.07"N, 98° 4'22.79"W), and the western shore north of the siphon (26° 5'45.82"N, 98° 4'21.99"W).

Bacteriological culture media

Liquid and solid minimal media (MM)

Liquid minimal media (MM) for bacterial growth contained 1g $(NH_4)SO_4$, 2.7g KH_2PO_4, and 10.95g $Na_2HPO_4 \cdot 12\ H_2O$ placed into a 2 liter flask, filled to 1 liter with deionized H_2O, and sterilized at 121°C for 15 minutes in an autoclave. 500 µL each of filter sterilized solutions

containing $FeSO_4 \cdot 7\ H_2O$ (2g L^{-1}), $Ca(NO_3)_2 \cdot 4\ H_20$ (6 g L^{-1}), and $MgSO_4 \cdot 7H_2O$ (40 g L^{-1}) were added per 100 mL of the prepared liquid minimal media according to Dercova *et al*. (2008a).

Solid minimal media was prepared by adding 5.37g $Na_2HPO_4 \cdot 12\ H_2O$, 1.30g KH_2PO_4, 0.50g NH_4Cl, 0.20g $MgSO_4 \cdot 7\ H_2O$, and 15g of bacteriological agar (Sigma-Aldrich, St. Louis, MO) to 1L of deionized water. Media was sterilized at 121°C for 15 minutes and poured into Petri dishes.

Solid Lysogeny broth (LB) media

Solid LB media was prepared by adding 20.6g of dehydrated LB medium (Sigma-Aldrich, St. Louis, MO) and 15g of bacteriological agar to 1L of deionized water. Media was sterilized at 121°C for 15 minutes and poured into Petri dishes.

Physiological saline

Physiological saline (0.85% w/v) was prepared by adding 8.5g NaCl to 1L deionized water. After NaCl was fully dissolved, 9 mL aliquots of saline were transferred to test tubes and sterilized at 121°C for 15 minutes.

Enumeration of bacteria in sediment samples

Total facultative bacteria in sediment samples

To estimate the total number of facultative bacteria living in the sediment, 1g sediment was aseptically removed from its conical tube and placed into a test tube containing 9 mL sterile saline to achieve a concentration of 10^{-1} of the original sample. This solution was diluted in a ten-fold fashion in saline until a concentration of 10^{-5} of the original was reached. 0.1 mL aliquots of

the 10^{-4} and 10^{-5} dilutions were spread on the surface of a solid LB medium plate in triplicate using a sterile glass cell spreader and incubated at 25°C for 4 days. Colony forming units (CFUs) that grew were visually counted and total facultative bacteria per gram of soil were enumerated using the formula: **(CFUs) / (dilution factor plated) (0.1 ml) = CFUs / gram of sediment.**

Total number of bacteria in sediments able to use biphenyl as sole carbon source

Biphenyl is the non-chlorinated parent compound of PCBs and the two chemicals are degraded using the same *bph* gene encoded enzymes. The total number of bacteria in sediment able to utilize biphenyl as the sole carbon source was estimated by aseptically removing 1g of sediment from its conical tube and placing the sample into a test tube containing 9 mL sterile saline to create a concentration of 10^{-1} of the original sample. This solution was diluted in a ten-fold fashion in saline until a concentration of 10^{-3} of the original was reached. 0.1mL of 10^{-2} and 10^{-3} dilution were spread on MM dishes in triplicate using a sterile glass cell spreader. Inoculated dishes were placed lid down, 250 mg biphenyl crystals (Sigma-Aldrich, St. Louis, MO) sprinkled inside the lid, and incubated at 25°C for 7-10 days. CFUs that grew were visually counted and total number of bacteria able to use biphenyl as a growth substrate was enumerated using the same formula used to enumerate total facultative bacteria.

Statistical analysis

The total number of facultative bacteria in each sample site was analyzed for statistical significance to other sample sites using t-tests for independent samples with the VassarStats Website for Statistical Computation (http://vassarstats.net/index.html). The same website was used to analyze the total number of biphenyl utilizing bacteria at sites for statistical significance.

Enrichment of biphenyl utilizing bacteria from sediment

Enrichment and isolation of biphenyl utilizing bacteria

The levels of some species of biphenyl utilizing bacteria in sediment samples are potentially too low to be isolated from natural sediments effectively. In order to maximize the diversity of biphenyl utilizing bacteria cultured from sediment samples on Petri dishes, biphenyl utilizing bacteria in sediment were first enriched in number by supplying sediment samples with biphenyl for an extended period of time. 10 g of sediment, 250 mg biphenyl crystals, and 100 mL of liquid minimal media were added to a 250 mL Erlenmeyer flask and placed into a rotary shaker set to 150 revolutions per minute (rpm) for 7 days at 25°C.

Enriched cultures were serially diluted in a ten-fold fashion to a concentration of 10^{-6} of the original using sterile saline. 0.1 mL aliquots of 10^{-5} and 10^{-6} dilutions were spread on MM dishes in triplicate. Inoculated dishes were placed lid down, supplemented with 250 mg biphenyl crystals inside the lid, and incubated for 7 days at 25°C. Well isolated bacterial colonies which showed different colony morphologies were picked up using a sterile inoculating loop and subcultured unto LB media for use in further tests.

Screening isolated bacteria for potential to degrade PCBs

Ability of isolates to cleave dibenzofuran

Dibenzofuran is a chemical structurally similar to biphenyl and polychlorinated biphenyls and is cleaved at the *meta*- position by the enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase, encoded by the *bphC* gene in the biphenyl degradation pathway. The resulting *meta*-cleavage product has yellow coloration and this coloration was used to visually identify bacteria which are potential PCB degraders.

Pure cultures of bacterial isolates from the enriched sediment samples were transferred to fresh MM dishes. 250 mg biphenyl crystals and 100 mg dibenzofuran crystals (Sigma-Aldrich, St. Louis, MO) were sprinkled unto the lid of the inverted Petri dish and incubated at 25°C for 3-7 days. Bacteria capable of cleaving dibenzofuran, causing a yellowing of the medium, were classified as potential PCB degraders. To ensure culture purity, dibenzofuran cleaving isolates were subcultured unto LB Petri dishes using streak plating technique. Once culture purity was ensured, the isolates were recultured unto MM media with biphenyl and dibenzofuran crystals added to reconfirm their dibenzofuran cleaving ability.

Characterization of potential PCB degrading bacteria

Morphology of isolates

Dibenzofuran cleaving isolates were subcultured onto LB Petri dishes and incubated for 24-36 hours at 25°C. Well isolated colonies were picked up with a sterile inoculating loop and mixed into a drop of nigrosin dye placed at the edge of a clean microscope slide. Another clean microscope slide was used to spread the drop of nigrosin across the first slide. The nigrosin was allowed to air dry and the resulting slide was observed under an oil immersion lens at 1,000x magnification to observe bacterial morphology.

Gram stain reaction

Bacteria were differentiated into Gram-positive and Gram-negative bacteria based on their reaction to the Gram-stain procedure. Gram-positive bacteria contain a cell wall which retains the crystal violet dye after attempted decolorization while the cell wall of Gram-negative

bacteria readily lose the crystal violet dye when exposed to the same decolorizer and stains red with the subsequent addition of safranin dye.

Isolates were grown on LB media for 24-36h at 25°C, picked up with a sterile inoculating loop, and mixed into a drop of deionized water placed on a microscope slide. The suspension was allowed to air dry and was heat fixed by quickly passing the slide through the flame of a Bunsen burner three times. Crystal violet dye was added to the smear of bacteria for 30 seconds and then rinsed off with deionized water. Next, Gram's Iodine was added for 10 seconds and rinsed off with deionized water. Samples were then decolorized with 95% ethanol until crystal violet dye was no longer removed from the smear and immediately rinsed with deionized water. Lastly, the bacterial smear was counter-stained with safranin for 30 seconds and rinsed with deionized water. Stained specimens were observed using a light microscope with an oil immersion lens at 1,000x magnification. Bacteria which retained crystal violet, staining them purple, were classified as Gram-positive. Bacteria which lost the crystal violet and were stained with safranin instead were classified as Gram-negative.

Presence of catalase

Catalase is a ubiquitous enzyme found in almost all organisms exposed to oxygen with the exception of a few genera of microorganisms, such as the bacterium *Streptococcus*. This enzyme catalyzes the decomposition of hydrogen peroxide (H_2O_2) to oxygen and water, protecting the organism from this reactive chemical. The presence of catalase was detected by placing a drop of aqueous 3% hydrogen peroxide solution on a clean microscope slide and aseptically adding a small amount of bacterial isolate to the drop. Isolates that had visible bubbling, indicating the release of oxygen, were recorded as catalase positive.

Presence of cytochrome c oxidase

Bacteria contain an electron transport chain much like the mitochondria of eukaryotes. Some bacteria lack cytochrome c oxidase, which reduces oxygen to form water, however, and the presence or absence of this enzyme can be used to distinguish between bacterial species. Bacteria that contain cytochrome c oxidase can oxidize certain indicator reagents, changing them into a purple compound. The indicator reagents used were a solution of 1% α-naphtol in 95% ethanol mixed with an equal volume of aqueous 1% *p*-aminodimethylaniline oxalate. Sterile cotton swabs were dipped into this mixture and the moistened swabs were used to pick up 24-36h old colonies of bacterial isolates. Isolates which turned purple on the swab were recorded as oxidase positive.

API® 20E Strips

API® 20E Strips (bioMérieux, Inc., Durham, NC) are designed for identification of bacteria in the family Enterobacteriacae and other non-fastidious, Gram-negative bacteria. The strips contain 20 separate compartments which test for the presence of a variety of enzymes and ability to ferment various carbohydrates. The enzymes tests are: β-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), citrate utilization (CIT), production of hydrogen sulfide (H_2S), urea hydrolysis (URE), tryptophan deaminase (TDA), tryptophanase (IND), acetoin production (VP), and gelatinase (GEL). The carbohydrate compartments test for the ability of the organism to ferment: glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY), and arabinose (ARA). Additional tests for nitrate reduction (NO_2) and nitrite reduction (N_2) were done, as well.

Isolates were subcultured unto LB Petri dishes and incubated at 37°C for 24 hours. Bacteria were picked up from the surface of the Petri dish using a sterile cotton swab and transferred to 10 mL of sterile, 0.85% (w/v) saline. The bacterial suspension was adjusted until it reached an absorbance value at 600 nm (A_{600}) of 0.5 ± 0.01. This suspension was distributed into each cupule of the strips according to the manufacturer's instructions. Inoculated API® strips were incubated at room temperature for 48 hours. At this time, extra reagents were added as described by the manufacturer and tests were read according to manufacturer instruction.

API® 20E Strip phenotypic tree

Results from the API® strips were converted into a binary system; a positive result for a test in the strip was set to a value of equal to 1 and a negative result was set to a value of equal to 0. The binary results of the API® strips for each isolate were compiled into a spreadsheet using the program NTedit v2.0. This program created a matrix which compared the binary results of each bacterium with the others. More specifically, the program compared the number of total positive results, and which positive results, each isolate contained to the positive results of other isolates. The results of these comparisons were made into a phenotypic tree based on the degree of phenotypic similarity the organisms displayed.

BIOLOG EcoPlates™

BIOLOG EcoPlates™ (BIOLOG, Hayward, CA) were used to test all dibenzofuran cleaving isolates for their carbon utilization profiles. EcoPlates™ are a 96 well microtiter plate containing 31 of the most useful carbon sources for soil community analysis, along with a water control, in separate wells. The carbon sources in the wells are: β-Methyl-D-Glucoside, D-

Galactonic Acid γ-Lactone, L-Arginine, Pyruvic Acid Methyl Ester, D-Xylose, D-Galacturonic Acid, L-Asparagine, Tween 40, i-Erythritol, 2-Hydroxy Benzoic Acid, L-Phenylalanine, Tween 80, D-Mannitol, 4-Hydroxy Benzoic Acid, L-Serine, α-Cyclodextrin, N-Acetyl-D-Glucosamine, γ-Hydroxybutyric Acid, L-Threonine, Glycogen, D-Glucosaminic Acid, Itaconic Acid, Glycyl-L-Glutamic Acid, D-Cellobiose, Glucose-1-Phosphate, α-Ketobutyric Acid, Phenylethylamine, α-D-Lactose, D,L-α-Glycerol Phosphate, D-Malic Acid, and Putrescine. These carbon sources are in triplicate in the microtiter plate and each EcoPlate™ can be used to test three separate organisms or one organism three times. Each well also contains a tetrazolium dye which changes color from clear to purple if the bacterial isolate is able to metabolize the particular carbon source found in the well. The intensity of the color change is proportional to how well the bacterial isolate can metabolize the particular carbon source.

Three different bacterial isolates were tested on each 96-well EcoPlate™ using the same bacterial suspensions made for the API® Strips. Each set of 32 wells containing the carbon sources and control were inoculated by filling them with 100 µL aliquots of bacterial suspension. Inoculated EcoPlates™ were incubated for 24h at 25°C and placed into a microplate reader set to a wavelength of 595 nm to quantify the intensity of the color change in each well. Readings were taken again following 48h and 72h of incubation time. Absorbance values were placed into a spreadsheet and corrected for background interference.

The absorbance values from the inoculated EcoPlates™ were gathered and analyzed in three separate ways. First, the total number of different carbon sources able to be used by the bacterial isolate was determined by the number of wells with positive absorbance readings after they were corrected for background interference. Secondarily, the substrate that was best used by

the bacterial isolate was noted. Lastly, the Total Activity of each bacterial isolate was determined by summing all the positive absorbance readings from the microplates.

EcoPlate™ phenotypic tree

The results from the EcoPlate™ system were changed to a binary system, compiled into a spreadsheet using the NTedit V2.0 program, and made into a matrix for comparison in the same way used for the API® strips. A phenotypic tree was created from the matrix based on the degree of similarity between the different isolates.

Scanning electron microscopy

Two representatives from the Gram-negative isolates and one representative from the Gram-positive isolates were selected for examination with scanning electron microscopy. Selected isolates were subcultured unto a LB plates and incubated at 37°C for 16-24 hours. Small samples of bacteria were picked up with a sterile inoculating loop and suspended in 500 µL of phosphate buffered saline (PBS). Bacterial suspensions were vacuum filtered through Whatman™ 13mm, 0.2 µm Nucleopore™ polycarbonate filters (Fisher Scientific, Waltham, MA) mounted on a Millipore™ Swinny Stainless steel, 13 mm filter holder (Millipore, Billerica, MA). Vacuum suction was applied until water was no longer visible on the filter surface.

The filters containing bacterial cells on their surface were transferred to glass vials containing 4 mL of 2.5% glutaraldehyde + 2% formaldehyde in sodium phosphate buffer (0.1 M, pH 7.0) and allowed to float on the surface of the fixative for one hour. The filters were then submerged in the fixative for 30 minutes. The filters were washed in sodium phosphate buffer (0.1 M, pH 7.0) for 5 minutes and this was repeated two more times in the same buffer. After

washing, the bacteria on the filters were serially dehydrated in 25, 50, 75, 95, 100, 100 and 100% ethanol for 5 minutes in each step. Dehydrated bacteria were then dried at the critical point using 100% ethanol and liquid CO_2. Dried bacteria were mounted unto aluminum stubs using non-conductive glue and were transferred to a Denton Vacuum Desk II sputter coater (Denton Vacuum, LLC., Mooreston, NJ). Bacteria were etched in argon gas at 150 millitorr of pressure and 2 mA of current for 3 seconds and sputter coated in argon gas at 50 millitorr of pressure and 45 mA with gold-palladium for 35 seconds. Coated samples were examined using a Zeiss Evo LS10 scanning electron microscope (Carl Zeiss LLC, Thornwood NY) using an accelerating voltage of 10 kV and a spot size of 300. Images were digitally saved using the SMARTSEM program and processed using Adobe Photoshop.

Amplification of *bphA1* from genomic DNA of dibenzofuran cleaving isolates

Primer design

The NCBI online protein database (http://www.ncbi.nlm.nih.gov/protein) was queried for organisms containing the *bphA1* protein. In total, 18 protein sequences were obtained from different Gram positive and Gram negative genera. For each protein, primary protein sequence, GenBank protein accession number and DNA coding sequence (CDs) was recorded. The 18 protein sequences were aligned using Clustal W set to default settings and examined for conserved regions using BoxShade through the San Diego Supercomputer Biology Workbench (http://workbench.sdsc.edu). BoxShade colors protein sequences using three different colors to differentiate between perfectly conserved regions (green), moderately conserved regions (yellow) and slightly conserved regions (blue). Highly conserved sequences of 5-8 amino acids (15-24 nucleotides) were noted and further analyzed for their possible use as primers.

Degenerate forward primers were constructed from highly conserved protein sequences using the Thatcher Development Software, LLC. Bioinformatics Reverse Translation Tool (http://www.gregthatcher.com/Bioinformatics/ReverseTranslate.aspx). Degenerate reverse primers were constructed from highly conserved protein sequences using the Harvard Reverse Complement tool (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html).

Forward and reverse primers were individually examined for their probability to form self-dimers and hairpins using the DNASTAR® PrimerSelect® program. Primers with ΔG values $\geq$ -10 kc/m for self-dimer formation and ΔG values $\geq$ -4.5 kc/m for hairpin formation were retained for further analysis. All possible forward and reverse primer pair combinations using retained primers were analyzed using the same programs for heterodimer formation. Primer pairs with ΔG heterodimer values $\geq$ -8.0 kc/m were chosen as optimal. Lastly, primer combinations which produced amplicons between 300-550 base pairs (bp) in length were chosen as ideal. The primer pairs which contained all desired attributes were ordered from Integrated DNA Technologies, Inc., Coralville, IA.

Extraction of genomic DNA from isolates

Genomic DNA of bacteria was extracted using phenol-chloroform methodology. Bacterial isolates were picked up from LB plates using sterile inoculating loops and placed into 200 uL of TE buffer (500 uL 0.5 mM EDTA; 2.5 mL 1M Tris-HCL; 250 mL sterile dH_2O) inside a 1.5 mL microcentrifuge tube until bacteria reached half-way to the 0.1 mL mark of the tube. Cells were collected into a pellet by spinning the tube for 10 min at 12,000 rpm and the supernatant was discarded. The pellet was resuspended in 900 µL of lysis buffer (1.6 mL 0.5M EDTA; 1 mL 1M Tris-HCl, pH 8.3; 5.13g sucrose) containing 10 mg/mL lysozyme and

incubated at 37°C for 30 minutes. After incubation, 50 µL proteinase K and 50 µL 20% aqueous SDS solution were added to the microcentrifuge tube. The tube was gently mixed and placed into a water bath set at 55°C for 30 minutes for Gram-negative isolates and overnight for Gram-positive isolates.

After incubation, the tube was centrifuged at 12,000 rpm for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. 900 uL buffered phenol (pH 8) was added to the supernatant and the tube was inverted several times. The tube was then centrifuged at 12,000 rpm for 2 minutes to separate the phases and 600 uL of the aqueous phase was transferred to a new microcentrifuge tube. 600 uL of phenol: chloroform: isoamyl alcohol (25:24:1) solution was added to the tube and mixed by inverting the tube several times. The phases were separated by centrifugation at 12,000 rpm for 2 minutes and 500 uL of the aqueous layer were transferred to a new tube. 500 uL of chloroform: isoamyl alcohol (24:1) were added to the aqueous phase, mixed by inverting the tube several times, and the phases separated by centrifugation at 12,000 rpm for 2 minutes. All of the aqueous phase was gathered and transferred to new tube.

3M sodium acetate was added in an amount that was equal to $1/10^{th}$ of the gathered aqueous phase and mixed by inverting the tube several times. 1000 uL ice-cold ethanol was added to the tube, inverted several times to mix, and placed in the freezer overnight. The tubes were then centrifuged at 12,000 rpm for 10 minutes and the ethanol discarded. Tubes were placed sideways with their lids open for 30 minutes to allow excess ethanol to evaporate. Lastly, the DNA pellet was resuspended in 100 uL of TE buffer.

Amplification of *bphA1* using Polymerase chain reaction (PCR)

In order to determine if bacterial isolates contained the *bphA1* gene, thus verifying their PCB degrading potential, genomic DNA of each isolate was used in PCR reactions containing the primer pairs designed for the *bphA1* gene. 25 uL PCR reactions were set up using 12.5 uL GoTaq® Green Master Mix 2X (Promega, Madison, WI) containing 400 uM dNTPs and 3 mM $MgCl_2$, 2.5 uL forward primer, 2.5 uL reverse primer, 0.4-1.0 uL genomic DNA, and 6.5-7.1 uL nuclease free H_2O.

PCR reactions were conducted using a Bio-Rad MyCycler™ thermocycler (Bio-Rad, Hercules, CA) using the following protocol: initial 4 minute denaturation at 95°C followed by thirty cycles of denaturation (30 seconds at 95°C), primer annealing (30 seconds at 40.7-54°C) and extension (30 seconds at 72°C). A final extension at 72°C was done for 30 seconds after cycles were completed and the PCR reaction was held at 4°C until used.

Visualization of *bphA1* PCR products

To verify the amplification of desired PCR products, agarose gel electrophoresis was conducted using a Bio-Rad Mini-Sub® Cell GT system. 1.0% Agarose gels were cast by adding 0.30g agarose (Sigma-Aldrich, St. Louis, MO) to 30 mL 1x TBE running buffer (10.8g Tris-Base; 5.5g Boric acid; 4 mL EDTA, pH 8) and microwaving the liquid gel until the agarose fully dissolved. After being allowed to cool for 5 minutes, 2 uL of ethidium bromide (10mg/mL) was added to the liquid gel and mixed by swirling. The gel was then poured into a casting tray and allowed to cool at 25°C for at least 1h before use. Solidified gels were placed inside the Mini-Sub® rig and 1x TBE running buffer was poured into the rig until the gel was covered by approximately 5 mm of 1x TBE running buffer.

To visualize the size of the amplicons, 12 uL of a 100 bp step ladder (7 uL ladder, 5 uL gel loading dye) was added to the first well of the gel and 10 uL of PCR reactions was pipetted into the other wells of the gel. An additional 2 uL of ethidium bromide was pipetted into the TBE buffer at the negative pole of the rig and gels were run at 80 V for 1h. Gels were visualized under UV light using a Fotodyne 60-2100 Bioimaging System (Fotodyne, Hartland, WI).

CHAPTER IV

RESULTS

Bacterial density in sediment samples

Number of facultative bacteria in sediment samples

The mean number of colonies that grew on the triplicate set of LB Petri dishes were used to calculate the colony forming units (CFUs) of facultative bacteria per gram of sediment using the formula: **(Mean CFUs) / (Dilution factor plated)(0.1 mL) = CFUs / g of sediment.** Calculations showed mean CFUs of facultative bacteria in the sample sites, from largest to smallest mean, were: West reservoir $\bar{x}$ = 4.11e6 (SD = 6.67e6) > Outlet siphon $\bar{x}$ = 2.98e6 (SD = 1.03e6) > East reservoir $\bar{x}$ = 8.41e5 (SD = 5.94e5) > FM1423 $\bar{x}$ = 6.94e5 (SD = 4.78e5) (Figure 1). Two sample t-tests for independent samples compared means to each other and results indicated the Outlet siphon site contained a statistically significant larger mean of CFUs than both the East reservoir ($p<0.001$) and FM1423 bridge ($p<0.001$).

Number of bacteria able to use biphenyl as a sole carbon source

The mean number of colonies growing on triplicate MM Petri dishes was calculated using the same formula used to calculate total facultative bacteria. Results indicated the mean CFUs in the sample sites, from largest to smallest mean, were: East Reservoir $\bar{x}$ = 6.37e5 (SD =

5.70e5) > Outlet siphon $\bar{x}$ = 5.46e5 (SD = 4.16e5) > FM1423 $\bar{x}$ = 9.56e4 (SD = 5.45e4) > West Reservoir $\bar{x}$ = 7.95e4 (SD = 8.80e4). Biphenyl utilizing bacteria are a subset of the total facultative bacteria in the Donna Irrigation System and roughly represented 10% of the facultative bacteria population (Figure 2). Means were compared to each other using a two sample t-test for independent samples. Results indicated the East reservoir contained a statistically significant higher mean of bacteria able to use biphenyl as a sole carbon source than the West reservoir ($p = 0.0028$) and the FM1423 site ($p = 0.003$). Furthermore, the Outlet siphon had statistically significant higher mean of bacteria able to use biphenyl as a sole carbon source than the West Reservoir ($p = 0.0045$) and the FM1423 site ($p = 0.0061$).

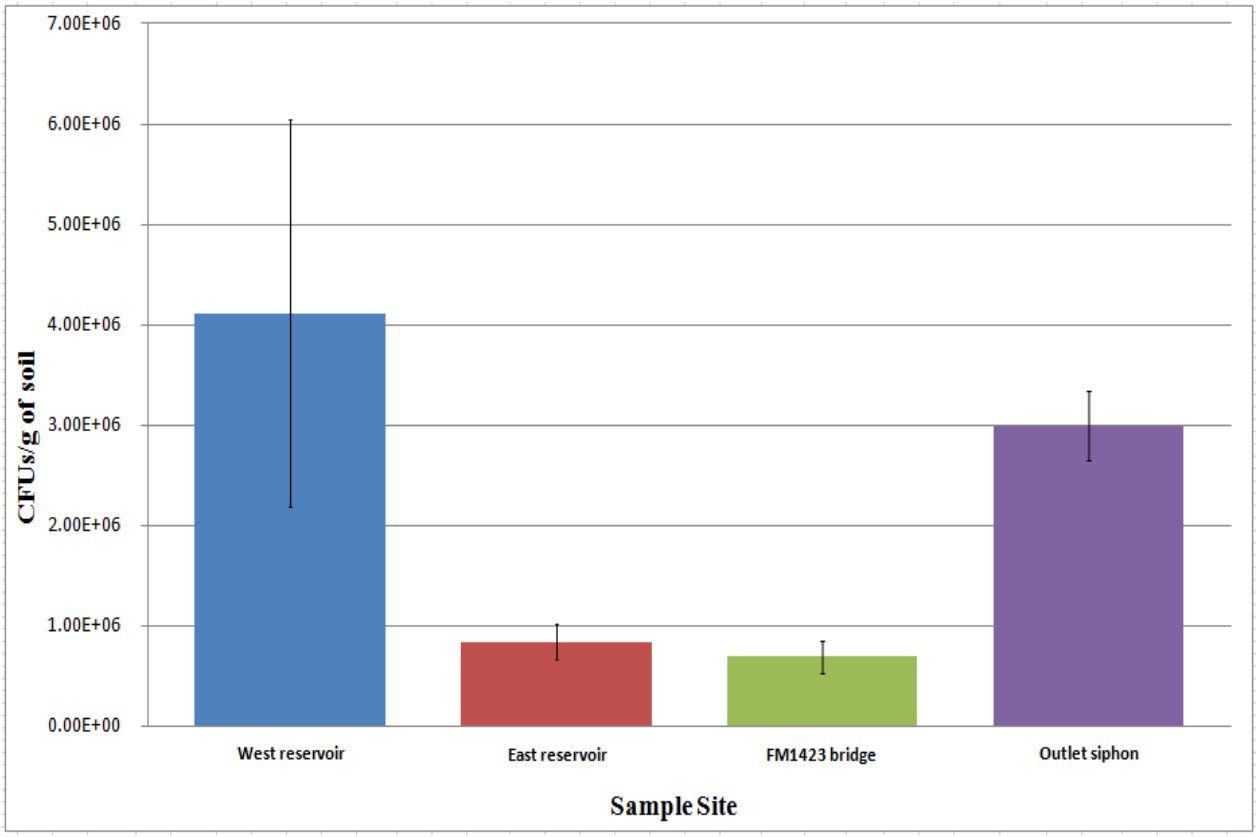

Figure 1. Mean colony forming units (CFUs) of facultative bacteria per gram of sediment of four sample sites in the Donna Irrigation System (error bars represent standard error). N = 12, 12, 9, 9, respectively.

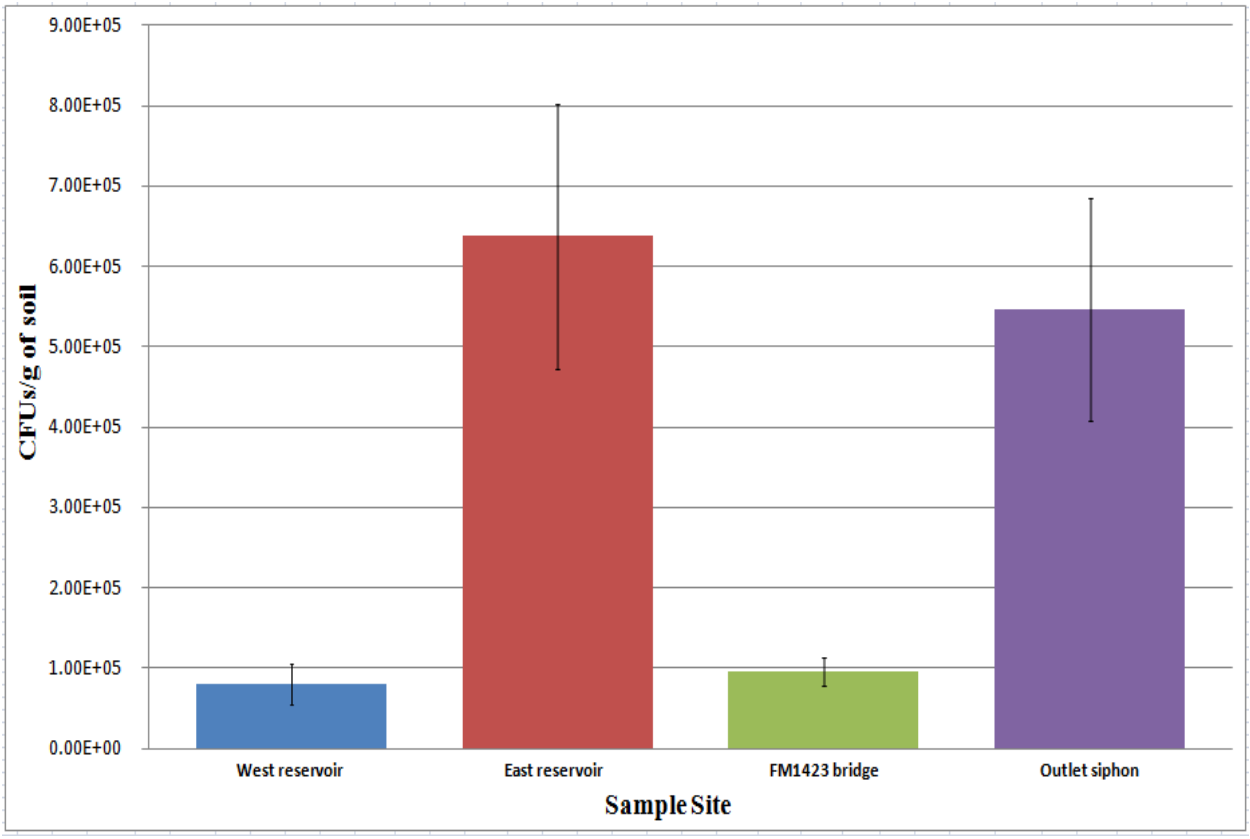

Figure 2. Mean colony forming units (CFUs) of biphenyl utilizing bacteria per gram of sediment of four sample sites in the Donna Irrigation System (error bars represent standard error). N = 12, 12, 9, 9, respectively.

Dibenzofuran cleaving isolates

There were large differences in the number of dibenzofuran-cleaving bacteria isolated from the different sample sites of the Donna Irrigation System. Twenty-six (26) dibenzofuran-cleaving isolates were grown from the Eastern reservoir (identified with a preceding "3" in tables), sixteen (16) from the western reservoir (identified with a preceding "1" in tables), one (1) from the FM1423 bridge site, and zero (0) from the outlet siphon site. In total, forty-three (43) dibenzofuran cleaving isolates were able to be isolated from the Donna Irrigation System (Tables 1, 2 and 3).

Characterization of bacterial isolates

Bacterial morphology

Negative staining with Nigrosin and observation using light microscopy showed two predominant bacterial morphologies for the isolates of both reservoirs: bacillus and coccobacillus. The western reservoir contained twelve (12) isolates with bacillus morphology and four (4) with coccobacillus morphology (Table 1). The eastern reservoir contained twenty-four (24) isolates with bacillus morphology and two (2) with coccobacillus morphology (Table 2). The sole isolate from the FM1413 bridge site was of coccobacillus morphology (Table 3).

Gram-stain reaction

Isolates showing bacillus morphology stained pink after being subjected to the Gram-stain reaction, indicating they had a Gram-negative cell wall structure. This result was obtained for all bacillus isolates, regardless of the reservoir they were isolated from (Tables 1-3). Conversely, all coccobacillus isolates stained purple after the Gram-stain reaction, indicating they had a Gram-positive cell wall. Again, this result was obtained regardless of the location they were isolated from (Tables 1-3). Thus, the western reservoir contained twelve (12) Gram-negative and four (4) Gram-positive isolates (Table 1), the eastern reservoir contained twenty-four (24) Gram-negative and two (2) Gram-positive isolates (Table 2), and the FM1423 bridge site contained one (1) Gram-positive isolate (Table 3).

Catalase and oxidase reactions

All isolates had visible bubbling after exposure to 3% aqueous H_2O_2, indicating the release of oxygen, and the presence of the catalase enzyme in all the bacteria (Tables 1-3).

Exposure of bacterial colonies to the cotton swab moistened with 1% α-naphtol in 95% ethanol mixed with an equal volume of aqueous 1% *p*-aminodimethylaniline oxalate indicated all Gram-negative isolates contained the cytochrome c oxidase enzyme while all Gram-positive isolates lacked the enzyme (Tables 1, 2 and 3).

Isolate #	Identifier	Morphology	Gram	Catalase	Oxidase
1	**1IC1 #1-1 S.2**	**Bacillus**	**-**	+	+
2	**1ISE1 #1**	**Bacillus**	**-**	+	+
3	**1ISW2 #1**	**Coccobacillus**	+	+	**-**
4	**1IC2 #3**	**Bacillus**	**-**	+	+
5	**1IC3 #4-2 S.2**	**Bacillus**	**-**	+	+
6	**1IC1 #1 S.1**	**Bacillus**	**-**	+	+
7	**1ISW2 #2**	**Coccobacillus**	+	+	**-**
8	**1IC3 #4-1 S.2**	**Bacillus**	**-**	+	+
9	**1IC3 #3 S.2**	**Bacillus**	**-**	+	+
10	**1IC1 #1 S.1**	**Bacillus**	**-**	+	+
11	**1IC3 #2 S.2**	**Bacillus**	**-**	+	+
12	**1IC1 #2 S.2**	**Bacillus**	**-**	+	+
13	**1IC3 #2 S.3**	**Bacillus**	**-**	+	+
16	**1IC3 #1 S.2**	**Coccobacillus**	+	+	**-**
17	**1IC1 #4-2 S.1**	**Coccobacillus**	+	+	**-**
18	**1IC1 #2**	**Bacillus**	**-**	+	+

Table 1. Morphology, Gram reaction, catalase reaction, and oxidase reaction of 16 dibenzofuran cleaving isolates from the Western reservoir of the Donna Irrigation System.

Isolate #	Identifier	Morphology	Gram stain	Catalase	Oxidase
21	3IC2 #13	Bacillus	-	+	+
22	3IC2 #19 S.1	Bacillus	-	+	+
23	3IC2 #16 S.2	Bacillus	-	+	+
24	3IC2 #16 S.1	Bacillus	-	+	+
25	3IC2 #10 S.2	Bacillus	-	+	+
26	3IC2 #28 S.3	Bacillus	-	+	+
27	3IC2 #19 S.2	Bacillus	-	+	+
28	3IC2 #28 S.2	Bacillus	-	+	+
29	3IC2 #10 S.3	Bacillus	-	+	+
30	3C1 #8 S.1	Bacillus	-	+	+
31	3C1 #8 S.2	Bacillus	-	+	+
32	3C1 #1	Bacillus	-	+	+
33	3C2 #4 S.2	Bacillus	-	+	+
36	3IC2 #7 S.3	Bacillus	-	+	+
37	3IC2 #5 S.1	Bacillus	-	+	+
38	3IC2 #5 S.3	Bacillus	-	+	+
39	3IC2 #5 S.2	Bacillus	-	+	+
40	3IC2 #3 S.2	Bacillus	-	+	+
41	3IC2 #6 S.2	Bacillus	-	+	+
42	3IC2 #4 S.1	Bacillus	-	+	+
43	3C2 #2 S. 2	Coccobacillus	+	+	-
44	3IC2 #5 S.4	Bacillus	-	+	+
45	3IC2 #4 S.2	Bacillus	-	+	+
46	3IC2 #10 S.4	Coccobacillus	+	+	-
47	3C1 #8 S.3	Bacillus	-	+	+
48	3C1 #1 S.2	Bacillus	-	+	+

Table 2. Morphology, Gram reaction, catalase reaction, and oxidase reaction of 26 dibenzofuran cleaving isolates from the Eastern reservoir of the Donna Irrigation System.

Isolate #	Identifier	Morphology	Gram stain	Catalase	Oxidase
50	FM1423	**Coccobacillus**	+	+	-

Table 3. Morphology, Gram reaction, catalase reaction, and oxidase reaction of the sole dibenzofuran cleaving isolate from FM1423 bridge sampling site.

API® 20E strips

Isolates taken from the western reservoir yielded negative results in most (19 of 22) tests in the API® strip system. The three tests that yielded positive results were: citrate utilization (CIT) (9 of 18 isolates), reduction of nitrate to nitrite (NO_2) (14 of 18 isolates) and reduction of nitrite to nitrogen gas (N_2) (1 of 18 isolates) (Table 4).

The sole isolate from the FM1423 bridge site was negative for all tests with the exception of reduction of nitrate to nitrite (Table 5).

The isolates of the eastern reservoir yielded fewer negative results (16 of 22) in the tests of the strips. The six tests that yielded positive results were: presence of arginine dihydrolase (ADH) (1 of 26 isolates), citrate utilization (14 of 26), glucose fermentation (GLU) (1 of 26), melibiose fermentation (MEL) (1 of 26), reduction of nitrate to nitrite (NO_2) (25 of 26) and reduction of nitrite to nitrogen gas (N_2) (1 of 26) (Table 6).

API® 20E phenotypic tree

Based on the branching pattern of the phenotypic created using the API® 20E strip results, isolates are at least 89% similar in their phenotypic characteristics. The next major branching in the phenotypic tree occurred at the 94% similarity mark, after which isolates show much more diversity. After this point, Gram-negative isolates were divided into seven different groups while the Gram-positive isolates remained grouped together (Figure 3).

#	Identifier	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2
1	**1IC1 #1-1 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
2	**1ISE1 #1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	**1ISW2 #1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
4	**1IC2 #3**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
5	**1IC3 #4-2 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
6	**1IC1 #1 S.1**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
7	**1ISW2 #2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
8	**1IC3 #4-1 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
9	**1ICE #3 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
10	**1IC1 #1 S.1**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
11	**1IC3 #2 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
12	**1IC1 #2 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
13	**1IC3 #2 S.3**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
16	**1IC3 #1 S.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
17	**1IC1 #4-2 S.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
18	**1IC1 #2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Table 4. API® 20E reactions for isolates from the Western reservoir. Positive results are highlighted in yellow.

#	Identifier	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2
50	**FM1423**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Table 5. API® 20E reactions for isolates from the FM1423 bridge site. Positive results are highlighted in yellow.

#	Identifier	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2
21	**3IC2 #13**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
22	**3IC2 #19 S.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
23	**3IC2 #16 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
24	**3IC2 #16 S.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
25	**3IC2 #10 S.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
26	**3IC2 #28 S.3**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
27	**3IC2 #19 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
28	**3IC2 #28 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
29	**3IC2 #10 S.3**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
30	**3C1 #8 S.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
31	**3C1 #8 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
32	**3C1 #1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
33	**3C2 #4 S.2**	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	**3IC2 #7 S.3**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
37	**3IC2 #5 S.1**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
38	**3IC2 #5 S.3**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
39	**3IC2 #5 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
40	**3IC2 #3 S.2**	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
41	**3IC2 #6 S.2**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
42	**3IC2 #4 S.1**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
43	**3C2 #2 S.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
44	**3IC2 #5 S.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
45	**3IC2 #4 S.1**	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
46	**3IC2 #10 S.4**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
47	**3C1 #8 S.3**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
48	**3C1 #1 S.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Table 6. API® 20E reactions for isolates from the Eastern reservoir. Positive results are highlighted in yellow.

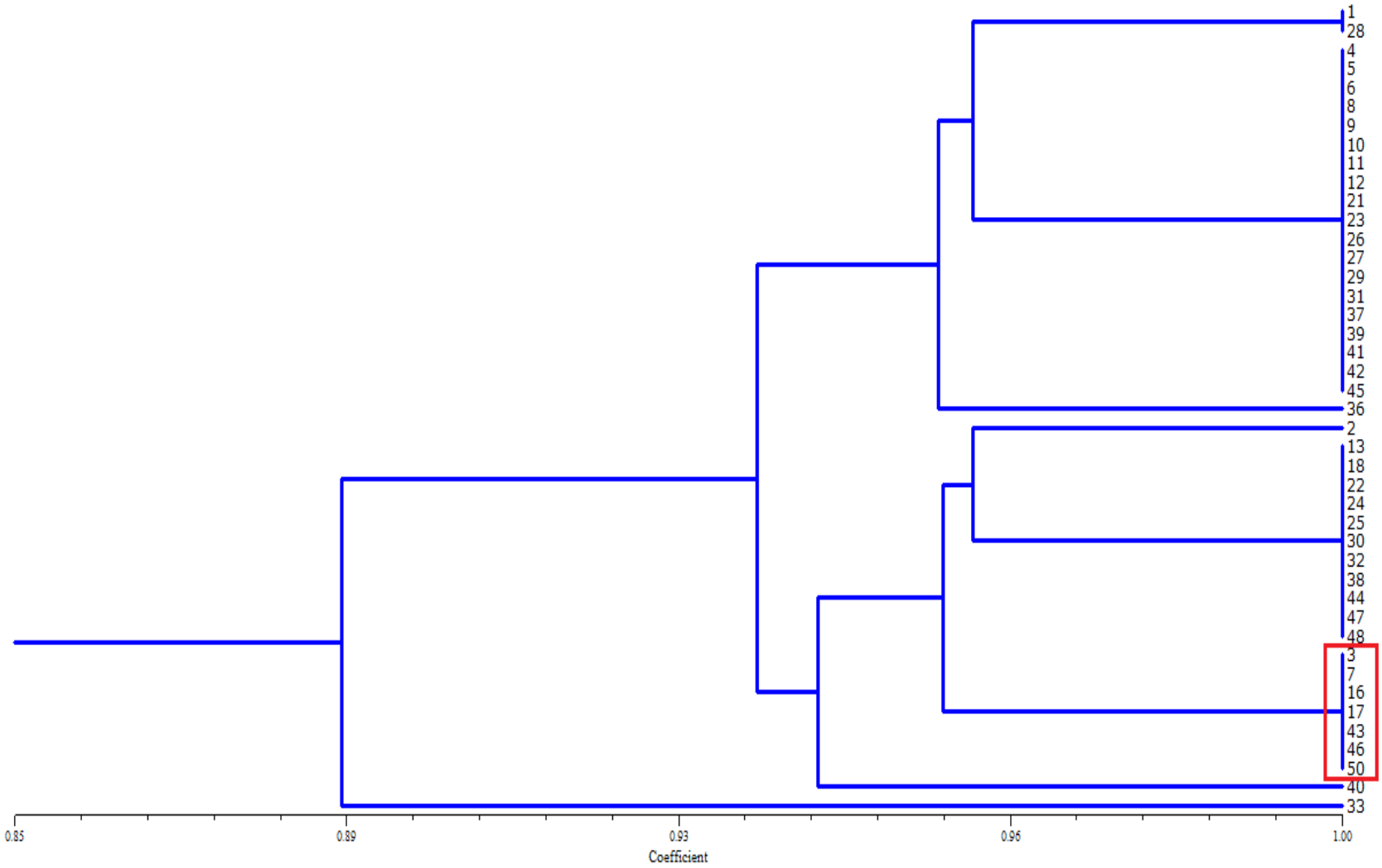

Figure 3. API® 20E phenotypic tree for all bacterial isolates from the Donna Irrigation System. Gram-positive isolates are highlighted with a red, rectangular box.

BIOLOG EcoPlates™

The mean number of different carbon substrates used by all bacterial isolates was $\bar{x}$ = 20.23 with a minimum of two (2) carbon sources used by a Gram-positive bacterium isolated taken from the West reservoir (isolate #16) and a maximum of thirty (30) carbon sources used by the sole Gram-positive bacterium isolated from the FM1423 bridge site (isolate #50). With the exception of isolate #16 from the West reservoir, Gram-positive bacteria used similar numbers of substrates regardless of their site of isolation. In contrast, Gram-negative bacteria appeared to have more variety in their substrate utilization numbers regardless of their site of isolation (Figure 4).

Of thirty-one (31) different carbon substrates on the BIOLOG EcoPlates™, six (6) were consistently the most highly metabolized by bacterial isolates after 72 hours of incubation: D-Galacturonic Acid (21 isolates), Pyruvic Acid Methyl Ester (11 isolates), Tween 80 (6 isolates), L-Serine (2 isolates), L-Asparagine (2 isolates) and Tween 40 (1 isolate) (Figure 5). All D-Galacturonic Acid, Pyruvic Acid Methyl Ester, L-Serine and Tween 40 metabolizing isolates were Gram-negative. The two L-Aspargine metabolizing isolates consisted of a Gram-positive and Gram-negative isolate and all Tween 80 metabolizing isolates were Gram-positive.

The mean Total Activity of all bacterial isolates was $\bar{x}$ = 9.48 with a minimum of 0.479 by isolate #16 from the West reservoir and a maximum of 17.043 by isolate #33 from the East reservoir (Figure 6). Total Activity of bacterial isolates was weakly positively correlated with total number of carbon sources used ($R^2 = 0.3808$) (Figure 7).

BIOLOG EcoPlate™ phenotypic tree

The branching pattern on the phenotypic tree created from BIOLOG EcoPlates™ suggested the isolates were only 54% similar based on their ability to metabolize the 31 carbon sources of the test. The BIOLOG tree also showed much more branching relative to the API® strip phenotypic tree, with major branching occurring at 58%, 63%, 64% isolate phenotypic similarity. Unlike the phenotypic tree constructed from API® strips, isolates on the BIOLOG phenotypic tree showed a large number of grouping patterns. Gram-positive isolates, which had shown great phenotypic similarity using the API® strip system, were divided into 5 distinct groups using the BIOLOG EcoPlates™ (Figure 8).

Scanning electron microscopy

Micrographs of the Gram positive bacterium (Isolate #3) showed a pleomorphic bacterium of either coccobacillus or bacillus morphology. Coccobacilli were approximately 0.6 μm in length while the bacilli were approximately 2.5 μm in length (Figure 9). Gram-negative bacteria (isolates #26 and #30) showed bacillus morphology but had clear differences in their bacterial cell dimensions. Isolate #26 displayed a consistent, slender rod appearance ranging from 1-3 μm in length and 0.3 μm in width (figure 10). In contrast, isolate #30 had much more pleomorphic rods, with some showing slight curvature, ranging from 0.7-2 μm in length and 0.5 μm in width (Figure 11).

Amplification of the *bphA1* gene

PCR primers for *bphA1*

Analysis of the aligned protein sequences for the *bphA1* gene of 18 bacteria revealed eight (8) highly conserved sequences which could be used as forward primers (Table 7) and seven (7) highly conserved sequences which could be used as reverse primers (Table 8). Only 3 of 8 potential forward primers, *bphA-F4, F6* and *F7,* had ΔG values for self-dimer formation less than -10 kc/m and were kept for further analysis (Table 7). Of 8 reverse primers, 5, *bphA1-R1, R2, R4, R5* and *R6,* had ΔG values for self-dimer formation less than -10 kc/m (Table 8). All possible combinations of forward and reverse primer pairs were then analyzed for their ΔG values for heterodimer formation. Primer pairs which had heterodimer ΔG values less than -8 kc/m were: *F4-R1, F4-R4, F6-R1, F6-R2, F6-R5, F7-R1* and *F7-R2* (Table 9). The amplicon length of these primer pairs was analyzed to narrow down the potential primers to those which had amplicon lengths between 300-550 base pairs. Primer pairs with desired amplicon lengths were: *F6-R2*, *F6-R5*and *F7-R2* (Table 9). A fourth primer pair, *F6-R1,* whose amplicon length was out of the desired range (1017 bp), was kept for comparison with the other primers.

PCR reaction and visualization of PCR products

The four selected primer pairs were all tested for their ability to amplify the *bphA1* gene from genomic DNA extracted from isolates #1, 2, 4, 5 and 6. The first two primer pairs tested, *F6-R2* and *F7-R2*, failed to amplify the gene from these DNA samples. The third primer pair tested, *F7-R1,* amplified the gene in DNA from isolates #2, 4 and 5. The last primer pair, *F6-R5*, amplified the gene in all 5 DNA samples and this primer pair was used for further PCR reactions (data not shown).

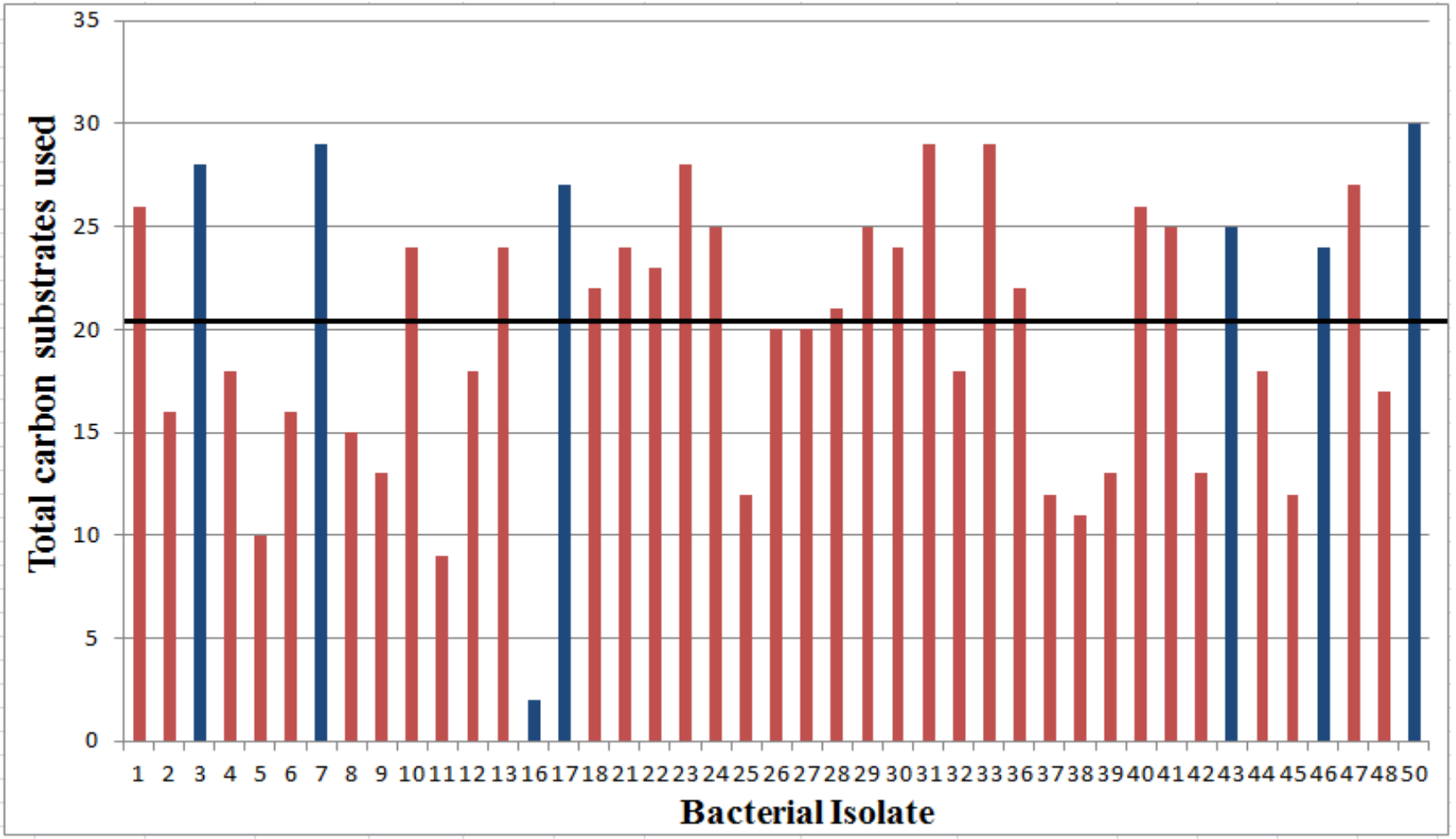

Figure 4. Total number of BIOLOG EcoPlate™ carbon substrates used by bacterial isolates. Mean number of substrates used ($\bar{x}$ = 20.23) is represented by the horizontal black line. Bars representing Gram-positive and Gram-negative isolates are colored blue and red, respectively.

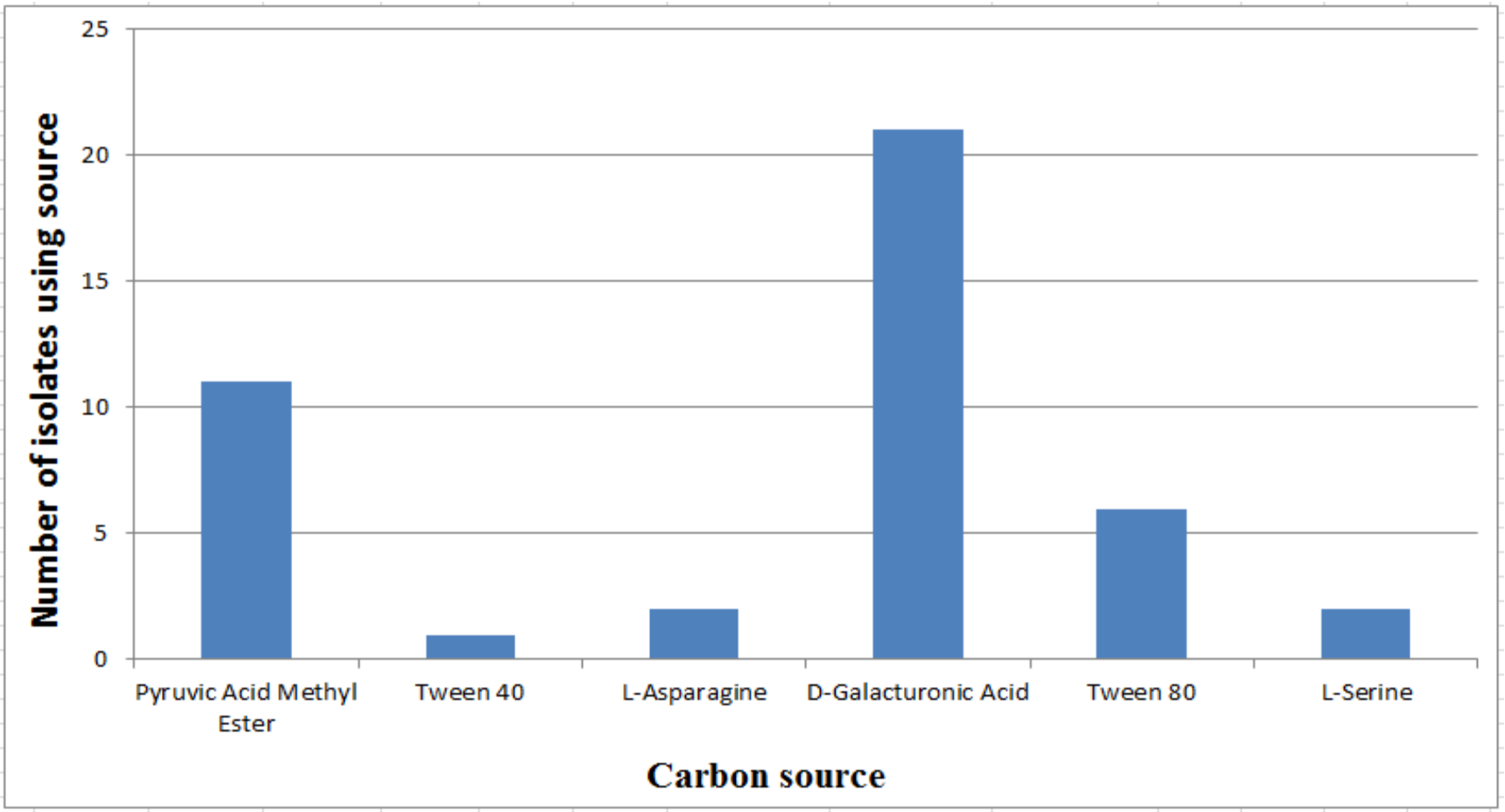

Figure 5. Number of bacterial isolates using the best metabolized carbon sources from BIOLOG EcoPlates™.

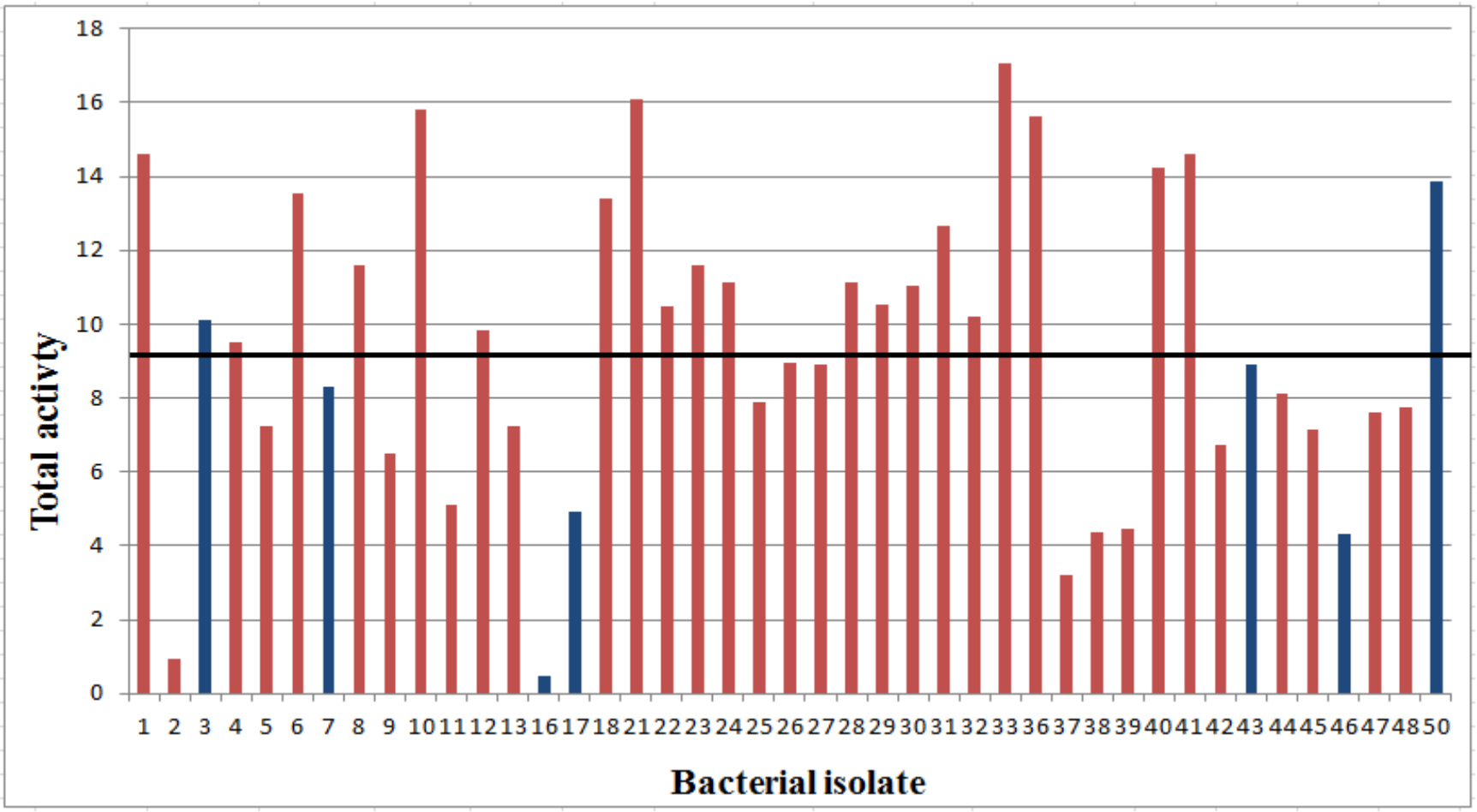

Figure 6. Total Activity (sum of all positive substrate metabolic activity) of bacterial isolates. Mean Total Activity ($\bar{x}$ = 9.48) is represented by the horizontal black line. Bars representing Gram-positive and Gram-negative isolates are colored blue and red, respectively.

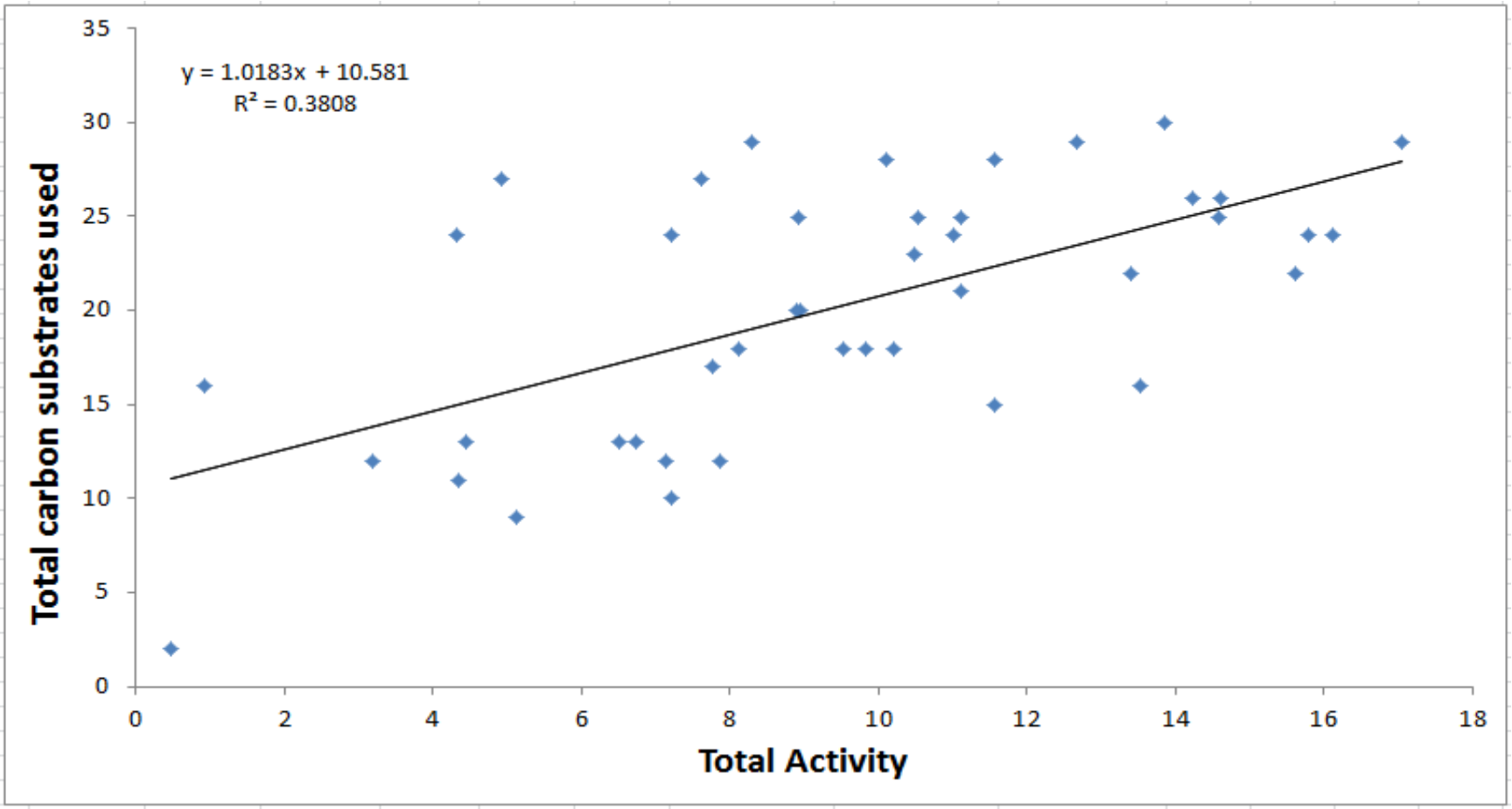

Figure 7. Scatterplot and linear regression model of total number of carbon substrates used vs. Total activity of bacterial isolates.

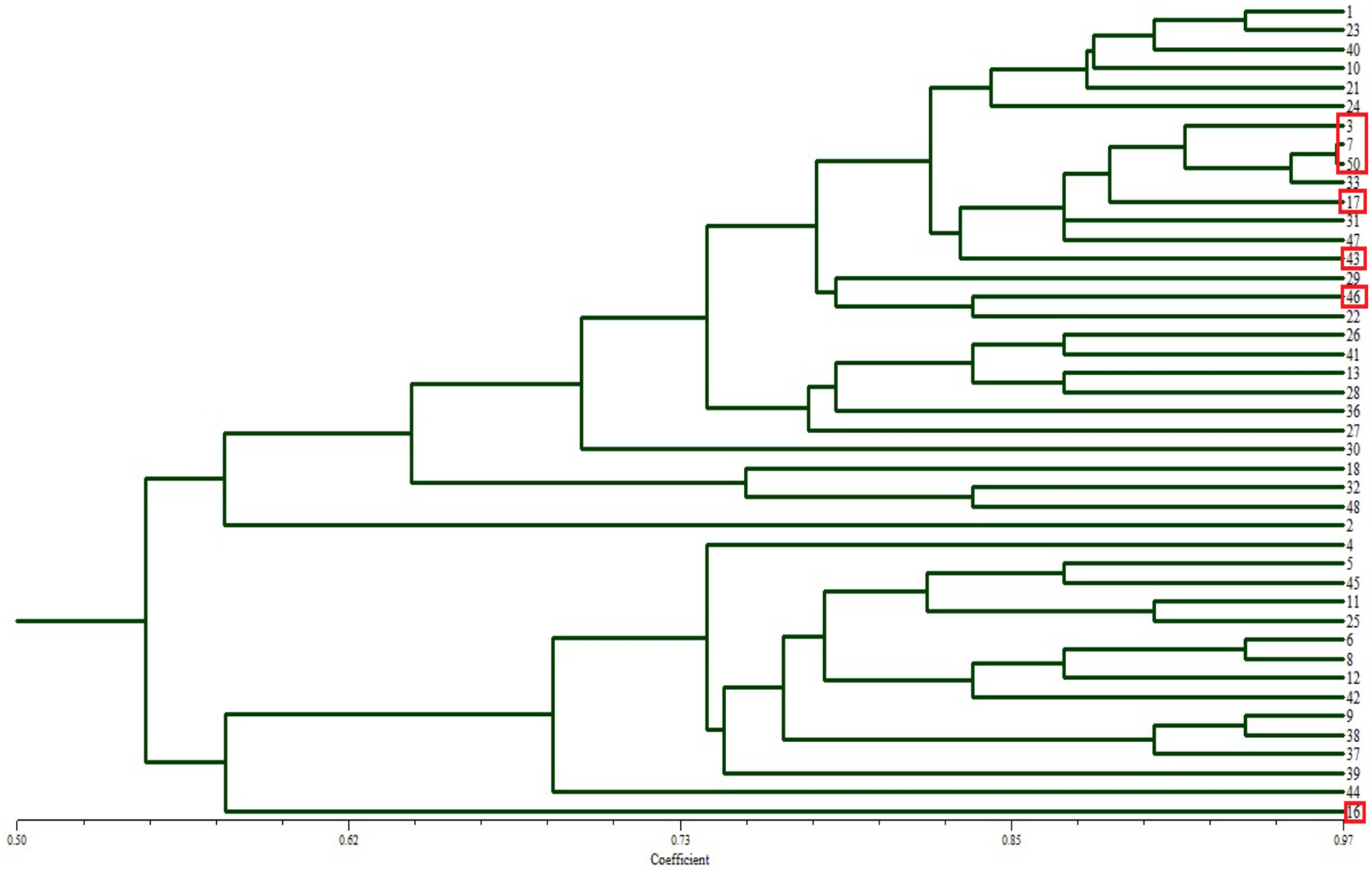

Figure 8. BIOLOG EcoPlate™ phenotypic tree for all bacterial isolates from the Donna Irrigation System. Gram-positive isolates are highlighted with red, rectangular boxes.

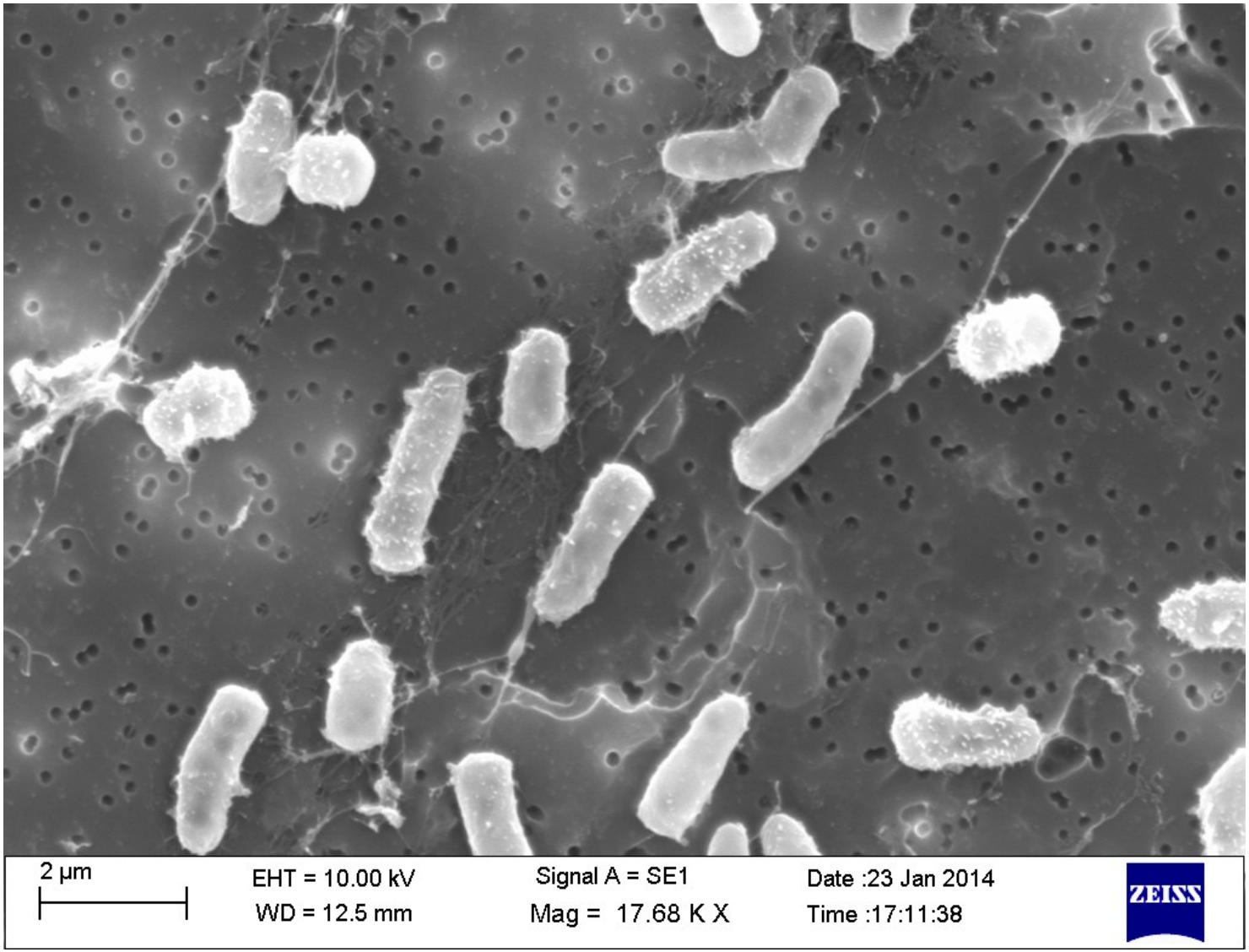

Figure 9. Scanning electron micrograph of a Gram-positive bacterium (isolate #3) isolated from the Western reservoir of the DIS.

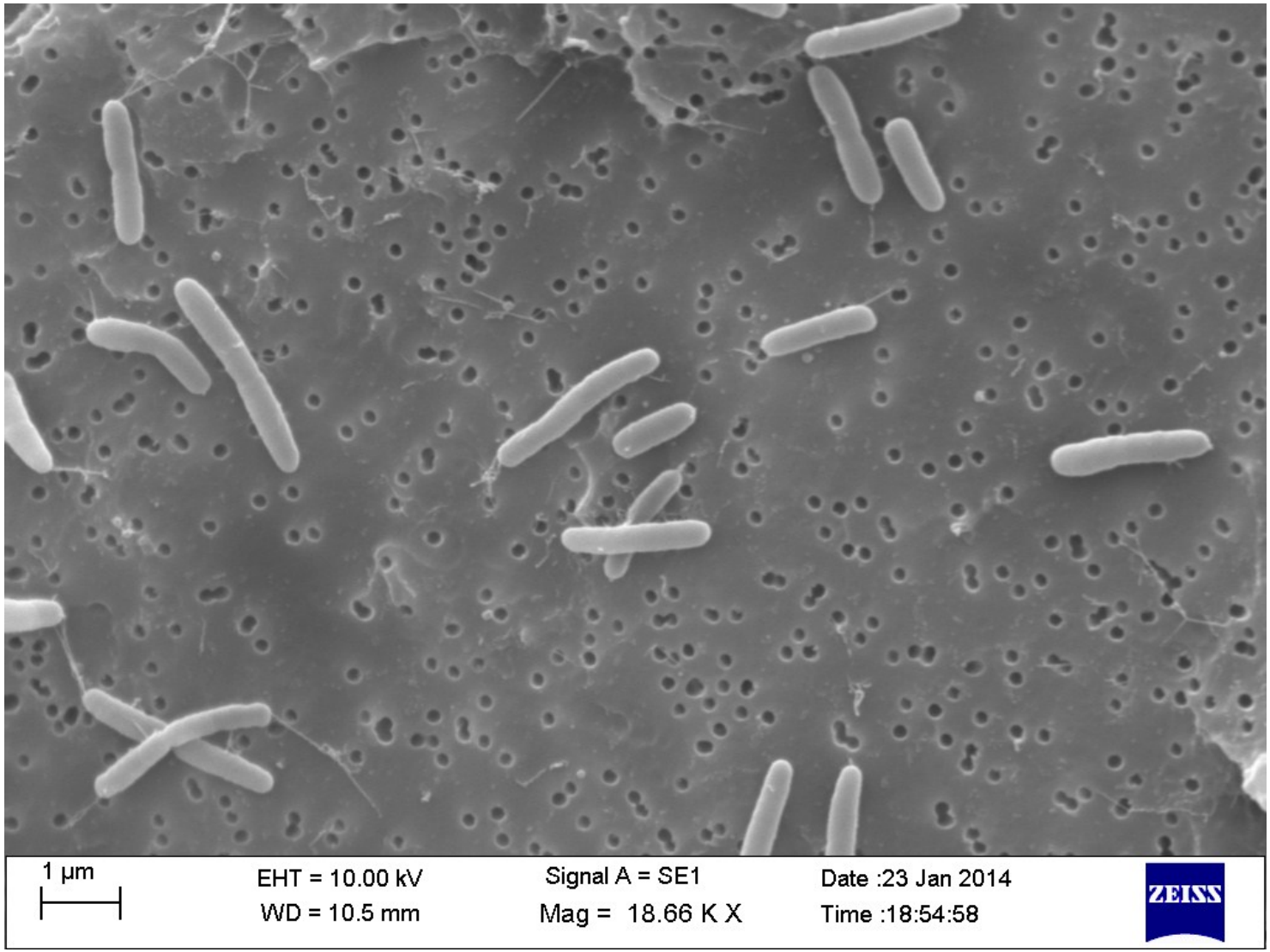

Figure 10. Scanning electron micrograph of a Gram-negative bacterium (isolate #26) isolated from the Eastern reservoir of the DIS.

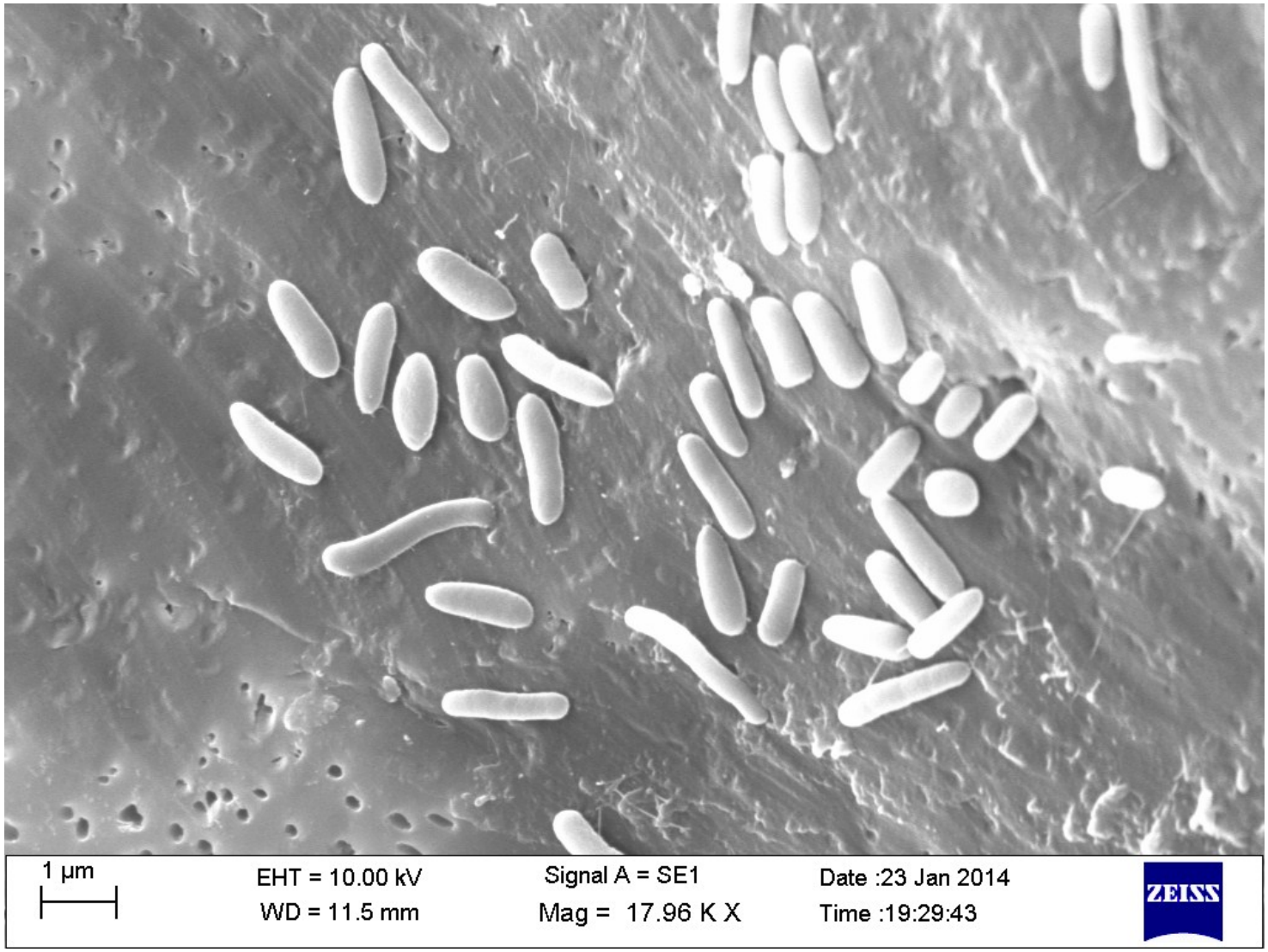

Figure 11. Scanning electron micrograph of a Gram-negative bacterium (isolate #30) isolated from the Eastern reservoir of the DIS.

Primer	Primer sequence (5' -> 3')	ΔG Self-dimer	ΔG Hairloop
bphA1-F1	GAR YTN GAR YTN GAR MGN GTN TTY	3' = -12.0 kc/m Internal = -7.8 kc/m	-5.2 kc/m
bphA1-F2	AAY CAR TGY MGN CAY MGN GGN ATG	3' = -10.5 kc/m Internal = -13.1 kc/m	-6.0 kc/m
bphA1-F3	TGY ACN TAY CAY GGN TGG	3' = -7.0 kc/m Internal = -12.8 kc/m	1.0 kc/m
***bphA1-F4**	**TAY AAR GGN YTN GTN TTY GCN**	**3' = -6.7 kc/m Internal = -7.8 kc/m**	**-1.6 kc/m**
bphA1-F5	TGG YTN YTN YTN GGN CAY	3' = -14.1 kc/m Internal = -9.9 kc/m	-2.0 kc/m
***bphA1-F6**	**ACN TAY ATG GGN GAR GAY**	**3' = -3.5 kc/m Internal = -2.0 kc/m**	**0.9 kc/m**
***bphA1-F7**	**TGG YTN YTN YTN GGN CAY GAR**	**3' = -9.9 kc/m Internal = -8.5 kc/m**	**-2.0 kc/m**
bphA1-F8	CAY MGN GGN ATG MGN ATH	3' = -13.4 kc/m Internal = -7.1 kc/m	-0.9 kc/m

Table 7. List of possible degenerate forward primers based on conserved sequences of 18 *bphA1* genes. Primers that were kept for further analysis are bolded and have an asterisk.

PCR was redone using genomic DNA extracted from isolates #1-9.With the exception of isolate #2, the *bphA1* gene was successfully amplified from the DNA of these isolates. Additionally, DNA from two cultures of *Escherichia coli* served as negative controls and also failed to amplify the *bphA1* gene. The amplicons of the successful PCR reactions traveled a distance of between 400 and 500 base pairs (based on a 100bp ladder) in the agarose gels, which matched the hypothesized 480 base pair amplicon size for this primer pair (Figure 12).

PCR conducted on genomic DNA extracted from isolates #10-13 and #16-18 successfully amplified the *bphA1*gene in all these samples. The amplicons of these reactions also traveled a a distance of between to 400 and 500 base pairs in the agarose gels, suggesting they were of the

Primer	Primer sequence (5' -> 3')	ΔG Self-dimer	ΔG Hairloop
bphA1-R1	**CCA RTT YTC NCC RTC RTC YTG**	**3' = -3.9 kc/m Internal = -2.0 kc/m**	**-0.7 kc/m**
bphA1-R2	**RAA YTG YTC NGC NGC RAA NCK**	**3' = -8.6 kc/m Internal = -7.8 kc/m**	**-4.1 kc/m**
bphA1-R3	NCC DAT NAC YTC NGT NCC	3' = -9.9 kc/m Internal = -7.2 kc/m	-2.8 kc/m
bphA1-R4	**NAR CAT NAC RTC CAT RTA**	**3' = -7.3 kc/m Internal = -6.3 kc/m**	**-2.5 kc/m**
bphA1-R5	**NGC RTG RTA CAT RTC**	**3' = -8.7 kc/m Internal = -5.5 kc/m**	**-0.3 kc/m**
bphA1-R6	**NGG NCC NCK NGG RTG CCA**	**3' = -8.2 kc/m Internal = -6.4 kc/m**	**-1.9 kc/m**
bphA1-R7	RCA RAA YTG YTC NGC NGC	3' = -10.2 kc/m Internal = -9.8 kc/m	-2.7 kc/m

Table 8. List of possible degenerate reverse primers based on conserved sequences of 18 *bphA1* genes. Primers that were kept for further analysis are bolded and have an asterisk.

correct hypothetical amplicon size for primer pair *F6-R5* (Figure 13).

The PCR reaction conducted on genomic DNA extracted from isolates #21-36 successfully amplified the *bphA1* gene in these samples, as well. Based on the migration of the amplicon on the agarose gel, amplicon size was determined to be between 400 and 500 base pairs and consistent with the hypothesized amplicon size (Figure 14).

PCR successfully amplified the *bphA1* gene from genomic DNA of isolates #37-48 and isolate #50. Similar to the other PCR reactions, the amplicons from this PCR reaction traveled a distance of between 400 and 500 base pairs in the agarose gel (Figure 15). Thus, 42 of 43

dibenzofuran-cleaving bacterial isolates, grown from Donna Irrigation System sediments, contained the *bphA1* gene required to begin PCB degradation within their genomic DNA.

Primer pairs	ΔG Heterodimer	Amplicon length
bphA1-F4 and *bphA1-R1*	-6.4 kc/m	738 bp
bphA1-F4 and *bphA1-R2*	-12.5 kc/m	177 bp
bphA1-F4 and *bphA1-R4*	-7.8 kc/m	738 bp
bphA1-F4 and *bphA1-R5*	-7.2 kc/m	198 bp
bphA1-F4 and *bphA1-R6*	-8.6 kc/m	603 bp
bphA1-F6 and *bphA1-R1*	-6.5 kc/m	1017 bp
bphA1-F6* and *bphA1-R2	**-7.9 kc/m**	**462 bp**
bphA1-F6 and *bphA1-R4*	-9.9 kc/m	369 bp
bphA1-F6* and *bphA1-R5	**-5.9 kc/m**	**480 bp**
bphA1-F6 and *bphA1-R6*	-11.3 kc/m	882 bp
bphA1-F7* and *bphA1-R1	**-7.9 kc/m**	**1071 bp**
bphA1-F7* and *bphA1-R2	**-6.4 kc/m**	**516 bp**
bphA1-F7 and *bphA1-R4*	-8.3 kc/m	426 bp
bphA1-F7 and *bphA1-R5*	-8.3 kc/m	531 bp
bphA1-F7 and *bphA1-R6*	-10 kc/m	936 bp

Table 9. List and description of all possible forward and reverse primer combinations. primers that were kept for use in PCR reactions are bolded and have an asterisk.

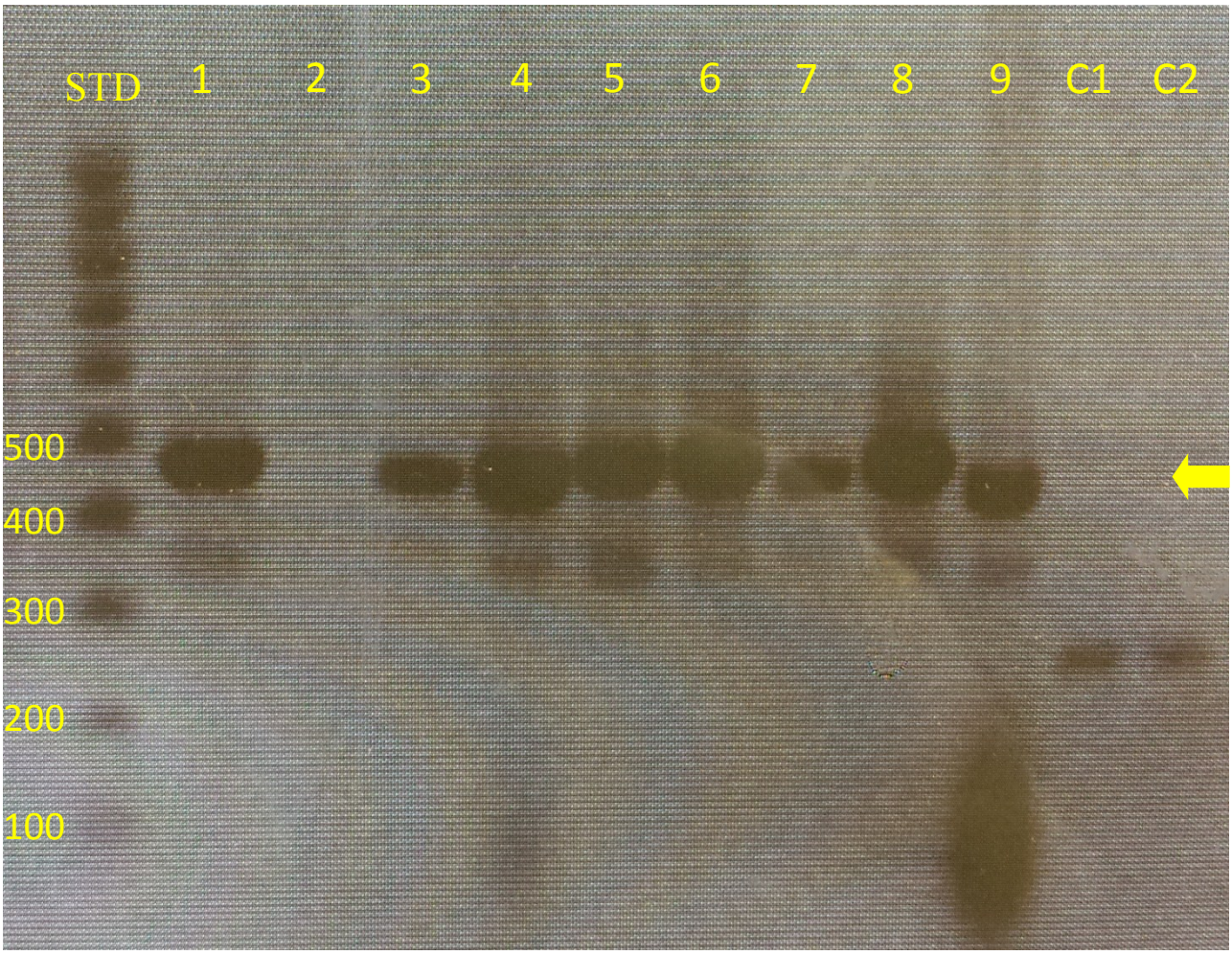

Figure 12. PCR amplification products of Western reservoir bacterial isolates #1-9 and two negative controls. Putative *bphA1* amplicons indicated by a yellow arrow.

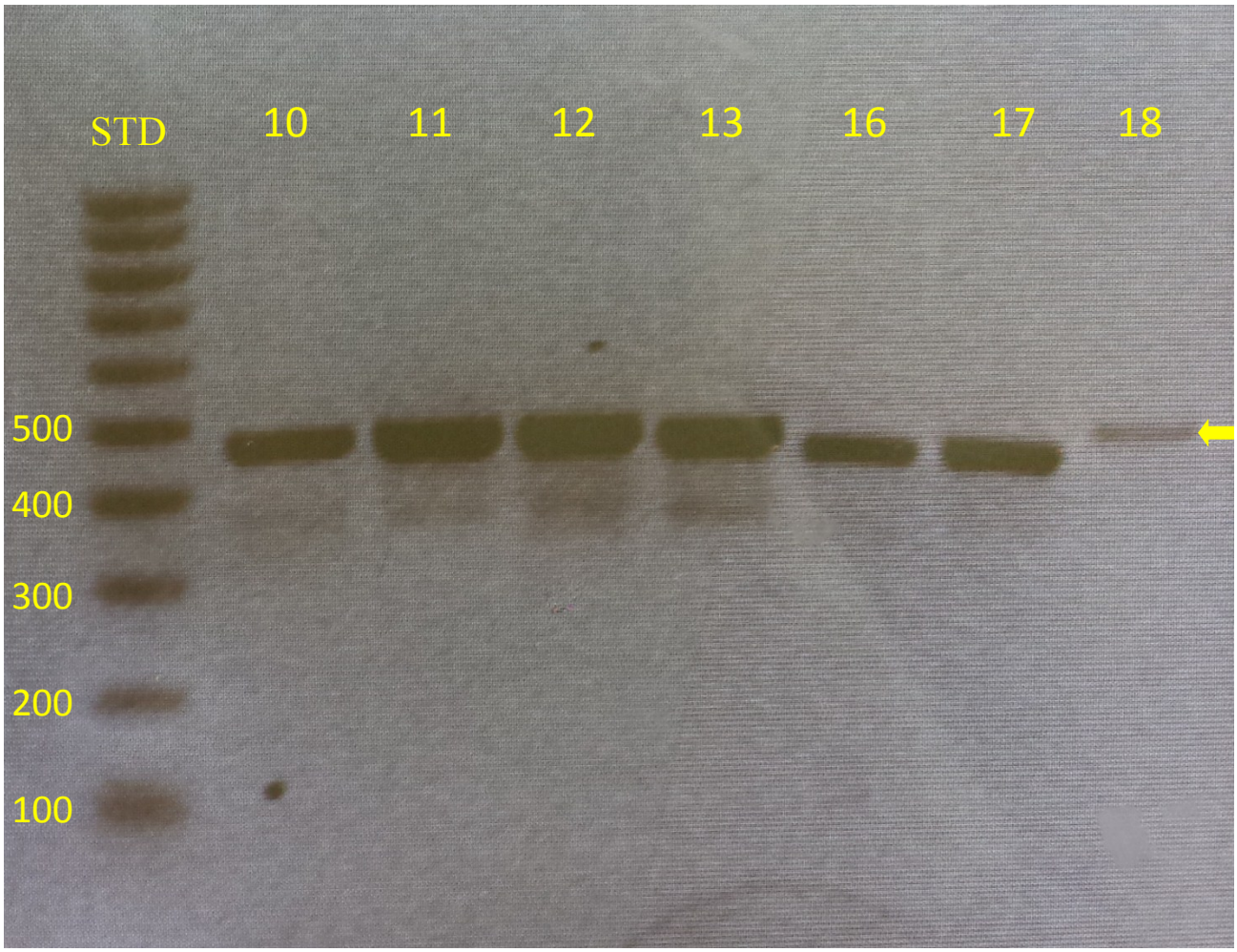

Figure 13. PCR amplification products of Western reservoir bacterial isolates #10-13 and isolates #16-18. Putative *bphA1* amplicons indicated by a yellow arrow.

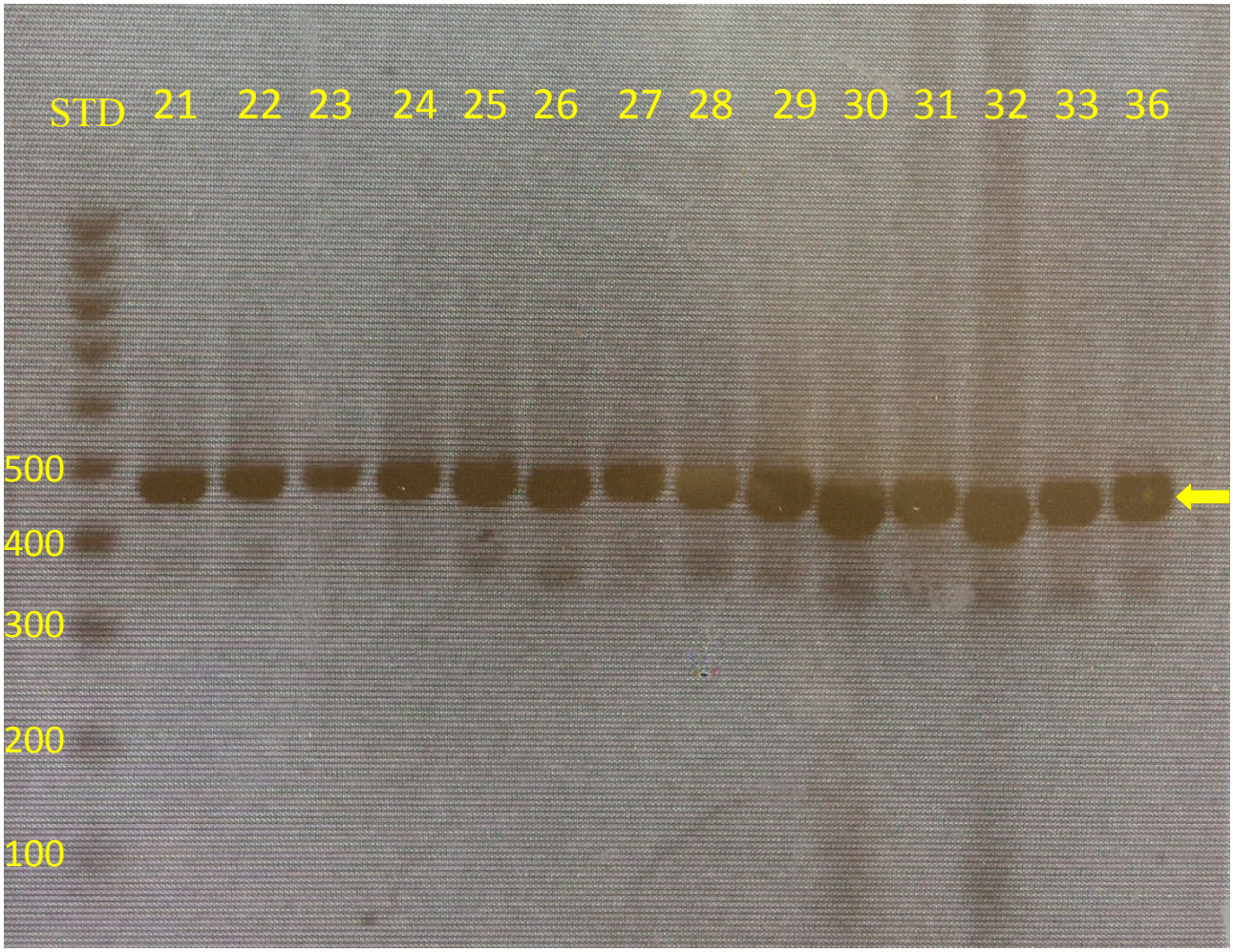

Figure 14. PCR amplification products of Eastern reservoir bacterial isolates #21-36. Putative *bphA1* amplicons indicated by a yellow arrow.

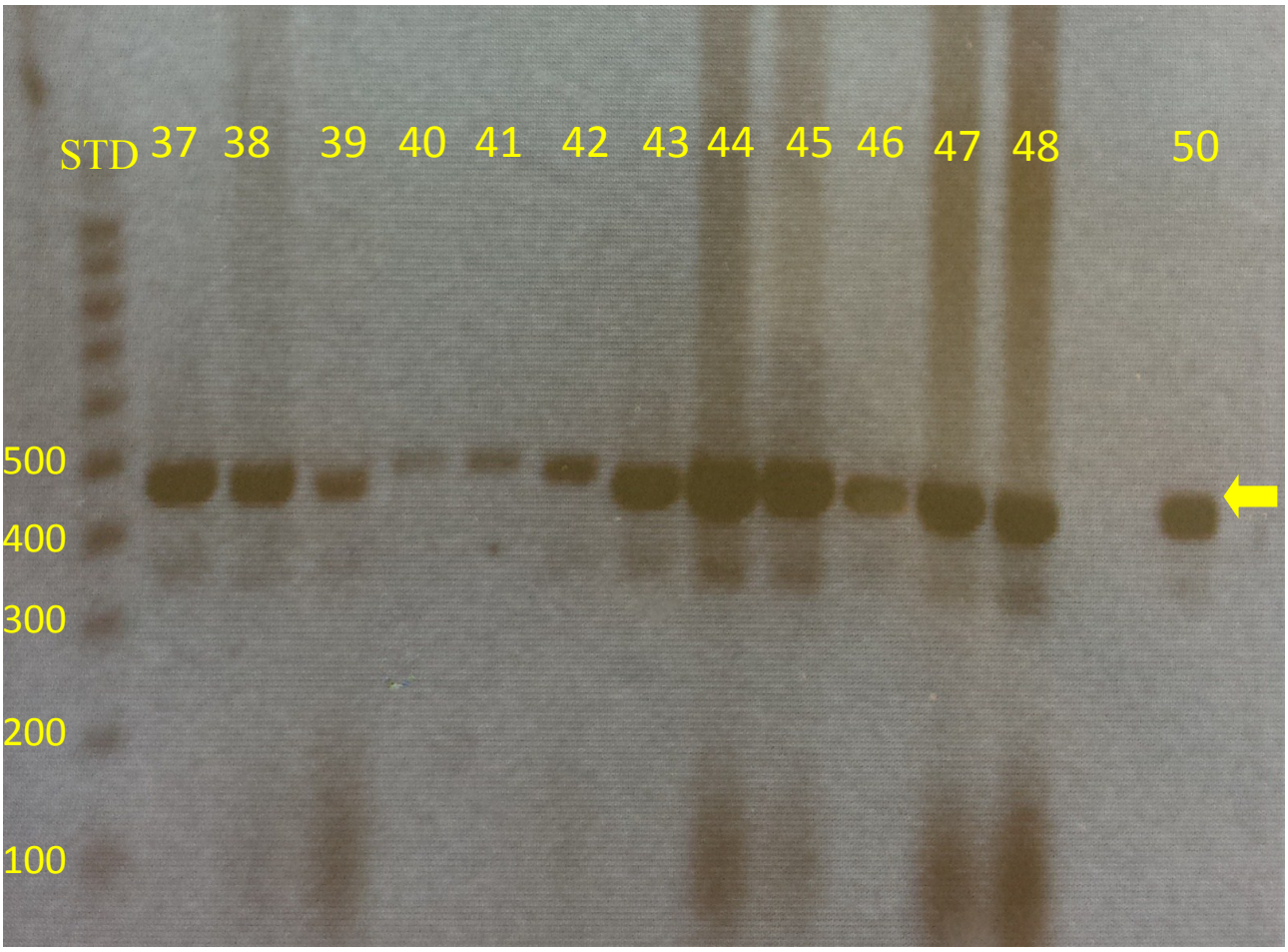

Figure 15. PCR amplification products of Eastern reservoir bacterial isolates #37-48 and FM1423 bridge site isolate #50. Putative *bphA1* amplicons indicated by a yellow arrow

CHAPTER V

DISCUSSION

Despite the large number of facultative bacteria isolated from the Eastern reservoir, statistical analysis concluded this number was not statistically significant to other locations in the reservoir, a result likely due to the large variance in these samples. The p-values of t-tests comparing the number of facultative bacteria in the Western reservoir to the FM1423 bridge site ($p=0.0524$) and East reservoir ($p=0.0594$) suggest increasing sample size, in order to reduce variance, could show a statistically significant difference between the eastern reservoir and other sites. The Outlet siphon did contain statistically significant higher numbers of facultative bacteria compared to the Western reservoir and FM4123 bridge sites but this data is very limited in its meaning. It has been estimated that less than 1% of bacterial species present in soil samples are able to be cultivated using traditional culture methods (Amann *et al*., 1995). Thus, the results for enumeration of total facultative bacteria represent a very small fraction of the actual facultative bacteria in sediment from the Donna Irrigation System.

Bacteria able to metabolize biphenyl are a subset of the facultative bacteria present in the reservoir sediments and their enumeration suggested the percentage of total bacteria they represent varied greatly from site to site. In, general, the number of biphenyl utilizing bacteria was approximately 25% of the facultative bacteria population. The Western reservoir, FM1423 bridge site and Outlet siphon had a relatively low percentage of bacteria able to use biphenyl, out of the total facultative bacterial population (1.93%, 13.77% and 18.28%, respectively) compared

the Eastern reservoir, which had a surprisingly high percentage (75.75%) of bacteria, out of total facultative bacterial population, able to utilize the chemical. There are limitations to these numbers, though. Bacteria able to utilize biphenyl as a carbon source are metabolically versatile organisms and presumably have the have the ability to grow well on LB media as well. These conditions select against a large number of bacteria; consequently, biphenyl utilizing bacteria are likely to represent a much lower percentage of facultative bacteria in actuality than is suggested by our culture-based methodology.

Screening of bacteria grown from sediment samples for their ability to cleave dibenzofuran yielded interesting results. Data from 2005-2006 fish samplings by the DSHS suggested the greatest PCB contamination was, in order from highest to lowest, Outlet siphon site > FM1423 site > Western reservoir > Eastern reservoir (ATSDR, 2007). These results were consistent with 1999-2000 suspended sediment samplings by the USGS which found PCB concentrations, in order from highest to lowest, to be Outlet > FM1423 bridge > Western reservoir > Eastern reservoir (EPA, 2007). Due to these findings, it was originally hypothesized the greatest number of dibenzofuran-cleaving isolates would be isolated from the outlet siphon, the site with the greatest PCB contamination, the least number of dibenzofuran-cleaving isolates would be isolated from the Eastern reservoir, the site of least PCB contamination. Testing for dibenzofuran-cleavage yielded opposite results, however, and the greatest numbers of dibenzofuran-cleaving bacteria were isolated from the Eastern reservoir (26) and West reservoir (16). Despite repeated sediment samplings of the FM1423 bridge and Outlet siphon sites, and testing of over 50 bacterial isolates for dibenzofuran cleavage from each location, only one (1) isolate capable of cleaving dibenzofuran was able to be grown from the FM1423 bridge site.

There are three possible hypotheses explaining these findings. The first hypothesis is higher PCB concentrations in the Outlet siphon and FM1423 bridge sites were sufficiently high to be too toxic for bacteria to degrade these chemicals. A second hypothesis is bacteria at these two sites had variants of *bph* genes which allow for growth on biphenyl but not cleavage of dibenzofuran. The third hypothesis is that bacteria need contaminated sediments to be relatively stable and stagnant in order for them to evolve mechanisms to degrade the chemicals. These hypotheses have specific weaknesses, however. For example, there is strong contradictory evidence for the hypothesis that PCB concentration at the FM1423 and outlet siphon was too high, and consequently toxic, to bacteria. Field and Sierra-Alvarez (2008) grew bacteria in laboratory conditions at a concentration of 700 ppm, an amount approximately 13,000 times higher than the 0.053 ppm of PCBs found in suspended sediment at the outlet siphon site of the DIS (EPA, 2007).

The second hypothesis has some support. Stemmer (1994) reported major differences in the ability to form the yellow *meta*-product from many biphenyl-related compounds, including dibenzofuran, in bacteria with variants of the *bphA1* gene. This observation was supported by our results as we isolated these microorganisms. Bacteria able to grow on minimal media supplemented with biphenyl, but not cleave dibenzofuran-supplemented media to produce a yellowing of the media, were isolated from multiple sediment samples from the FM1423 bridge and Outlet siphon. These results were particularly confounding since isolates able to cleave dibenzofuran were so readily isolated from the Eastern reservoir that sample size had to be limited to 26 isolates. In the western reservoir, only 1 isolate out of 30 tested for dibenzofuran cleavage was able to grow on biphenyl but not turn the media yellow when exposed to dibenzofuran. This resulted in a pattern of increasing ability to cleave dibenzofuran as one

traveled away from the source of contamination near the outlet siphon. It is a possibility that most bacteria from the FM1423 and Outlet siphon sites contained variants of *bphA1*, which are not capable of producing a yellow coloration in media, but it is not known why these bacteria would be localized to the FM1423 and Outlet siphon sites given the DIS is a connected system.

A factor which may be of importance is that the overwhelming majority of dibenzofuran cleaving isolates from the reservoirs (39 of 42 isolates) did not come from the main body of the reservoirs but from canal sites adjacent to the reservoirs (isolates identified with a "C" in their name in tables). Upon further investigation, these canals were actually drainage ditches of the reservoirs and sediment taken from these sites was clearly different in density and composition compared to sediment taken from other sites. The difference in sediment composition might be largely explained by stagnant conditions in these drainage ditches. These conditions are not seen anywhere else in the DIS, which has a relatively fast flow rate keeping the waters murky with suspended sediment. The stagnant conditions of these ditches, which slowly build up bottom sediment from contaminated suspended sediments, might be a factor in the ability of organisms to evolve mechanisms to metabolize biphenyl-related compounds. Given these results, it is clear this project isolated a fraction of potential PCB degraders in the DIS and future research will be needed to further investigate this location.

Regardless of the reason as to why not all bacteria cleaved dibenzofuran, the isolation of 43 bacterial isolates able to cleave the chemical was the first breakthrough in this project. The ability to cleave dibenzofuran into its yellow *meta*- product is encoded by the *bphC* gene, the third gene in the same metabolic pathway used to break down biphenyl and PCBs (Wesche *et al*., 2005). The isolation of bacteria able to produce a yellowing of minimal media supplemented

with dibenzofuran, and thus containing the *bphC* gene, was the first indication that potential PCB degraders were located in the reservoir.

Simple characterization tests of isolates gave an initial and rudimentary indication of the diversity of the potential PCB degraders in sediment samples. While two morphologies, coccobacillus and bacillus, predominated, there were clear differences seen in the shape of the bacilli isolates in negative stains. This initially suggested at least three species of potential PCB degraders were present in the isolates capable of degrading dibenzofuran: a coccobacillus, a long bacillus and a short bacillus. This was confirmed by scanning electron micrographs which showed Gram-negative isolates contained two distinct types of bacillus: a longer, slender bacillus and a more pleomorphic, thicker bacillus. Gram-staining results indicated that all bacilli were Gram-negative and all coccobacillus were Gram-positive. This indicated that, at the very least, two different species of bacteria in the reservoir were potential PCB degraders.

The more complex characterization tests, such as API® 20E strips, indicated greater diversity was present in our samples than negative staining and Gram-staining first indicated. Isolates #33 and #40, for example, were unique in their API® result pattern and contained the enzyme arginine dehydrolase and were able to ferment glucose, respectively, indicating these two organisms were of different strains compared to the other Gram-negative bacteria. The API® system phenotypic tree placed all Gram-positive isolates in the same branch, suggesting these were highly related, possibly to the species level. Not all Gram-negative isolates were placed together on the phenotypic tree, however, suggesting isolates #1 and 28, #36, #2, #40 and #33 could be different strains or species of Gram-negative bacteria compared to the other Gram-negative, which grouped together into large groups of 10 bacteria or more.

The BIOLOG EcoPlate™ system gave a variety of useful information for the bacterial isolates. Compared to Gram-Negative bacteria, which showed varying abilities to metabolize the 31 carbon sources, most Gram-positive bacteria had the metabolic capacity to use most of the carbon sources in the assay. The exception to this was Gram-positive isolate #16, which metabolized the least number of sources of all the isolates. This indicates this organism is less related to the other Gram-positive bacteria than previously believed, and might be a different strain or species within the same genus. Gram-negative bacteria had much more variety in their ability to metabolize the different carbon sources of the assay, though some isolates metabolized the exact same number of sources. This further indicated a variety of strains of bacteria in the DIS were potential PCB degraders. This data alone does not rule out the possibility that these isolates belong to the same species or genera, however.

The best carbon sources used by bacterial isolates yielded interesting results. Overall, microorganisms were best able to metabolize relatively common carbon sources such as: pyruvic acid methyl ester (11 of 43 isolates) and D-galacturonic acid (21 of 43 isolates). These two compounds are the methylated ester of pyruvate and the oxidized state of D-galactose, which are important sources of energy for cells via aerobic respiration and an important carbon source for bacteria which live on decaying plant material, respectively (Martens-Uzunova and Schaap, 2008). Several bacteria were able to use the amino acids L-asparagine (2 of 42 isolates) and L-serine (2 of 42 isolates) as their best metabolized source. Though not unusual in the sense that these amino acids are commonly found in nature, it is of interest that they were the best utilized carbon source. The most surprising carbon utilization result was the use of Tween 40 by Gram-negative isolate #1 and Tween 80 by 6 of 7 Gram-positive isolates as the best metabolized carbon sources. The last Gram-positive bacterium, isolate #3, used Tween 80 as the best carbon

sources during the first 48 hours but used L-asparagine as the best carbon source at 72 hours. Tween 40 and Tween 80 are synthetic compounds used as emulsifiers and it is not known why these bacterial isolates would use these compounds so effectively relative to other natural sources of energy. As a whole, the carbon sources best metabolized by bacterial isolates further suggest that a variety of bacterial species or strains of potential PCB degraders are present in the sediments of the DIS.

The Total activity of bacterial isolates was weakly positively correlated with total number of carbon sources able to be metabolized. Bacteria generally follow one of two metabolic strategies: 1) they are generalists able to metabolize a variety of carbon sources relatively poorly or 2) they are specialists able to metabolize a few carbon sources relatively well. The weak positive correlation obtained from our data suggests bacterial isolates are overall generalists, a finding that is supported by their ability to grow on minimal media supplemented with biphenyl while being able to grow on general bacteriological media (LB) at the same time.

The phenotypic tree created from BIOLOG Ecoplate™ data provided further support for the hypothesis that the DIS contains a variety of PCB degrading bacteria. Unlike the API® phenotypic tree, which grouped the bacterial isolates into 7 distinct groups, the Ecoplate™ phentotypic tree placed the bacteria into a large number of groups with varying levels of phenotypic similarity, indicating bacterial isolates are much diverse than other characterization tests initially indicated. Gram-negative groupings suggest upwards of 28 bacterial strains are present in these bacteria. The first major branching-off at the tree (54% phenotypic similarity) divided the Gram-negative bacteria into two major groups and strongly suggests these two groups contain different species of Gram-negative bacteria. A second major branching at 56% phenotypic similarity suggests isolates #2 is a different species from the other Gram-negative it

was grouped with. Gram-positive bacterial isolates yielded equally surprising resulted. All Gram-positive bacteria had been grouped together in the API® system but were divided into 5 groups in the BIOLOG system consisting of isolates #16, 17, 43 and 46 alone and isolates #3, 7 and 50 grouped together. Isolate #16 contained a 56% phenotypic similarity to other Gram-positive bacteria, strongly suggesting this isolate belongs to a different species. The most surprising result, however, was the close grouping of Gram-positive bacteria with Gram-negative bacteria. Gram-positive isolates #7 and #50 were approximately 96% phenotypically similar to Gram-negative isolate #33. Gram-positive isolates #43 and #46 contained approximately 82% phenotypic similarity to several Gram-negative isolates. This finding suggests bacteria from the reservoir have adapted to similar conditions and may be undergoing horizontal gene transfer. The BIOLOG Ecoplate™ phenotypic tree, along with other characterization tests conducted on dibenzofuran cleaving isolates, provides a preponderance of evidence which indicates a variety of genera, species and strains of bacterial isolates with the potential to degrade PCB are present in the sediments of the Donna Irrigation System.

Of 15 possible primer pair combinations, only 3 met the established criteria for primer selection. The 4th primer pair chosen exceeded desired amplicon size but was kept in order to test the ability of the primers to amplify the *bphA1* gene. Primer pairs F6-R2 and F7-R2 failed to amplify the gene from the genomic DNA tested while primer pair F7-R1 amplified the gene in 3 of 5 DNA samples and the amplicons were of the correct hypothetical size of ~1,000 bp. There are multiple reasons as to why these three primer pairs failed to amplify the gene in the genomic DNA of bacterial isolates. The first is that our bacteria did not contain a similar enough *bphA1* sequence to that of the primers in order for the primers to bind to the DNA. While primers were designed from 18 different bacterial isolates, which included both Gram-positive and Gram-

negative bacteria, and intended to be very general, the *bphA1* gene is known to vary by as many as 20 amino acids. The second possible reason for the failure of these primers is that primer annealing temperature was not optimal for their PCR reaction. While this can be optimized through trial and error, this was not done since a 4th primer pair, F6-R5 still required testing.

Primer pair F6-R5 successfully amplified the *bphA1* gene in all 5 initial DNA samples and annealing temperature optimization for the other primers was not attempted. Subsequent PCR reactions with primer pair F6-R5 successfully amplified the *bphA1* gene from 42 of 43 genomic DNA samples extracted from the dibenzofuran cleaving isolates with the exception of Gram-negative isolate #2. Genomic DNA from two *Escherichia coli* cultures was used as a negative control also failed to amplify the gene. It is not known why the final PCR reaction, which produced the pictures used in this manuscript, did not amplify the *bphA1* gene in sample #2, as two preliminary PCR reactions amplified the gene for this bacterial isolate. It is possible the extracted genomic DNA from this sample had degraded by the time the final PCR reaction was conducted, preventing amplification of the gene. In the 42 samples which amplified the *bphA1* gene, the amplicon size was verified using agarose gel electrophoresis. When compared to a standard DNA marker, the amplicon size matched the hypothesized amplicon size (480 bp) for this primer pair. The presence of the *bphA1*, the gene required to initialize aerobic degradation of PCBs, in the genomic DNA of dibenzofuran cleaving isolates provided verification that these bacteria are PCB degraders.

Our research showed that bacteria which had evolved the ability to degrade PCBs were present in the Donna Irrigation System and contained two of the required genes for PCB degradation, *bphA1* and *bphC*. There were limitations to this study, such as the limited scope of site sampling, however. The reservoirs and canals of the DIS cover a huge area and sampling

was limited to the southern shore of the reservoirs and the shores of the main irrigation canal due to lack of a boat and/or access to the area. Better sampling methodology, such as use of a boat to sample the center of the reservoirs and main irrigation canal might yield greater variety of aerobic PCB degrading isolates. This study focused exclusively on bacteria able to grow aerobically and neglected anaerobic bacteria, which also play an important role in PCB degradation. Future research could focus on isolating and examining the anaerobic bacteria which are undoubtedly catalyzing anaerobic dechlorination of PCBs in the reservoir. This study also focused exclusively on isolates able to cleave dibenzofuran and produce a yellow *meta*-product in their growth media, an ability encoded by the *bph* genes. Bacteria with *bph* gene variants may not produce this yellow *meta*- product from dibenzofuran, however, even if they are capable of metabolizing biphenyl and PCBs. Thus, this study isolated only a fraction of potential PCB degraders in the Donna Irrigation System and future research should examine bacteria which do not cleave dibenzofuran for the presence of *bph* genes. Lastly, while characterization tests strongly suggest multiple genera, species and strains of bacteria are aerobically degrading PCBs in the DIS, 16S rDNA sequencing will be required to determine the identity of these isolates. This work has been started and will be published in the future. If a unique isolate is identified, whole genome sequencing could be conducted to provide detailed information on this novel PCB degrading bacterium which could hopefully help remediate the problem that PCB contamination has created worldwide.

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APPENDIX

APPENDIX

Congener- Defined as the IUPAC as one of two or more substances related to each other by origin, structure of function. When applied to PCBs, each congener has either a differential amount of chlorines attached or the positions of its chlorines varies to that of other congeners.

Aroclor 1254- The chemical mixture contaminating the Donna Irrigation System. Aroclor 1254 is a combination of 20-60 congeners of PCBs with an average weight percentage of chlorine of 54%.

Chloracne- The most common symptom of exposure to PCBs. It resembles outbreaks of acne on the face, neck, torso and genitals.

Yusho- A Japanese word meaning "oil disease". This word is now widely used to describe the October, 1968 mass poisoning of residents of Kyushu, Japan who consumed PCB contaminated rice oil.

TSCA- Toxic Substance Control Act of 1976 which led to the banning of PCB manufacturing, processing and distribution by 1978.

Bioremediation- The use of microorganisms to completely breakdown a hazardous substance, lower its bioavailability or reduce its toxicity.

Colony Forming Units (CFUs)- An estimation of how many viable cells are present in a sample. A colony is defined as a single bacterium or group of bacteria which gave rise to a visible cluster of bacteria. Colonies are presumed to come from a single ancestor and to be genetically identical.

LB media- Abbreviation for "lysogeny broth medium". This medium a tryptone, yeast extract and NaCl. Originally used as a growth medium for recombinant strains of *Escherichia coli*, this media is also routinely used as a general growth media for bacteria.

MM- Abbreviation for "minimal media". This medium contains essential elements required for bacterial growth but lacks a carbon source. Theoretically, no organism should be able to grow on this medium unless an outside carbon source is added.

Dibenzofuran- A chemical with a structure very similar to PCBs. The *bphC* gene, also responsible for biphenyl and PCB degradation, can cleave the rings of this chemical and cause a yellow coloration to form in minimal media

Bacterial morphology- The shape a particular bacterium exhibits. Bacillus bacteria are shaped like elongated rods while coccobacillus bacteria are shaped as slightly elongated circles.

Gram-positive bacteria- Bacteria which have a thick layer of peptidoglycan facing the environment and only one phospholipid bilayer as part of their cell wall. This cell wall structure retains the crystal violet dye of the Gram-stain procedure after decoloration with ethanol and leaves the bacteria stained purple.

Gram-negative bacteria- Bacteria which have a thin layer of peptidoglycan in between two phospholipid bilayers as part of their cell wall. This cell wall structure does not retain the crystal violet dye after exposure with ethanol and will subsequently stain red with other stains.

PCR- Abbreviation for "polymerase chain reaction". This procedure uses known primers of DNA to amplify selected regions of DNA by several orders of magnitude. This procedure is extremely useful when only a small amount of DNA is present by magnifying its amount.

16s rDNA- Gene which encodes for the small subunit of the bacterial ribosome. This gene is widely used to identify and differentiate bacteria based on its DNA sequence.

BIOGRAPHICAL SKETCH

Sergio Cepeda was born in Rio Bravo, Tamaulipas, Mexico on August 18, 1987. At the age of 6, his family moved to San Juan, TX to give him and his two brothers the opportunity to learn English. At the age of 8, his family moved to McAllen, TX where he finished his grade school education at James Nikki Rowe High School as a member of the National Honor Society.

In the fall of 2006, he started his Bachelor of Science degree in pre-medicine at the University of Texas-Pan American. After his freshman year, he transferred to the University of Texas-Austin to study microbiology. He transferred back to the University of Texas-Pan American his final year, where he graduated Magna Cum Laude with a Bachelor's of Science degree in Biology in December 2010.

He began his Master of Science in Biology degree in spring 2011 under the supervision of Dr. Luis Materon, Dr. Kristine Lowe and Dr. Michael Persans. Throughout his studies, he worked part-time in family businesses and also served as a graduate laboratory teaching assistant for the General Microbiology and Research Methods courses. He was active in research since his undergraduate degree and helped in several master's theses and co-authored a scientific publication during his graduate school years. In July 2014, he completed his Master's degree and now resides at 2912 Kerria Avenue, McAllen, TX.