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Examining Plasmid Transfer of pJP4 in a Mixed Community Biofilm Formed in a Microcosm Simulating a Porous Aquifer

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**EXAMINING PLASMID TRANSFER OF pJP4 IN A MIXED COMMUNITY
BIOFILM FORMED IN A MICROCOSM SIMULATING A POROUS AQUIFER**

by

Floriana Postelnik

Bachelor of Science, Honors Biology
McMaster University
2010

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presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the program

of Molecular Science

Toronto, Ontario, Canada, 2012

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EXAMINING PLASMID TRANSFER OF pJP4 IN A MIXED COMMUNITY BIOFILM FORMED IN A MICROCOSM SIMULATING A POROUS AQUIFER

Floriana Postelnik

Master of Science, Molecular Science, Ryerson University, 2012

ABSTRACT

In this work, a lab-scale microcosm was designed and used to simulate a porous groundwater aquifer. A minimal media acted as the non-selective pressure for plasmid transfer, while a minimal media with gentamicin acted as the selective pressure. PCR, plate counts and confocal laser scanning microscopy were used to monitor transconjugant and donor persistence in the microcosm. The donor was identified through *gfp* on the chromosome and red fluorescence was used to identify transconjugants through *dsRed* on pJP4. The donor persisted for 73 h in a minimal media environment when 1×10^9 cells were inoculated into the microcosm. Biofilm thickness increased in response to gentamicin addition, potentially increasing interactions between donor and recipient for increased rate of plasmid transfer. A higher number of transconjugants was identified when selective pressure was used due to increased transfer of pJP4. In conclusion, an inhibitory concentration of gentamicin provided sufficient selective pressure for pJP4 transfer.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CFU	Colony Forming Unit
CLSM	Confocal Laser Scanning Microscope
DGGE	Denaturing Gradient Gel Electrophoresis
EPS	Extracellular Polymeric Substances
G	Gentamicin
GFP	Green Fluorescence Protein
HGT	Horizontal Gene Transfer
Inc	Incompatibility
IPTG	Isopropyl-B-D-1-thiogalactopyranoside
K	Kanamycin
LB	Luria-Bertani
NC	Negative Control
OD	Optical Density
PCR	Polymerase Chain Reaction
PVC	Polyvinyl Chloride
RPM	Revolution Per Minute
RT	Room Temperature
TSB	Tryptic Soy Broth

CHAPTER 1: INTRODUCTION

1.1 PROJECT SIGNIFICANCE AND MOTIVATION

Groundwater is an important contributor of fresh water all over the world, especially in Canada. Almost 10 million Canadians rely on groundwater for their water supply, which is around 30% of the total population (Rutherford, 2004). Moreover, groundwater is a crucial part of the hydrological cycle that is used to sustain surface waters such as lakes (Rutherford, 2004). As important as groundwater is to Canadians, a lot of the time it is polluted by human activity. Groundwater contains numerous contaminants from agricultural and industrial processes, which, as an important supply of drinking water for Canadians, poses a great health risk. Another issue with groundwater contamination is groundwater flowing into other bodies of water, which can produce toxic effects in biological organisms as well as undergo bioaccumulation, which is the accumulation of substances in organisms. Bioaccumulation of toxic substances in organisms such as fish can pose major health concerns when they are consumed by humans.

Moreover, a majority of contaminants are very persistent and will remain in the environment unless they are removed or degraded. Microbial degradation of contaminants has been showing great promise in eradicating contaminants from ecological sites. One way of acquiring degradative capabilities is through horizontal gene transfer (HGT). HGT of catabolic genes has been detected in various environments, such as soils and wastewater (Singh *et al.* 2006). HGT of catabolic genes represents an important part of acquiring new catabolic properties that help indigenous bacteria degrade existing contaminants (Trefault *et al.* 2004).

Even though there have been studies on horizontal gene transfer in porous media, there is a lack of research on plasmid transfer in biofilms in subsurface environments and plasmid transfer without any selective pressure. With the construction of a microcosm reflecting a

porous aquifer, the distribution of plasmid-donating microorganisms among the recipient community can be investigated and plasmid transfer can be examined to divulge the extent of catabolic gene transfer below ground in a microbial biofilm, both with and without selective pressures. In this project, the antibiotic gentamicin was used as a selective pressure and the plasmid under investigation was pJP4, which encodes genes for 2,4-dichlorophenoxyacetic acid (2,4-D) degradation. The long-term goals of the research conducted would be to examine the feasibility of groundwater remediation using bioaugmentation strategies.

1.2. OBJECTIVES AND THESIS OUTLINE

The overall objective of this project was to examine the transfer of the plasmid pJP4 to a mixed microbial community formed in a microcosm simulating a porous aquifer (Chapter 2). The first objective was to design and construct a lab-scale microcosm simulating a porous aquifer. The second objective was to analyze donor persistence in the microcosm. The third objective was to examine plasmid transfer under selective (antibiotics) and non-selective conditions to determine how each condition affects pJP4 transfer to a recipient community. The presence of antibiotics has been found to increase HGT. Thereby, antibiotics were used as the selective pressure instead of 2,4-D due to the presence of the *tfdB* gene in the soil microbial community, where 2,4-D may not exert sufficient selective pressure. The last objective was to measure the biofilm thickness on glass bead taken from both selective and non-selective pressure experiments to elucidate changes in biofilm thickness under different environmental conditions and their effect on plasmid transfer.

In order to complete these objectives, mating experiments were initially conducted to find a suitable recipient community. A lab-scale microcosm was designed and constructed. Plasmid transfer was then examined in a microcosm under both selective and non-selective environments to examine donor interaction with the recipient community and the extent of catabolic plasmid transfer in a mixed community biofilm. After inoculation of the microcosm with microorganisms, effluent samples were collected at regular intervals as well as glass bead samples, which were collected from two individual ports extending from the microcosm. Effluent samples were plated to get the total heterotrophic plate count. DNA from the effluent samples was extracted and PCR for *gfp* was used to verify donor presence. A dissection scope with green and red fluorescence filters was also used to count donor colonies and visualize

transconjugant cells, respectively. Lastly, samples of glass bead were examined for biofilm formation under CLSM using a nucleic acid stain and the biofilm thickness measured using ImageJ. CLSM was also used to visualize donor and transconjugants cells using green and red fluorescence, respectively.

It was expected that the use of an antibiotic as the selective pressure would reduce the microbial community in the effluent to species that are resistant to gentamicin. Moreover, it was also expected that more transconjugants would be identified when using selective pressure.

1.3. BACKGROUND

1.3.1. Groundwater

Groundwater is not synonymous with subsurface water. In order to define groundwater, one must first make reference to the two major vertical zones carrying water beneath the ground. The two major zones are denoted as the unsaturated (vadose zone) and the saturated zones (Bear and Cheng, 2010 and Younger, 2007). Groundwater refers to water found in the saturated zone, as shown in Figure 1.1, which comes from surface waters filtering downward through the unsaturated zone (Bear and Cheng, 2010). As can be further seen in Figure 1.1, the water that moves down through the ground becomes saturated above a deposit of bedrock and is roofed by a layer called the phreatic surface, or the water table (Bear and Cheng, 2010). The water table is not a fixed foundation; it represents an invisible line in the earth where, just below, water will move up in a well (Younger, 2006). Groundwater occupies the free space, called pores, between soil grains or rocks (Younger, 2006).

Groundwater eventually returns to the surface as it moves into rivers and lakes, among other bodies of water (Bear and Cheng, 2010). The unsaturated zone encompasses both water and air, making this area unsaturated with water. The unsaturated zone can be subdivided again into three more areas, the soil water zone, the intermediate zone and the capillary zone (Bear and Cheng, 2010). The intermediate zone is the area where water moves down during infiltration and is situated between the soil water zone and the capillary fringe (Bear and Cheng, 2010). The capillary fringe is the area right above the water table and, even though it is part of the unsaturated zone, is actually, most of the time, fully saturated with water (Younger, 2006). The capillary fringe is part of the unsaturated zone because it tends to either provide or withhold water from the saturated zone (Younger, 2006).

Figure 1.1 The unsaturated and saturated zones in the ground. The saturated zone contains the groundwater, above which is the water table or phreatic surface; above the water table is the capillary fringe. The impervious bedrock is seen beneath the saturated zone (Adapted from Bear and Cheng, 2010).

1.3.2. Aquifers

An aquifer is an important formation related to groundwater, which stores and transmits groundwater (Bear and Cheng, 2010). An aquitard is also a saturated formation, but it does not allow free movement of water (Younger, 2006). There are two types of aquifers, unconfined and confined aquifers. An unconfined aquifer is defined as an aquifer that is bounded by the water table above and an impervious layer below it, while a confined aquifer is defined as an aquifer that is bounded by an aquitard above it and an impervious layer below it (Bear and Cheng, 2010 and Younger, 2006).

1.3.3. Soil

Soil is defined as granular particles that come in a variety of shapes and sizes (Bear and Cheng, 2010). Most soil comes from weathering of the Earth's crust (Powrie, 1997). Soils consist of minerals, which can be made up of a variety of different elements such as oxygen and aluminum (Powrie, 1997). In general, there are spaces between soil particles that are filled up by either water or air particles (Powrie, 1997). Soil particles are classified according to their size, with clay having a small particle size, < 0.002 mm, and sand being significantly larger in size,

with a range in diameter from 0.05 mm – 2mm (Powrie, 1997 and Paul, 2007). Silt particle size comes between clay and sand, with a range in diameter from 0.002 – 0.05 mm (Paul, 2007). The granules that make up gravel are even larger than sand particles, with a very variable range of sizes (Paul, 2007). In sand or gravel, water tends to move quite freely as opposed to clay particles, since clay particles are able to fill up more space, leaving little room for any movement (Powrie, 1997).

1.3.3.1. Antibiotic Resistance in Soil and Water

One gram of soil can contain anywhere from 10^7 - 10^9 microorganisms, with a high diversity of species (Wright, 2010). The microorganisms best known for producing antibiotics are the actinomycetes (D'Costa *et al.* 2006). D'Costa *et al.* 2006 sampled a variety of soil samples, where a number of the identified bacteria were the actinomycetes *Streptomyces*. They tested 480 strains for their resistance to natural antibiotics, semi-synthetic as well as synthetic antibiotics. The study found that all the isolated strains were multi-resistant to around 7-8 antibiotics. Resistance was found for almost all of the 21 antibiotics tested, such as tetracycline, erythromycin, and chloramphenicol, among others (D'Costa *et al.* 2006). Samples from the Michigan and Ohio wastewater treatment plants and drinking water also contained strains that were resistant to antibiotics. Resistance was found to amoxicillin and gentamicin, as well as 5 other antibiotics (Wright, 2010). Resistance, as well as multiple-resistances to a variety of antibiotics, can come about through environmental contamination with antibiotics.

1.3.3.1.1. Environmental contamination with antibiotics

Antibiotics are highly prevalent in the environment due to their extensive use to treat human and animal infection. Antibiotics are excreted, mostly unchanged, from human or animal bodies and end up either in wastewater treatments plants or else are directly excreted into the environment (Martinez, 2009). Antibiotics are also frequently used in agriculture, where they are sprayed directly onto infected plants. As well as topical applications of antibiotics, antibiotics are also produced in the environment by microorganisms, where HGT allows the propagation of these antibiotic resistance genes throughout the microbial community (Martinez, 2009).

1.3.3.1.2. Aminoglycosides

A group of antibiotics called aminoglycosides, which include gentamicin and kanamycin, are produced by the actinomycetes *Streptomyces* or *Micromonospora*. This line of antibiotics inhibits protein synthesis (Jana and Deb, 2006). Aminoglycosides interact with the 30S ribosomal unit, disturbing the proofreading process and halting protein synthesis. The secondary effects caused by this disturbance include membrane damage and the consequent alteration of the ionic concentration within the cell (Jana and Deb, 2006). Resistance to this class of antibiotics can come about in one of three ways, a reduction in the intracellular levels via either a membrane that is no longer permeable to the drug or an efflux pump that actively pumps the drug out, a mutation in the region the antibiotic originally bound to or an inactivation of the antibiotic (Jana and Deb, 2006).

1.3.4. Groundwater and Soil Contamination

As significant as groundwater is as a source of drinking water for Canadians, since it is not a visible part of the landscape, often, its misuse goes unnoticed. Groundwater is often contaminated by pollutants that are released by humans. These contaminants include arsenic, BTEX (benzene, toluene, ethylbenzene and xylene), pathogens, , pesticides and herbicides, hydrophobic organic contaminants or mixed contaminants from industrial or agricultural practices, which lead to concerns about groundwater quality (Wang and Mulligan, 2006 and Farhadian *et al.* 2008 and Santamaria and Toranzos, 2003).

1.3.4.1. Arsenic

One major groundwater contaminant is arsenic. Arsenic is a naturally occurring element, found in certain rocks, that is also a known toxin for humans (Wang and Mulligan, 2006). As well as being released by erosion of rock containing arsenic, it is also released by human use (Wang and Mulligan, 2006). Several parts of the world, including Canada and China, have reported high amounts of arsenic in their groundwater, going above the limit of 10 µg/L, where toxic effects can be seen above 1.2 mg /L or 0.2 mg /L in children (Wang and Mulligan, 2006). The symptoms of acute toxicity include stomach cramps and muscle pains (Wang and Mulligan, 2006). Exposure to lower concentrations of arsenic can lead to chronic effects, such as cancer (Wang and Mulligan, 2006).

1.3.4.2. BTEX

BTEX are volatile aromatic hydrocarbons that are found in petroleum, which are released into the environment from oil industries and leaks from petroleum tanks (Farhadian *et al.* 2008

and Jindrova *et al.* 2002). Since BTEX compounds are generally very water-insoluble, they are not easily remediated when they contaminate groundwater resources (Jindrova *et al.* 2002).

BTEX present with acute and chronic toxicity and are generally very persistent (Jindrova *et al.* 2002 and Nadim *et al.* 2000). These compounds also diffuse rapidly in groundwater and are highly toxic (Jindrova *et al.* 2002).

1.3.4.3. Pathogens

Another, non-chemical, contaminant of groundwater is pathogens. Major quantities of enteric pathogens can be released into the environment through the disposal of biosolids. Landfilling of biosolids is believed to have caused an increase in enteric infections within the human population as a large number of pathogens are introduced into the soil (Santamaria and Toranzos, 2003). Another method of introducing pathogens into the soil is the use of manure in agriculture (Santamaria and Toranzos, 2003). Leaching of pathogens from the original site of application may cause groundwater contamination, since pathogens are able to move swiftly through saturated soils (Santamaria and Toranzos, 2003). Enteroviruses have been isolated from river samples in Quebec and groundwater contaminated with pathogens has been linked to infections in both the United States and the United Kingdom (Santamaria and Toranzos, 2003). Food products grown in contaminated soils may interact with the pathogens as they come into contact with the ground, and, if the products are unpasteurized, can also lead to human infections after ingestion (Santamaria and Toranzos, 2003).

1.3.4.4. Pesticides and Herbicides

Another major class of contaminants of groundwater is pesticides, where there are more than 500 different preparations (Arias-Estevez *et al.* 2008). Though pesticide use has been increasing in the last few years, their toxicity against non-target organisms, including humans, has not been addressed. Less than 0.1% of pesticides actually makes it to the intended organism and instead winds up in the environment as a contaminant, many of which are highly persistent. The spread of pesticides into groundwater can be attributed to initial soil contamination (Arias-Estevez *et al.* 2008). The persistence of a pesticide in soil is defined by its interactions with soil particles; the 2, 4-D herbicide usually attaches weakly to soils and would therefore be preferentially transported to groundwater. Pesticide transport through soil is dependent on both pesticide and soil properties, where weak attachment to soil particles and the conditions during the application, i.e., rainfall, may be to blame for rapid transport through the unsaturated zone (Arias-Estevez *et al.* 2008).

1.3.4.4.1. 2, 4-dichlorophenoxyacetic acid

The herbicide 2, 4-D, one of the most frequently used herbicides in the world, is used as an herbicide for broadband leaves; it is commonly used in homes, agriculture and forests (Bukowska, 2005). Through its widespread use, 2,4-D has been found in a variety of terrestrial and aquatic systems, such as forests, groundwater and freshwater (Chinalia *et al.* 2007 and 2,4-D in drinking water, 2003). The half-life of 2,4-D can vary from several days to weeks in soil environments depending on soil conditions (2,4-D in drinking water, 2003). In its target organisms, which are broad-leafed weeds, it increases RNA, DNA and protein synthesis, causing uncontrollable growth, eventually destroying the plants (Chinalia *et al.* 2007). Its toxicity in

non-target organisms can range from teratogenicity to immunotoxicity, neurotoxicity and hepatotoxicity (Chinalia *et al.* 2007). 2,4-D effects the membrane structure by interacting with the membrane and weakening it, resulting in increased susceptibility to attack by free-radicals (Chinalia *et al.* 2007). These membrane interactions can lead to the disruption of the electron transport chain, potentially leading to apoptosis (Chinalia *et al.* 2007). Non-target organisms can admit 2,4-D into the system by absorption through the skin, inhalation or ingestion. Although 2,4-D is considered a toxin to higher organisms, several microorganisms use it as a carbon and energy source, such as *Pseudomonas* and *Chromobacter* (Bukowska, 2005).

1.3.5. Bioremediation

Bioremediation is defined as the use of biological agents to treat contaminants as opposed to other conventional method of treatment (Singh *et al.* 2006). Waste generation is an inevitable part of human life, with some generated waste being highly toxic (Juwarkar *et al.* 2010). There are various types of bioremediation techniques, one of which is called intrinsic or natural attenuation, which is the use of indigenous microorganisms to remove contaminants at the site without the addition of any exogenous materials that can support microbial growth (e.g. nutrients or electron acceptors). Intrinsic bioremediation, although it may be the least expensive method of bioremediation, may not always produce high rates of biodegradation, allowing contaminant spread (Juwarkar *et al.* 2010). Bioremediation is generally very advantageous, one reason being that it can often be done on site, reducing any additional costs of exporting the contaminated medium off site. The microorganisms capable of degrading the pollutant must be in contact with the contaminant and be working under the right environmental conditions for successful bioremediation to take place (Juwarkar *et al.* 2010).

1.3.5.1. In Situ Bioremediation

In situ bioremediation describes xenobiotic, which is any substance that is present at a higher concentration than is natural in the environment, treatment directly at the site. There are various mechanisms that can be employed when conducting in situ bioremediation, one of which is biostimulation. Biostimulation is the addition of materials, such as nutrients or electron acceptors, which is used as a stimulant for existing microbes to degrade contaminants present in the environment (Sinha *et al.* 2009). Another mechanism, bioventing, is the transport of oxygen to areas below the surface (Juwarkar *et al.* 2010). This treatment requires that organisms adept at degrading the contaminant be present and functioning under aerobic conditions. Air-sparging is very similar to bioventing, except that oxygen is pumped into the saturated zone to treat groundwater contamination. Natural attenuation is also an in situ approach, except that with natural attenuation, nothing is being added. However, with natural attenuation, meticulous monitoring is required to ensure that existing microorganisms are degrading the contaminant (Juwarkar *et al.* 2010).

Bioaugmentation is the physical addition of microorganisms capable of degrading the contaminant or genes encoding degradative enzymes; this method is also frequently used in ex situ bioremediation, which is the removal of a site for treatment (Juwarkar *et al.* 2010). Often, introducing microorganisms into the soil is inefficient due to the fact that they may be unable to adjust to their environment, both due to biotic and abiotic factors, with a loss of proliferation and incomplete degradation of the contaminant (Thompson *et al.* 2005). To minimize or eliminate this issue, a few solutions can be employed. One of the solutions to this problem is to pre-adapt the bacterial strain to the environmental conditions prior to its addition to the environment (Fantroussi and Agathos, 2005). Another solution is to introduce genetically engineered strains

that do not accrue any toxic products (Fantroussi and Agathos, 2005). The last solution is to introduce strains carrying relevant degradative genes, usually encoded on a plasmid vector, which can be transferred to the indigenous bacterial strains living in the contaminated zone (Fantroussi and Agathos, 2005).

1.3.5.1.1. Transfer of Catabolic Genes in Bioaugmentation

Often, bacteria carrying catabolic genes are unable to survive for a prolonged period of time when introduced into a new environment, and thereby disappear quickly (Fantroussi and Agathos, 2005 and Bathe *et al.* 2004). These bacteria, however, can transfer catabolic genes to other indigenous bacteria living in the area, giving them an ability to degrade the surrounding contaminant. The incompatibility group P1 catabolic plasmids are very promising mobile genetic elements, carrying catabolic genes, as they have a broad host range (Bathe *et al.* 2004). This technology of bioaugmentation via gene transfer is a feasible method that involves engineering an indigenous population to digest the surrounding contaminant, as opposed to introducing exogenous bacteria carrying chromosomally-encoded catabolic genes, which may have a low survivability in a new environment; they may not live long enough to degrade the entire contaminant.

1.3.5.1.2. Catabolic genes

Catabolic genes are found on a variety of mobile genetic elements: plasmids, chromosomes and transposable elements. Plasmids are self-replicating genetic materials that exist outside of the chromosome and encode genes that are termed non-essential, such as antibiotic resistance. Plasmids that encode enzymes capable of degrading environmental

contaminants are called degradative plasmids (Shintani *et al.* 2010). Plasmids are categorized based on their Inc (Incompatibility) groups, which is a naming system based on plasmid replication and partitioning machinery, where plasmids with the same Inc groups cannot coexist within one cell (Shintani *et al.* 2010). Usually genes that degrade human-made synthetic compound are encoded on plasmids that have a low copy number and a broad host-range (Jimenez *et al.* 2002). Catabolic genes are usually located at non-essential sites of the plasmid (Dennis, 2005). Catabolic genes can also be found in the genome, such as the genes involved in polycyclic aromatic hydrocarbon degradation in the bacterium *Mycobacterium vanbaalenii* (Kim *et al.* 2008). Transposable elements can also carry catabolic genes (Top and Springael, 2003). These elements can transfer between DNA sequences without needing any homologous sequences (Tan, 1999). Transposons have the ability to excise themselves out of the genome and insert themselves into the genome of the same host or the genome of a new host after conjugal transfer (Salyers *et al.* 2005). Catabolic genes can be used in bioremediation of contaminated sites, such as in ex situ bioremediation.

1.3.5.2. Ex Situ Bioremediation

Ex situ bioremediation is the removal of the contaminated medium (e.g. soil or groundwater) for treatment outside of the site. Sometimes ex situ bioremediation is not a viable mechanism, which may be due to physical barriers that would make excavation very difficult, as well as costly. Moreover, exposing the contaminant to the surface could potentially be hazardous, as it could become volatile, polluting the surrounding air as well as potentially spreading from the site to other areas (Juwarkar *et al.* 2010). While microorganisms or their

genes are utilized in both in situ or ex situ bioremediation approaches, immobilizing microbial cells onto a surface to increase cell density may improve the success of bioremediation.

1.3.6. Biofilms

Though bacteria are often described as single, isolated cells, in the natural environment, they often adhere to surfaces as aggregates in a slimy coat that they secrete (Singh *et al.* 2006), which can comprise almost 90% of the total dry mass (Flemming and Wingender, 2010). This aggregation of cells is termed a biofilm. A biofilm, which is mostly composed of water, consists of polypeptides, lipids, nucleic acids and sugars as well as matter from the extracellular environment, such as nutrients; these are called the extracellular polymeric substances (EPS). The EPS works to keep the community in close contact (Flemming and Wingender, 2010). A biofilm can be formed by a single bacterial strain or multiple strains of bacteria, along with eukaryotic organisms such as protozoa and fungi (Singh *et al.* 2006). The formation of a biofilm is a complex process that begins with surface attachment, changes in cell morphology and altered gene expression. After the cells attach to a surface, the biofilm becomes established and matures (Singh *et al.* 2006). Cell detachment, however, can be observed throughout all stages and does not depend on maturation (Bester *et al.* 2009). A biofilm is a great adaptive force, with unique properties that are not inherent to planktonic cells, such as the ability to withstand shear force and antibiotics, among other factors (Singh *et al.* 2006).

1.3.6.1. Biofilms in Bioremediation

Biofilms have a great potential in bioremediation strategies due to their resilient nature, which comes from the matrix that they are encased in. Moreover, the degradation of a

xenobiotic can be greatly accelerated in a biofilm as the cells can interact more closely and communicate more efficiently to enhance degradation (Singh *et al.* 2006). As well, the biofilm matrix offers protection for cells for better survivability than planktonic cells. There is also a benefit that comes from the interactions between various microbes in the biofilm (Singh *et al.* 2006). The EPS can also sorb, in varying degrees, to different organic and inorganic molecules (Carlson and Silverstein, 1998) giving the microorganisms in the biofilm better access for degradation of xenobiotics. Many enzymes are encased in the biofilm matrix and they function to degrade biopolymers for energy sources (Flemming and Wingender, 2010). Gene transfer occurs at a high frequency in biofilms thanks to their densely packed structure and collection of cells (Singh *et al.* 2006), whereby catabolic genes can be transferred from one cell to the next. These genes, as mentioned before, are very useful in contaminant degradation and thus equip bacterial cells with the ability to degrade a surrounding contaminant. Quite frequently, cells within the genus *Pseudomonas* possess a variety of catabolic genes.

1.3.7. *Pseudomonas putida*

The Pseudomonads belong to the gamma class of *Proteobacteria* and can be found in very diverse environments, such as soil and water (Loh and Cao, 2008). This group is widely applied in bioremediation studies due to its capability to degrade numerous xenobiotics (Loh and Cao, 2008). *Pseudomonas putida* is a strain that is frequently used in bioremediation studies. Not only do strains of *P. putida* have the capabilities of degrading the xenobiotics themselves, the relevant degradative plasmid harbored by *P. putida* can be transferred to microorganisms native to a specific environment contaminated with the xenobiotic (Niu *et al.* 2009).

1.3.7.1. pJP4 and *Pseudomonas putida* SM1443

The strain *Pseudomonas putida* SM1443 carries a *lacI^q* gene and, using transposon mutagenesis, was tagged with 2 *gfp* genes under a *psbA* promoter, giving the strain the ability to fluoresce green (Fig. 1.2). The strain also carries rifampicin and kanamycin resistance (Bathe *et al.* 2004) and contains a plasmid called pJP4, which was originally discovered in *Ralstonia eutropha* JMP134 isolated from a soil environment by Don and Pemberton (1981). This plasmid carries a number of different genes capable of degrading both 2,4-D and 3-chlorobenzoic acid (Trefault *et al.* 2004). There are 6 *tfd* genes that are involved in 2,4-D degradation; *tfdA-tfdF* (Fig. 1.3). These genes degrade 2,4-D to β -ketoadipate (Fig. 1.3). The gene *tfdB* codes for a 2,4-dichlorophenol hydroxylase that transforms 2, 4-dichlorophenol, previously converted from 2,4-D by *tfdA*, to dichlorocatechol. This gene is also transcribed on its own, while the rest of the genes are found in an operon (Fulthorpe *et al.* 1995). These *tfd* genes have been found in organisms other than *Ralstonia eutropha* and have a similar if not identical sequence to those found on the pJP4 plasmid (Fulthorpe *et al.* 1995). These *tfd*-like genes can be found either on plasmid backbones or on the chromosome (Vallaey's *et al.* 2006). A study conducted by Vallaey's *et al.* (2006) tested the ability of soil microorganisms to degrade 2,4-D. They found that previously untouched soil, no added 2,4-D, could harbor the ability to degrade the herbicide, whether partially or fully, and some of the isolated strains showed a positive PCR product for *tfdA* and *tfdB*. Moreover, in addition to degradation of 2,4-D and 3-chlorobenzoic acid, pJP4 carries mercury resistance (Trefault *et al.* 2004).

Figure 1.2 The donor *Pseudomonas putida* SM1443 and the transconjugant. The donor has rifampicin and kanamycin resistance encoded on its chromosomal genes, as well as 2 *gfp* genes and a *lacI^q* gene to inhibit the lac operated *dsRed* gene located on the pJP4 plasmid, inhibiting red fluorescence; the plasmid also contains gentamicin resistance and 2,4-D degradation genes. The transfer of the pJP4 plasmid will allow the transconjugant to fluoresce red as well as carry gentamicin resistance.

The modified plasmid pJP4 also carries a gene for gentamicin resistance, transferred via a transposon, as well as a P_{lac} -*dsRed* cassette, making *dsRed* lac-operated. DsRed can give *Pseudomonas putida* SM1443 red fluorescence, detectable in the red spectrum of visible light, if the *dsRed* gene is induced by IPTG (Bathe *et al.* 2004) (Fig. 1.2). Without IPTG, the lac repressor protein, encoded by the *lacI^q* gene, will repress *dsRed* gene expression. Transconjugants of pJP4 will fluoresce red and will consequently carry a gene for gentamicin resistance (Fig. 1.2). Due to accumulation of DsRed, transconjugants can also be differentiated on an agar plate as red colonies.

Figure 1.3 The degradation pathway of 2,4-D. The degradation of 2,4-D leads to the end product β -ketoadipate through a series of enzymatic reaction using 6 enzymes, *tfdA-F*. The enzymes involved in the breakdown of 2,4-D are *tfdA*, 2,4-D/ α -ketoglutarate dioxygenase, *tfdC*, chlorocatechol dioxygenase, *tfdD*, chloromuconate cycloisomerase, *tfdE*, dienelactone hydrolase and *tfdF*, maleylacetate reductase (Adapted from Schwartz, 2009 and Plumeier *et al.* 2002).

1.3.7.2. DsRed

The DsRed protein, a multimer existing in a tetrameric state, was formally isolated from the coral *Discosoma*. DsRed takes 4-5 d at room temperature (RT) to fully mature, however, approximately 2 weeks in the fridge is required for the colonies to fully develop a red color. Early in the maturation of DsRed, it has a tendency to express green fluorescence when it is excited (Baird *et al.* 2000). Green fluorescence peaks at 7 h and then rapidly declines over 2 d

(Baird *et al.* 2000). Even though it shows slow maturation, it is relatively insensitive to pH changes that vary from 4.2 to 12 (Baird *et al.* 2000). The excitation maxima of DsRed is 558 nm and the emission maxima is 583 nm (Baird *et al.* 2000, Figure 1.4). DsRed can be used as a selective marker in genetic transfer of catabolic genes in a simulated environment created using a microcosm.

Figure 1.4 Excitation and emission spectra of DsRed and maturation of DsRed. The image on the left shows dashed lines indicating the excitation and emission spectrum of DsRed in bacterial cells. The image on the right shows the maturation of DsRed. Open symbols show green fluorescence and closed symbols show red fluorescence (Baird *et al.* 2000)

1.3.8. Microcosm

Microcosms are a valuable tool in ecological studies that attempt to reproduce a field environment to study natural processes or the effects of a pesticide on an ecosystem (Edward, 2002). As well, the effects of introducing a genetically modified microorganism can be studied and its use in bioremediation purposes analyzed (Hill and Top, 1998). Moreover, the microorganism's survivability and interaction with the community can be assessed and the results can be extrapolated to processes occurring in the environment (Hill and Top, 1998). The soil that is used to pack the microcosm is either collected from the relevant site or commercially purchased, after which the microorganisms are added to the microcosm. While microcosms can

be relatively small, such as test tubes, these microcosms cannot accurately represent real environmental conditions. A 100-g soil microcosm is, according to Hill and Top (1998), fairly replicable and can give a pretty accurate representation of external processes. Closed-batch as well as continuous-flow microcosms can be used, where media can be delivered to the microcosm continuously (Hill and Top, 1998). Microcosms can also be very large, with some using more than 600-g of soil (Hill and Top, 1998).

CHAPTER 2: ANALYSIS OF TRANSFER OF PLASMID pJP4 IN A LAB-SCALE MICROCOSM UNDER SELECTIVE AND NON-SELECTIVE PRESSURE

2.1 – INTRODUCTION

Groundwater contamination arises from two main sources, point source pollution and diffuse pollution (Lapworth *et al.* 2012). Point source pollution comes from sewage treatment plants, septic tanks and industrial wastes, while diffuse sources of pollution come from regions that are geographically extensive and the source of pollution hard to pin-point (Lapworth *et al.* 2012). A wide variety of compounds have been detected in groundwater ranging from pharmaceuticals such as analgesics and antibiotics or components in skin care products like parabens, to industrial compounds like bisphenol-A and detergents (Lapworth *et al.* 2012). Some diffuse sources of groundwater pollution are pesticides or fertilizers from agricultural sites. Groundwater contamination not only causes a loss of a water supply, but since groundwater is part of the hydrological cycle, contaminants can run into surface waters.

Bioaugmentation is a method of bioremediation where microorganisms capable of degrading xenobiotics are introduced into a contaminated area. One of the ways to circumvent the potential problem that arises when exogenous microorganisms are introduced into a new environment, i.e. their inability to survive, is gene bioaugmentation. Genetic bioaugmentation is a method of introducing exogenous genes to an indigenous population, providing the native population with the ability to degrade the surrounding xenobiotic.

Although transfer of catabolic plasmids has been shown to be an effective mechanism of bioremediation, the transfer of genes has not been widely investigated in biofilms in subsurface environments, especially without any selective pressure. In this study, the overall objective was

to investigate the transfer of pJP4 in a mixed microbial community in a microcosm simulating a porous aquifer. The specific objectives were to examine biofilm thickness in relation to environmental changes. Moreover, the transfer of pJP4, with or without any selective pressure, was monitored in a mixed microbial biofilm in the microcosm, and donor persistence was assessed to ensure that the donor survived sufficiently long enough for transfer to occur.

2.2 – MATERIALS AND METHODS

Overall Experimental Design

Figure 2.1 A flow chart showing the techniques used to characterize the microbial behavior in a microcosm simulating a porous aquifer. For the microcosm inoculated with recipient and donor, further analyses were conducted to examine plasmid transfer, which was monitored via CLSM and sequencing.

Strains and Growth Conditions

Pseudomonas putida SM1443 (pJP4::dRed) (Bathe *et al.* 2004) was grown in 100% LB (Luria-Bertani) broth (Difco™, BD, Sparks, MD, USA). The medium contained gentamicin sulfate (IBI Scientific, Peosta, IA, USA), in a final concentration of 25 µg/mL, and kanamycin sulfate, in a final concentration of 100 µg/mL (Teknova, Hollister, CA, USA). *P. putida* was grown for 2 d at room temperature ($22 \pm 2^\circ\text{C}$) on a shaker at 290 RPM. The recipient strains are described in Table 2.1.

Table 1.1 List of recipients or recipient microbial communities used in mating with donor strain *P. putida* SM1443 and their source

Recipients	Source or reference	Growth conditions
<i>Escherichia coli</i>	ATCC 15939	100% LB
Soil sample	Collected from Ryerson University	100% R2A
Groundwater sample	Aquifer in Cambridge, Ontario	5% TSB
<i>Pseudomonas putida</i>	ATCC 12633	100% LB
Peat sample	Graciously provided by Karen Terry	100% R2A
Wastewater sample	Municipality of Peel wastewater treatment plants	10% R2A

Media Preparation

LB agar (BD, Sparks, MD, USA) was made at 100% strength; the solution was autoclaved at 121°C for 1.5 h. The composition of the LB broth is 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per 20 g/L of water. For antibiotic plates, a final concentration of 25 µg/mL of gentamicin sulfate and a final concentration of 100 µg/mL of kanamycin sulfate was added. R2A broth (HiMedia, India) was made at 100% strength. R2A agar (EMD chemicals, Germany) plates were made also made at 100% strength. Both R2A broth and agar were autoclaved at 121°C for 1.5 h. The typical composition of R2A agar in g/L is yeast extract 0.5; protease peptone 0.5; casein hydrolysate 0.5; glucose 0.5; starch soluble 0.5; sodium pyruvate

0.3; dipotassium hydrogen phosphate 0.3; magnesium sulfate 0.05; agar-agar 12.0. TSB (Tryptic Soy Broth) (EMD Chemicals, Germany) was made at 5% strength and also autoclaved at 121°C for 1.5 h. The composition of TSB broth in g/L is peptone from casein 17.0; peptone from soymeal 3.0; D (+) glucose 2.5; sodium chloride 5.0; and dipotassium hydrogen phosphate 2.5.

Mineral media MMN/MMO was synthesized, as described by Boon *et al.* (2003), starting with the 100 x phosphate buffer, which was made using 141.96 g/L of Na₂HPO₄ and 136.09 g/L of KH₂PO₄; the phosphate buffer was autoclaved. The 200 x MMN buffer was made using 19.7 g/L of MgSO₄, 1.18 g/L of CaCl₂ * 2H₂O, 0.23 g/L of H₃BO₄, 0.56 g/L of FeSO₄ * 7H₂O, 0.23 g/L of ZnSO₄ * 7H₂O, 0.34 g/L of MnSO₄ * H₂O, 0.08 g/L of CuSO₄ * 5H₂O, 0.05 g/L of CoCl₂ * 6H₂O, 0.02 g/L of Na₂MoO₄ * 2H₂O, 0.64 g/L of Na₂EDTA; MMN buffer was filter sterilized. A 1 M solution of NH₄Cl was autoclaved. The phosphate buffer and MMN buffer were added to make a 1x solution; NH₄Cl was added to a final concentration of 1 mM and glucose was added to a final concentration of 0.9 g/L.

Mating of Donor and Recipient

The donor, *Pseudomonas putida* SM1443 (pJP4::dsRED) was grown at RT for 2 d as indicated above and the recipients were grown for 2 d as indicated in Table 2.1. A 1 mL sample of each was removed from each culture and placed in sterile centrifuge tubes. The tubes were centrifuged for 5-10 minutes at 14, 000 – 16, 000 x g in the centrifuge (Thermo Electron Corporation, SORVALL legend micro 17R centrifuge). The supernatant was discarded and the pellet re-suspended in 500 µL of saline (7% NaCl). The samples were centrifuged for 5-10 minutes at 14, 000 – 16, 000 x g and the supernatant discarded. The pellet was re-suspended in 500 µL of saline. Two hundred µL of the donor culture was removed and put in a fresh tube. Two hundred µL of the recipient culture was then mixed with the donor and vortexed for 10

seconds. A 100 μL drop of the mating mixture was added to an R2A or LB plate. As controls, a 5-20 μL drop of the donor and recipient cultures was also added to new R2A or LB plate. The plates were incubated at room temperature for 5 d. They were then put in the fridge at 4°C for 2-3 weeks. CLSM was used to check for transconjugants and controls were investigated for autofluorescence.

Microcosm design

The microcosm was made out of polyvinyl chloride (PVC) by the Engineering and Research Support Facilities (Ali Hemmati) as shown in Figure 2.2. The PVC tube has a length of 12.2 cm and a diameter of 3.3 cm (Figure 2.2). To close the microcosm at the top, a twist top was made out of gray PVC (Figure 2.2). The inner part of the twist top has a diameter of 5 cm. A mesh with holes <0.5 mm was used at the bottom of the microcosm to keep the glass beads (2mm) made from soda lime (VWR®, Mississauga, ON, Canada) from escaping as effluent (Figure 2.2). Two sampling ports were also drilled at the sides of the microcosm, the first 4.3 cm from the top and the other 3 cm from the bottom and 1.5 cm from the mesh (Figure 2.2). The sampling ports were also made out of PVC (Figure 2.2). The screws at the sampling ports can be removed to withdraw samples from the microcosm (Figure 2.2).

Figure 2.2 Microcosm design and construction. a) the top of the microcosm and the two detachable pieces that are screwed in at the top, b) the outer piece, fitted between the top of the microcosm and the twist top, will hold the silicon tubing; the outer diameter of the screw is 1.0 cm and the inner diameter is 0.35 cm, c) the bottom side of the outer piece has a diameter of 2.2 cm, d) shows the top of the microcosm together with the outer piece and the twist top put together, e) the top of the microcosm once the outer piece and the twist top are removed and f) the assembled microcosm with a length of 12.2 cm and a width of 3.2 cm.

Preliminary Microcosm Setup for Soil Microbial Community

The microcosm was set up as shown in Figure 2.3. A Watson Marlow Sci-Q 205U/CA multichannel pump (Fisher-Scientific Limited, Nepean, ON, Canada) was used to pump the media into the microcosm. A 1 h treatment with 100% ethyl alcohol was used to sterilize the microcosm. The microcosm was then washed with sterilized dH₂O for a minimum of 24 h and flushed with 1 x MMN media (0.9 g/L glucose) for 1.5 h prior to inoculation. Glass bead were

added to the microcosm. Approximately 1×10^8 cells of the recipient, after washing with saline, was inoculated into the microcosm. After inoculation, the bottom silicone tube was clamped for 30 minutes. The clamp was removed after 30 minutes and the 1 x MMN glucose media ran for 1.5 h at approximately 10 mL/h before sampling. The minimal glucose media was run through the system for 13 d. Glass bead were collected on days 1, 2, 3, 7, 8, 9, 10 and 13. Five to ten mL of effluent was collected into sterile 15 mL centrifuge tubes and kept in the -20°C fridge for later analysis. The collected effluent was serially diluted and spread-plated onto R2A and LB plates to get a total heterotrophic plate count and on R2A-G, R2A-K and LB-G,K in order to select for any antibiotic resistant strains. A red nucleic acid stain, SYTO 62 (Molecular Probes®, Eugene, OR, USA), was used to visualize the biofilm under CLSM and measure the biofilm thickness. To measure biofilm thickness, the Pythagorean Theorem was used on biofilm thickness images (Figure 2.4). Three images were used per sample time and three sections of the biofilm in each image were used (Figure 2.4).

Figure 2.3 Microcosm setup. Setup showing the effluent supplied with sterile MMN-glucose media (0.9 g/L of glucose) being pumped into the microcosm at 10 mL/h (1.6RPM). The effluent was collected in a closed sterile flask.

Figure 2.4 Measuring thickness of biofilm adhered to glass beads. A sample was stained with SYTO62 and scanned using the 633 nm laser with CLSM. Three images were acquired per sample time. ImageJ was used to count the distance (in pixels) between X_2 and X_1 and between Y_2 and Y_3 . The Pythagorean Theorem was used to determine the hypotenuse distance, which represented the biofilm thickness. For each image, biofilm thickness was measured three times and averaged. As well, for each sample, three images from three different beads were used and averaged. The values for each sample time were then transformed into μm (1 pixel is equal to $0.45\mu\text{m}$).

Donor Persistence

A 1 h treatment with 80% ethyl alcohol was used to sterilize the microcosm. The microcosm was then washed with sterilized dH_2O three times and flushed with 50% LB for 1 h prior to inoculation. The autoclaved beads were then added. A sample of the recipient donor culture, grown in 40 mL of LB with the same antibiotic concentrations as previously mentioned, was centrifuged at $16,000 \times g$ and the pellet was washed with saline and inoculated into the microcosm at approximately 10 mL/h. A 1 mL, washed sample, of the same culture was used to determine the total cell count inoculated into the microcosm using an LB plate. A

spectrophotometer (Eppendorf BioSpectrometer®, Hamburg, Germany) was used to measure the OD of the sample at 660 nm prior to inoculation or serial dilution. After the donor fully entered the microcosm, the microcosm was clamped for 1 h and the pump shut off. After 1 h, it was unclamped and the LB media was pumped at approximately 10 mL/h. Effluent samples were taken at 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h and 4 h. The samples were centrifuged at 16,000 x g for 10 minutes and washed with saline prior to serial dilution. The serially diluted samples were plated onto LB plates. The plates were counted after two days of incubation at room temperature. DNA extraction was performed on the samples and the *gfp* gene amplified for gel electrophoresis.

Donor Persistence with Soil Inoculum

The same procedure for cleaning and adding beads was used as in the donor persistence experiment. Before the soil community was inoculated, 50% LB was run for 1 h. Around 7×10^7 cells of the soil culture, after washing with saline, were inoculated into the microcosm. The OD was measured prior to inoculation and a 1 mL sample was serially diluted to determine the total count. The microcosm was clamped for an hour after the soil culture fully entered the microcosm and the pump shut off. Afterward, 50% LB was run through the microcosm at 10 mL/h for 2 d. On the third day, the donor, *P. putida* SM1443, was washed with saline and inoculated into the microcosm at 10 mL/h; around 7×10^7 cells were inoculated. The OD of the culture was determined prior to inoculation and the culture serially diluted to determine the number of donor cells inoculated. After the entire solution containing the donor entered the microcosm, the microcosm was clamped for 1 h and the pump shut off. After 1 h, the clamp was removed and the pump turned back on at 10 mL/h. Effluent samples were taken at 0 h, 0.5 h, 1

h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h and 28 h. The effluent samples were washed with saline and serially diluted onto LB double antibiotic plates (kanamycin and gentamicin). Donor colonies were counted using a dissection scope (Leica, model MZFLIII) with fluorescence capabilities and *gfp* PCR was used to verify donor presence.

Microcosm Operation for Soil and Donor With or Without Selective Pressure

A 1 h treatment with 80% ethyl alcohol was used to sterilize the microcosm. The microcosm was then washed with sterilized dH₂O three times. The sterilized beads were then added. The media, MMN with 0.9 g/L of glucose, was run for 1 h prior to inoculation. A sample of the recipient culture was washed with saline and approximately 7×10^7 cells of the recipient culture were pumped into the microcosm. After the recipient culture fully entered the microcosm, the bottom silicone tube was clamped for 1 h and the pump turned off. The clamp was removed after 1 h and the pump turned back on at 10 mL/h. MMN with 0.9 g/L of glucose was run through the system for 4-5 d. Glass bead samples were collected on a regular basis. Three to six mL of effluent was removed into sterile 15 mL centrifuge tubes and kept for further analyses. DNA was extracted from the effluent. A nucleic acid stain, SYTO62, was used to stain the biofilm on the glass bead to calculate biofilm thickness using CLSM images. On day 5 or 6 of its operation, a culture of the donor (*P. putida* SM1443) was washed with saline and approximately 1×10^9 cells of the donor were pumped into the microcosm; the bottom silicone tube was clamped for 1 h and the pump shut off. After 1 h, the clamp was removed and the pump turned back on. The media was changed to 50% R2A for 25 h. After 25 h, the media was either changed to MMN with glucose (0.9 g/L) or MMN with glucose (0.9 g/L) and 100 µg/L of gentamicin. Three to six mL of effluent was removed into sterile 15 mL centrifuge tubes after 1

h, 4 h, and then every 24 h after for 1 week. Glass bead were extracted on a regular basis. To examine biofilm thickness, the biofilm on the glass beads was stained with SYTO62 and visualized under the CLSM. The biofilm thickness was calculated using the same approach as outlined in the microcosm experiment using solely the soil microbial community. A biofilm slurry was also made in order to analyze the isolated biofilm under CLSM for red and green fluorescent cells. In order to acquire a biofilm slurry, 200-300 μ L of saline was added to the glass bead samples and the samples were vortexed for 5 minutes. Afterwards, the solution was removed and centrifuged at 16,000 x g for 5 minutes to acquire a pellet; the pellet was re-suspended in 10 μ L of saline. DNA was extracted from the effluent and the biofilm; *gfp* was PCR-amplified from these samples to identify donor presence. *dsRed* was PCR-amplified to identify either donor or transconjugants; *dsRed* presence, but no *gfp*, could potentially indicate transconjugants. The collected effluent was serially diluted and spread-plated onto R2A and LB plates (for total count), as well as R2A-G and LB-G,K plates (to select for donor or transconjugant cells). The transconjugants growing on LB-G,K plates were isolated and the DNA extracted to perform PCR and confirm the presence of the *dsRed* gene. The 16S-rRNA gene was also amplified and the PCR products were used for sequencing of the transconjugants. The DNA from putative donor cells on LB-G,K was extracted and *gfp* was PCR-amplified to ensure donor presence.

Statistical Analysis

A simple correlational analysis with a probabilities test was used to examine the relationship between plate counts, as well as between plate counts and biofilm thickness measured on glass bead samples. Analysis of variance (ANOVA) was also conducted to

examine any significant differences between counts. A pairwise comparison using the Tukey significance test was used with ANOVA. SYSTAT®13 was used for all statistical analyses.

DNA extraction, PCR and Gel Electrophoresis

Plasmid extraction was performed using the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Indianapolis, IN, USA). DNA extraction was performed using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). The eluate was stored at -20°C. The PCR reactions were conducted using 0.5 µl of Nova Taq™ Taq Polymerase (Biolabs, Ipswich, MA, USA) with a concentration of 5 U/µl, 10 µl of 10 x Taq DNA polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂), 0.625 µl of BSA (Biolabs), 1 µL of forward primer (25 µM) and 1 µl of the reverse primer (25 µM), 8 µl of dNTPs (5 µL of each mixed with 38 µL of dH₂O), 27.875 µL of dH₂O and 1 µL of template DNA. For the *dsRed* gene, the forward primer was 5'-TTGAAGACGGTGG CGTCGTTAC3-' and the reverse primer was 5'-CCTTTCAACACGCCATCACGAG-3' (Bathe *et al.* 2004). The initial denaturing step was performed at 94°C for 15 minutes, and the 32 cycles began with the denaturing step at 94°C for 30 seconds, then primer annealing at 62°C for 1 minute and extension at 72°C for 2 minutes. The final extension step was at 72°C for 2 minutes. The size of the PCR product was 261 bp. Different primers for *dsRed* were also used. The forward primer was 5'-TTGCCATTTGCT TGGGATA-3' and the reverse primer was 5'-CTCGGTTCTTTCATA CTGCTC-3' (Ma and Bryers, 2012). The initial denaturation step was performed at 95°C for 1 minute, followed by 35 cycles of 45 seconds at 92°C, 45 seconds at 52°C, and 45 seconds at 72°C. A final primer extension was performed for 10 minutes at 72°C. The size of the PCR product was 480 bp. For the *tfdB* gene, the forward primer is 5'-GCTGACGGCGTGTCG

TCGCG-3' and the reverse primer is 5'-GATGGACGATGCGC GACGTA-3' (Neilson *et al.* 1992). The initial denaturing step was performed at 94°C for 15 minutes, and the 32 cycles began with the denaturing step at 94°C for 30 seconds, then primer annealing at 62°C for 1 minute, then extension at 72°C for 2 minutes. The final extension step is at 72°C for 2 minutes. The size of the PCR product was 205 bp. For the *gfp* gene, the forward primer is 5'-TTTCAAGA GTGCCATGCCCCGAAGG-3' and the reverse primer is 5'-CTGGTAAAAGGAC AGGGCC ATCGC-3' (Bathe *et al.* 2004). The initial denaturing step was performed at 94 °C for 2 minutes, and the 30 cycles began with the denaturing step at 94°C for 30 seconds, the annealing step at 60°C for 45 seconds and the extension step at 72°C for 1 minute. The final extension was at 72°C for 2 minutes. The size of the PCR product was 341 bp. For 16S rRNA, the F1 primer with a sequence of 5'-GAGTTTGATCCTG GCTCAG-3', and U519R, with a sequence of 5'-GTATTACCGCGGCTGCTGG-3', was used, producing a fragment size of 518 bp (Yeung *et al.*, 2011). DNA denaturation was performed at 96°C for 5 minutes. Ten cycles were run, starting with a temperature of 94°C for 1 minute, 65°C for 1 minute and 72°C for 3 minutes. Afterward, 20 cycles began with a temperature of 94°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes.

Gel electrophoresis was performed using 1%-2% agarose made out of agarose (FroggaBio, Toronto, ON, Canada) and 1 x TAE buffer (242 g Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA, diluted to 1L with H₂O). SYBRsafe (0.83 µl/50mL) (Molecular probes, Eugene, OR, USA) was added to the heated solution when it reached 55°C. Once the gel solidified, ~15 minutes, the samples (5 µL of sample with 1 µL of loading dye) were loaded onto the gel, along with a DNA ladder (MBI Fermentas, Amherst, NY, USA). The gel was run at 85 Volts for 45 minutes.

Sequencing

16S rRNA was first PCR-amplified using extracted DNA. PCR-amplified DNA was then quantified and 50 ng of DNA was sent for sequencing to the Hospital for SickKids (MARS building) in Toronto, Ontario. Sequencing was conducted using the Applied Biosystems SOLiD 3.0 System. The BLAST algorithm from the nucleotide NCBI database was used to check for similar nucleotide sequences for 16S rRNA in bacteria and archaea (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Confocal Laser Scanning Microscope

Green and red fluorescent cells were visualized using CLSM (LSM410 Carl Zeiss, Jena Germany). Three different lasers were used, 488 nm (Argon), 543 nm (HeNe1), and 633 nm (HeNe) lasers. For green fluorescence, to visualize donor cells, the 488 nm laser was used to excite the chromophore, while an IR 500-550 emission filter was used to capture the emitted signals. For red fluorescence, to visualize transconjugants, the 543 nm laser was used to excite the chromophore, while a 560 long pass filter used to capture emitted signals. For SYTO 62, a 633 nm laser was used to excite the chromophore, while a 650 long pass filter was used to capture the emitted signals. The microscope was used in transmission mode to visualize cells with no fluorescence. The images were obtained using the x10 lens (for glass beads) or the x 63 water immersion lens (for glass slides).

Fluorescence Dissecting Microscope

A dissecting microscope (Leica, model MZFLIII) was used for colony counts. A green fluorescent protein filter/blue excitation (480 nm) was used to detect colonies fluorescing green.

A red fluorescent protein filter/green excitation (546 nm) was used to detect red fluorescent colonies.

2.3 – RESULTS

Selecting a Recipient Community for pJP4

To select a recipient community for pJP4, several mating experiments were conducted where the recipients from Table 2.1 were mixed with the donor (*P. putida* JM1443). After being incubated, only the donor mating with the mixed community from the soil and the donor mating with *P. putida* 12633 showed areas of red patches, indicating transconjugants. The *P. putida* 12633 and donor mixture had several closely situated colony patches that turned a visibly red/pink color after the incubation period (Figure 2.5A and 2.5B). The mating experiment with the soil taken from Ryerson University and the donor strain led to a few secluded areas of pink patches, whereas the rest of the patch remained unchanged (Figure 2.5C). All the color changes were noted to exist on the outskirts of the mating patch. The other conjugations did not show any change in color within the mating patch, especially the desired red or pink color that would indicate transconjugants. The controls, which included the recipients listed in Table 2.1 or the donor, were plated separately on LB or R2A plates and did not show any color change after the same incubation period.

Figure 2.5 Mating experiments of donor and recipients. The mating patch of the conjugation between *P. putida* 12633 and the *P. putida* SM1443 donor strain on LB plates contained red patches after 5 d at room temperature and 2 weeks in the fridge (A). The mating patch of the conjugation between *P. putida* 12633 and *P. putida* SM1443 strain on R2A plates contained pink patches after 5 d at room temperature and 2 weeks in the fridge (B). The mating patch of the conjugation between a Ryerson University soil sample and the *P. putida* SM1443 donor strain on LB contained red patches after 5 d at room temperature and 2 weeks in the fridge (C).

CLSM for the controls indicated no autofluorescent signals (Appendix A: Figure 5.1A for soil control). The control scan for the donor control yielded only green fluorescent cells (Appendix A: Figure 5.1B). In regards to the mating patch scans, all the scans showed both green and red fluorescence, to varying degrees. For the soil and *P. putida* 12633 mating experiments, the scan using transmission mode and red and green fluorescence showed a high number of red and green fluorescent cells, for transconjugants and donor, respectively (Figure 2.6 for soil mating). The scans for *E. coli*, the wastewater, groundwater and peat community conjugation experiments showed a very small number of red transconjugants and many green donor cells (Appendix A: Figure 5.2A and Figure 5.2B). It was expected that transconjugants would be visualized for all the samples used.

Figure 2.6 CLSM image taken of donor conjugation with Ryerson University soil. CLSM images taken of the mating patch where soil community from Ryerson University was conjugated with *P. putida* SM1443 showed both green and red fluorescence after 5 d at room temperature and 2 weeks in the fridge. The image was taken at 630x magnification. Sequencing determined the transconjugant belonged to the genus *Pseudomonas* (Appendix E: Table 5.4).

Preliminary Microcosm Setup for Soil Microbial Community Biofilm Thickness and Plate Counts

The general trend was an increase in plate count numbers from 1.5 h to 144 h; the counts dropped dramatically at 168 h, where they did not return to their peak (Figure 2.7). Furthermore, the total counts on R2A and LB plates had a similar trend, where the total counts on the R2A and LB plates had a positive correlation of 0.962 and a p-value of 0.001, indicating a strong correlation between the counts.

Figure 2.7 Total counts on R2A or LB from preliminary microcosm setup with only soil microbial community. The CFU/mL was calculated from R2A and LB plates after spread-plating serially diluted effluent samples from 1.5 h to 288 h collected from the microcosm supplied with 1 x MMN glucose media (0.9 g/L).

The biofilm thickness, measured using CLSM images, closely resembled the pattern seen in the total counts for the same time period (Figure 2.7 and Figure 2.8). There was a general increase in biofilm thickness on glass beads until 144 h in both the top and bottom ports, and then a decrease at 168 h, where the biofilm thickness remained stable until 288 h (Figure 2.8). The biofilm thickness measured on the glass beads taken from the top port had a correlation of 0.841 and 0.951, with p-values of 0.018 and 0.001, to the total counts on R2A and LB, respectively. The biofilm thickness measured on glass beads from the bottom port had a correlation of 0.676 and 0.711, with p-values of 0.095 and 0.073, to the LB and R2A total plate counts, respectively. The biofilm thickness from the top and bottom ports had a correlation of 0.728, with a p-value of 0.063. From this data, the biofilm thickness measured from the top port can be said to be highly correlated to the total heterotrophic plate counts on LB and R2A. The biofilm thickness measured from glass beads taken from the bottom and top ports were slightly

correlated, though the correlation was not significant. As well, the biofilm thickness from the glass beads taken from the bottom port was slightly correlated to the total count on LB and R2A, but the correlations were also not very significant.

Figure 2.8 Biofilm thickness measured from microcosm setup with the addition of only the soil microbial community. The biofilm was stained with SYTO62 and visualized using CLSM at 400x. The biofilm thickness was measured using the Pythagorean Theorem. Three images were analyzed per sample, with three points used for each image.

A large number of colonies were noted when effluent samples from 168, 192, 216 and 288 h were spread-plated on antibiotic plates. Colonies resistant to gentamicin, kanamycin or doubly-resistant to both antibiotics were noted. A high number of gentamicin resistant colonies were cultured: 2.4×10^6 to 1.11×10^7 CFU/mL. As well, a large number of kanamycin and doubly resistant microorganisms were cultured: 2.6×10^6 to 6.1×10^6 CFU/mL for kanamycin resistant species and 2.0×10^6 to 6.7×10^6 CFU/mL for the doubly resistant species.

PCR Detection of *gfp*, *dsRed* and *tfdB* genes for Soil Microbial Community Alone Microcosm Setup

After PCR-amplification of the *tfdB* gene for the collected effluent samples, bands were visible for all the extracted effluent samples, as well as the pre-inoculated soil culture, on the agarose gel after electrophoresis (Figure 2.9).

Figure 2.9 Gel electrophoresis for *tfdB* for soil alone microcosm setup. A 1.5% gel ran for 45 minutes at 85 volts was used to show the PCR-amplified *tfdB* gene from both donor and effluent samples. The expected band size was 205 bp. The bands below the expected size were primer dimers. The lanes in the gel were: A) 100 bp ladder; B) *P. putida* SM1334 (donor); C) *P. putida* 12633; D) pre-inoculated soil culture; E) 1.5 h; F) 24 h; G) 48 h; H) 144 h; I) D 168 h; J) 192 h; K) 216 h; L) 288 h

Thereby, some of the bacteria in the inoculated soil sample and the collected effluent samples possessed some form of the *tfdB* gene, the gene that encodes a protein required in the transformation of 2, 4-dichlorophenol to dichlorocatechol; the second step in 2,4-D degradation. After further PCR-amplifications of the *gfp* and *dsRed* genes, none of the bacteria in the inoculated soil sample and the collected effluent samples had a band, meaning that these genes were not present in the soil community. These genes were only present in the donor *P. putida* SM1443 (Appendix C: Figure 5.8).

Donor Persistence

With the microcosm having a pore volume of 27.5 mL (total volume of 70 mL) and the microcosm being run at 10 mL/h, if the donor is unable to grow or attach to glass beads within the microcosm, the donor should be washed out after approximately 2.75 h. However, after 2.75 h, the donor was still evident in the effluent samples, with a plate count of 3.5×10^5 CFU/mL (Figure 2.10). Moreover, the graph showed an incline in CFU/mL at 4 h with a count of 1.58×10^6 CFU/mL (Figure 2.10).

Figure 2.10 Donor persistence experiment using donor alone. Donor cells (2×10^7) were inoculated into the microcosm and 50% LB was pumped for 4 h. Effluent samples were removed at 0.5-1 h intervals and spread-plated onto LB-gentamicin/kanamycin plates.

Two types of colonies were evident on the LB plates, small cream-colored colonies and medium-sized white-colored colonies that were irregularly shaped, indicating that a contamination of the microcosm had occurred. The two types of colonies were compared to the original serially diluted un-inoculated donor plate and visualized under the CLSM for green fluorescence. The small cream-colored colonies had the same morphology compared to the original plates and were the only colonies to fluoresce green under the CLSM. These were then

confirmed as the donor. Both colony types were included in the PCR for the *gfp* gene to further verify for the donor.

A DNA extraction for all the effluent samples was performed and the *gfp* gene was amplified for gel electrophoresis. In the gel image, all the effluent samples showed a band for *gfp*; the 3 h effluent sample showed a very faint band (Figure 2.11). The negative control, *P. putida* 12633, showed no band, as was expected. The small-cream colored colony also showed a band, while the other medium-sized white colony, representing the contaminant, did not (Figure 2.11). Thereby, the PCR for the *gfp* gene confirmed the presence of the donor in the microcosm after 4 h, which indicated that it was not washed out after 2.75 h, and the PCR also confirmed that the small cream-colored colonies were in fact the donor cells growing on the LB plates.

Figure 2.11 PCR-amplified *gfp* gene for donor persistence experiment. A 1.5% gel ran at 85 volts for 45 minutes showed the amplified *gfp* gene for the effluent samples in the donor persistence experiment. A product of 341 bp was expected. Larger than expected bands may be DNA extraction or PCR artifacts. The lanes in the gel were: A) 100 bp ladder, B) donor (*P. putida* SM1443), C) *P. putida* 12633, D) 0 minutes, E) 30 minutes, F) 1 h, G) 1.5 h, H) 2 h, I) 2.5 h, J) 3 h and K) 4 h, L) medium-sized white colony, M) small cream-colored colony

The generation time for *P. putida* SM1443 was calculated as follows:

$$\mu = (\log_{10}N_0 - \log_{10}N_1)/0.301t$$

μ = Index of growth rate

N_0 = Number of cells at time 0

N_1 = Number of cells at t minus

t= Time

$$\mu = [(\log_{10}(1 \times 10^8 \text{ CFU/mL}) - \log_{10}(3.6 \times 10^7 \text{ CFU/mL})) / 0.301(2.75 \text{ h})]$$

$$\mu = 0.54 \text{ CFU/h}$$

Generation time (g) = 1/ μ

$$g = 1/0.54$$

$$g = 1.85$$

The generation time for *P. putida* SM1443 is 1.85 h/CFU, which indicates that the donor can double in 1.85 h.

Donor Persistence with Soil Inoculum

The next experiment explored donor persistence with soil inoculum, which was inoculated into the microcosm prior to the inoculation of the donor. The soil microbial consortium was inoculated into the microcosm 2 d prior to donor inoculation. Around 7×10^7 cells from the soil sample were inoculated into the microcosm. As well, around 7×10^7 donor cells were inoculated 2 d after soil inoculation. The donor showed an ability to persist in the microcosm for 28 h as confirmed by *gfp* PCR (Figure 2.12).

Figure 2.12 Amplified *gfp* for donor persistence with soil inoculum. A 1.5% gel ran at 85 volts for 45 minutes showed the amplified *gfp* gene for the effluent samples. The expected product size was 341bp. The larger than expected bands may be DNA extraction or PCR artifacts. The lanes in the gel were: A) 100 bp ladder, B) donor (*P. putida* SM1443), C) *P. putida* 12633, D) soil, E) 0 h, F) 0.5 h, G) 1 h, H) 1.5 h, I) 2 h, J) 2.5 h, K) 3 h, L) 4 h and M) 28 h

Using plate counts, the donor could also be visualized through green fluorescence using the dissection scope. The donor cell count declined from 0.5-1.5 h; it stabilized after 1.5 h (Figure 2.13). The total count on LB plates, with gentamicin and kanamycin, showed a similar pattern to the donor count (Figure 2.13).

Figure 2.13 Donor and total CFU/mL on double antibiotic plates (kanamycin and gentamicin) to elucidate donor persistence with soil inoculum. The graph shows the counts on a log₁₀ scale. Donor was inoculated 2 d after the soil community was inoculated. Donor colonies were counted using a dissection microscope with a filter for a green fluorescence protein after 3 d of growth on LB-G,K plates at room temperature.

The correlation between the total count on the double antibiotic plates and the donor count on the double antibiotic plates was 0.895 with a p-value of 0.006, indicating that the total count and the donor count were highly correlated (Figure 2.13).

Microcosm Run with Soil and Donor with or without any Selective Pressure Persistence of Donor

Two experiments were performed, one using selective pressure (MMN-glucose media with 100 µg/mL of gentamicin) and one using no selective pressure (MMN-glucose media). The

donor was counted on double antibiotic plates (gentamicin and kanamycin) where previously serially diluted effluent samples were plated. For both experiments, the donor persisted throughout the experiment; 98 h after its inoculation into the microcosm (Fig. 2.14).

Figure 2.14 Donor CFU/mL from non-selective pressure experiment in comparison to donor CFU/mL from selective pressure experiment. The graph shows the donor colony count on a log₁₀ scale from serially diluted effluent samples taken from selective and non-selective pressure experiments. The mixed community from the soil sample was grown in the microcosm for 4-5 d before the inoculation of the donor (*P. putida* SM1443). Fifty percent R2A was used for 25 h and later switched to either MMN-glucose (0.9 g/L) for no selective pressure or MMN-glucose with 100 µg/mL of gentamicin for selective pressure. Green fluorescing donor colonies were counted on LB-G,K plates using a dissection scope. ^a Indicates data that have a p-value less than 0.05.

For both experiments, 50% R2A was used as influent for 25 h prior to a media switch to either MMN-glucose media (non-selective) or MMN-glucose media with 100 µg/mL of gentamicin (selective). For both experiments, the donor count (CFU/mL) remained stable from 4-24 h when 50% R2A was used (Figure 2.14). When the media was switched, the donor count rapidly declined for both conditions (Figure 2.14). However, at 98 after donor inoculation, the donor count increased to 2.75×10^4 CFU/mL for the selective pressure experiment, a 6x increase,

while the donor count for the non-selective pressure experiment continued to decline to very minimal levels (700 CFU/mL), a decrease of 3x (Figure 2.14). Moreover, at 98 h after donor inoculation, the p-value was under 0.05 when comparing the selective and non-selective pressure experiments, indicating a significant difference between the two counts (Figure 2.14).

Figure 2.15 PCR-amplified *gfp* from non-selective and selective pressure experiments. A 1.5% agarose gel was run with PCR-amplified *gfp* for soil and donor experiments using no selective pressure (A) and selective pressure (B). The expected band size was 341bp. Larger than expected bands may be DNA extraction or PCR artifacts. D indicates the donor and NC indicates dH₂O. Effluent samples 1, 4, 24, 48, 72, and 98 h after donor inoculation were PCR-amplified. No bands were seen at 24, 48, 72 and 98 h for the non-selective pressure experiment (A). For the experiment using selective pressure, only one band was present for the sample taken 1 h after donor inoculation, while the other samples, 4, 24, 48, 72 and 98 h after donor inoculation, did not have a band (B).

To further identify the donor cells in the effluent, *gfp* was PCR-amplified for the effluent samples taken from the non-selective and selective pressure experiments. Bands were seen for only 2 effluent samples, taken 1 and 4 h after donor inoculation, for the non-selective pressure experiment, and 1 band was seen for only 1 effluent sample, taken 1 h after donor inoculation, for the selective pressure experiment (Fig. 2.15).

Bands were expected to be evident for all sample times, as donor colonies were evident on all selective plates visualized by the fluorescence detecting dissection scope. Thereby, to

further verify for the presence of the donor in the effluent, for the selective pressure experiment, the donor colonies were picked off the plates, their DNA extracted, and PCR-amplified for *gfp*. Bands for *gfp* were seen for all sample times (Fig. 2.16A). Moreover, *gfp* performed on DNA extracted from the biofilm removed from glass beads for the selective pressure experiment showed bands for all sample times, except for the biofilm taken 4 h after donor inoculation from the top port (Figure 2.16B).

Figure 2.16 PCR-amplified *gfp* for selective pressure experiment using donor colonies picked off plates (A) and extracted biofilm from glass beads (B). Individual donor colonies (visualized through green fluorescence using a dissection microscope) were picked off and their DNA extracted for PCR-amplification of *gfp*. D is the donor and NC is dH₂O. Samples used were h after donor inoculation (A). *gfp* was also PCR-amplified for the selective pressure experiment using biofilm extracted from glass bead. The order for the wells is: 1) 100 bp ladder, 2) donor, 3) dH₂O, 4) 4 h TP, 5) 4 h BP, 6) 24 h TP, 7) 24 h BP, 8) 48 h TP, 9) 48 h BP, 10) 72 h TP, 11) 72 h BP, 12) 98 h TP, 13) 98 h BP (B). The expected band size was 341bp. The PCR products were run for 45 minutes on a 1.5% agarose gel. Larger bands were either DNA extraction or PCR artifacts (A and B). *TP = top port, BP =bottom port, the h indicates time after donor inoculation

Transfer of pJP4 under Selective and Non-selective Pressure Conditions

To examine transfer of the plasmid pJP4 under selective and non-selective pressure conditions, the DNA from the effluent samples taken from the selective and non-selective pressure experiments was extracted and the *dsRed* gene amplified. For the non-selective pressure experiment, bands for *dsRed* were seen 1, 4, and 24 h after donor inoculation, while 2 bands, 1 and 4 h after donor inoculation, were seen for the selective pressure experiment (Figure 2.17). In comparison to the *gfp* PCR for the non-selective and selective pressure experiments, bands were only seen 1 and 4 h after donor inoculation for the non-selective pressure experiment and 1 h after donor inoculation for the selective pressure experiment (Figure 2.15). Thereby, for both selective and non-selective pressure experiments, one extra band was seen for *dsRed* compared to *gfp* (Figure 2.15 and Figure 2.17).

Figure 2.17 PCR-amplified *dsRed* for non-selective and selective pressure experiments. A 1.5% agarose gel was run with PCR-amplified *dsRed* for soil and donor experiments using no selective pressure (A) and selective pressure (B). The expected band size was 480bp. Effluent samples 1, 4, 24, 48, 72, and 98 h after donor inoculation were PCR-amplified. D indicates the donor, NC indicates dH₂O, P indicates *P. putida* 12633 and D4 refers to day 4 (selective pressure experiment), which was time prior to donor inoculation. Bands were seen at 1, 4 and 24 h after donor inoculation for the non-selective pressure experiment (A), while only 2 bands, 1 and 4 h after donor inoculation, were seen for the selective pressure experiment (B).

Both *gfp* and *dsRed* PCR amplifications were performed for the biofilm extracted from glass bead samples taken from both the top and the bottom ports for the non-selective pressure experiment. No bands were seen for *gfp*, while two bands were seen 48 and 72 h after donor inoculation; the samples came from the bottom port (Figure 2.18).

Figure 2.18 PCR-amplified *gfp* and *dsRed* for non-selective pressure experiment using biofilm from glass bead. The expected band size for *gfp* is 341bp and for *dsRed* it is 480bp. Gels with a 1.5% agarose concentration showed PCR-amplified *gfp* (A) and *dsRed* (B) for DNA extracted from biofilm on glass beads taken from both the top and bottom ports. Larger than expected bands may be DNA extraction or PCR artifacts. The order for the wells is: 1) 100 bp ladder, 2) *P. putida* SM1443 (donor), 3) dH₂O, 4) 4 h TP, 5) 4 h BP, 6) 24 h TP, 7) 24 h BP, 8) 48 h TP, 9) 48 h BP, 10) 72 h TP, 11) 72 h BP, 12) 98 h TP, 13) 98 h BP (AB). No bands were seen for the PCR-amplified *gfp* gene (A). Two bands were seen for the PCR-amplified *dsRed* gene, at 48 and 72 h, for the bottom port (B). *TP = top port, BP =bottom port, the h indicate time after donor inoculation

To further identify transconjugants, double antibiotic plates (gentamicin and kanamycin), where serially diluted effluent samples were plated, were examined under the dissection scope using a filter for red fluorescent proteins. Patches of red fluorescing cells were visible under the dissection scope after 1 week of incubation at room temperature; most of these red fluorescent patches were associated with the donor, which fluoresced green. The patches were re-streaked to acquire pure cultures. Once a pure culture was obtained (Figure 2.19), it was once again verified

for red fluorescence under the dissection scope to ensure the entire culture was fluorescing red. Transconjugants were isolated for the non-selective pressure experiment (4, 24, 48, 72 and 96 h after donor inoculation) and for the selective pressure experiment (1, 4, 24, 48 and 72 h after donor inoculation). The DNA of these transconjugants was extracted and the 16S rRNA PCR-amplified for sequencing. The sequences were BLASTed, and, at 98-99% identity, each of the isolated transconjugants belonged to the genus *Stenotrophomonas*, (Table 2.2 and Table 2.3).

Figure 2.19 Red fluorescent cells taken from an isolated transconjugant culture from non-selective pressure experiment. The transconjugant was isolated from a 214 h sample (72 h after donor inoculation) that was serially diluted on an LB-G,K plate. The image was taken at 630x. The transconjugant shown here is a *Stenotrophomonas maltophilia* strain ATCC 19861 (Appendix E: Table 5.4)

Table 2.2 Transconjugants isolated from effluent samples from non-selective pressure experiment. PCR products were sequenced at SickKids. NCBI BLAST algorithm was used to identify the isolated transconjugants.

Sample time	Transconjugant strains	Identity (%)
143 h	N/A	N/A
146 h	<i>Stenotrophomonas maltophilia</i> IAM 12423	99
166 h	<i>Stenotrophomonas maltophilia</i> IAM 12423	99
190 h	<i>Stenotrophomonas maltophilia</i> IAM 12423	99
214 h	<i>Stenotrophomonas maltophilia</i> IAM 12423, <i>Stenotrophomonas maltophilia</i> ATCC 19861	99
238 h	<i>Stenotrophomonas maltophilia</i> IAM 12423	98

Moreover, for the selective pressure experiment, a slightly higher variety of microorganisms were isolated as transconjugants. For the selective pressure experiment, transconjugants were isolated for 5 sample times, and out of those 5, 3 different species were identified (Table 2.3), while, out of 8 isolated transconjugants, only 2 different species were identified for the non-selective pressure experiment (Table 2.2). Thereby, a slightly greater diversity of transconjugants was identified for the selective pressure experiment.

Table 2.3 Transconjugants isolated from effluent samples from selective pressure experiment. PCR products were sequenced at SickKids. NCBI BLAST algorithm was used to identify the isolated transconjugants.

Sample time	Transconjugant strain	Identity (%)
120 h	<i>Pseudomonas hibiscicola</i> ATCC 19867	98
123 h	<i>Pseudomonas hibiscicola</i> ATCC 19867	99
143 h	<i>Stenotrophomonas maltophilia</i> ATCC 19861	99
167 h	<i>Stenotrophomonas maltophilia</i> IAM 12423	99
191 h	<i>Stenotrophomonas maltophilia</i> ATCC 19861	99
217 h	N/A	N/A

The biofilm slurry, extracted from glass beads that were sampled after donor inoculation, was also examined under CLSM using the 543 nm laser (Figure 2.20 and Figure 2.21).

Figure 2.20 Biofilm extracted from glass beads examined under CLSM for non-selective pressure experiment. CLSM was used to visualize red cells using the 543 nm laser. The images were taken at 630x. One to three red cells were seen per image, indicating transconjugants. Three red cells were visualized for the biofilm sample taken at 4 h from the bottom port (A), 2 red cells for the biofilm sample taken at 24 h from the bottom port (B), one red cell for the biofilm sample taken at 72 h from the bottom port (C) and one red cell taken at 98 h from the top port (D). Samples indicate time after donor inoculation. .

Figure 2.21 Biofilm extracted from glass beads examined under CLSM for selective pressure experiment. CLSM was used to visualize red fluorescence cells using the 548 nm laser. The images were taken at 630x. Two red cells were visualized for the biofilm sample taken at 24 h from the top port (A) and more than 50 red cells were visualized for the biofilm sample taken at 48 h from the bottom port (B). Seven red cells were visualized for the biofilm sample taken at 72 h from the bottom port (C) and 2 red cells for the biofilm sample taken at 98 h from the bottom port (D). Samples indicate time after donor inoculation.

For the non-selective pressure experiment, only a few red fluorescent cells were evident in the biofilm slurry, with less than 10 red cells identified per sample extracted from either the top or bottom ports (Table 2.4). For the selective pressure experiment, more than 10 red cells were visualized, per sample time, in the biofilm slurry after gentamicin addition (Table 2.4). As well, over 50 red cells were evident in the biofilm slurry 48 h after donor inoculation, which was also 23 h after gentamicin addition (Table 2.4 and Figure 2.21). In total, a greater number of red cells, indicating transconjugants, were evident for the selective pressure experiment when compared to the non-selective pressure experiment (Table 2.4, Figure 2.20 and 2.21).

Table 2.4 Semi-quantitative evaluation of red cell abundance in CLSM images (3-4 per sample) taken of the biofilm extracted from glass bead samples for both the selective and non-selective pressure experiments (data is given after donor addition).

Time	Number of red cells (selective)	Number of red cells (non-selective)
4 h (top)	—	—
4 h (bottom)	—	+
24 h (top)	+	—
24 h (bottom)	—	+
48 h (top)	+	+
48 h (bottom)	+++	—
72 h (top)	++	+
72 h (bottom)	++	—
98 h (top)	—	+
98 h (bottom)	++	+

Legend: “—” no red cells detected, “+” <10 red cells, “++” 10-50 red cells, “+++” >50 red cells

Plate Counts and Biofilm Thickness from Selective and Non-selective Pressure Experiments

To examine biofilm thickness and total heterotrophic plate counts, effluent and glass bead samples were taken at regular intervals for both selective and non-selective experiments.

Effluent samples were plated on either LB or R2A to get a total heterotrophic plate count (Figure

2.22), while glass bead samples were stained with SYTO62 and CLSM was used to take images of the biofilm (Figure 2.23).

Figure 2.22 Total counts from effluent on R2A and LB plates for the non-selective pressure experiment. The soil microbial consortium was inoculated into the microcosm and MMN-glucose (0.9 g/L) ran for 142 h. At 142 h, the donor was added into the microcosm and the media changed to 50% R2A for 25 h. At 167 h, the media was switched back to MMN-glucose (0.9 g/L).

For the non-selective pressure experiment, both plate counts and biofilm thickness varied widely throughout the experiment (Figure 2.22 and Figure 2.23). Plate counts on LB varied from 7.05×10^7 to 3.6×10^8 CFU/mL and biofilm thickness varied from 17 μ m to 65 μ m (Figure 2.22 and Figure 2.23). Even though there was a lot of variation, biofilm thickness and total counts remained high throughout the experiment.

Fig. 2.23 Biofilm thickness at various sample times from the non-selective pressure experiment. SYTO62 was used to stain the cells on the glass bead. Biofilm thickness was measured using the Pythagorean Theorem using images taken by CLSM. The experiment was conducted after the soil microbial consortium was inoculated into the microcosm and MMN-glucose (0.9 g/L) was run for 142 h. At 142 h, the donor was inoculated into the microcosm and the media switched to 50% R2A for 25 h. At 167 h, the media was switched back to MMN-glucose (0.9 g/L).

For the selective pressure experiment, the biofilm thickness remained relatively stable in comparison to the plate counts, which changed dramatically throughout the experiment, especially after the addition of gentamicin (Figure 2.24 and Figure 2.25). The total counts on R2A varied from 2.76×10^8 to an all-time low of 3.43×10^5 CFU/mL 47 h after gentamicin addition, while the biofilm thickness remained high throughout the experiment, especially after gentamicin addition, where biofilm thickness increased and hovered close to $30\mu\text{m}$ (Figure 2.24 and Figure 2.25).

When comparing the total counts between the selective and non-selective pressure experiments, there was a similar pattern in total counts after donor inoculation at 1, 4 and 24 h. The correlation between the two experiments was 0.995 and 0.899 comparing the total counts on LB from the non-selective pressure experiment to the total counts on R2A and LB for the

selective pressure experiment, respectively. The correlations were high, though they were not significant, which was probably due to the small sample size.

Figure 2.24 Total counts on LB and R2A from effluent samples taken from the selective pressure experiment. The soil microbial consortium was inoculated into the microcosm and MMN-glucose (0.9 g/L) media ran for 119 h. The donor was added at 119 h and the media changed to 50% R2A for 25 h. At 144 h, the media was changed to MMN-glucose (0.9 g/L) with 100 µg/mL of gentamicin.

Also, after the change in media to either MMN-glucose or MMN-glucose with gentamicin for the non-selective and selective pressure experiments, the biofilm thickness for both experiments, top and bottom ports, remained very high respective to each experiment. The average thickness from the top and bottom ports was 29.92 µm and 31.20 µm for the selective pressure experiment after gentamicin addition, and the average thickness from the top and bottom ports was 39.63 µm and 50.99 µm for the non-selective pressure experiment after media change from 50% R2A to MMN-glucose (Figure 2.22 and Figure 2.24).

Figure 2.25 Biofilm thickness at various sample times from the selective pressure experiment. Biofilm thickness was measured using the Pythagorean Theorem using images taken by CLSM. Cells were stained with SYTO62. The experiment was started with the inoculation of the soil microbial consortium and the use of MMN-glucose (0.9 g/L) for 119 h. At 119 h, the donor was inoculated into the microcosm and the media switched to 50% R2A for 25 h. At 144 h, the media was changed to MMN-glucose (0.9 g/L) with 100 μg/mL of gentamicin.

Furthermore, total counts on either LB or R2A from either the selective or non-selective pressure experiments were compared to the counts from single (R2A-G) or double antibiotic plates (LB-G,K). For the non-selective pressure experiment, there was a significant difference between the total heterotrophic plate counts on LB to both the counts on the single antibiotic or double antibiotic plates, with p-values of under 0.05 comparing total counts on LB to both the single or double-antibiotic plate counts (Figure 2.26). However, most plate counts between the single and double antibiotic plates had a p-value of over 0.05, indicating no significant difference in the count between the two different types of plates. The only times the p-value was under 0.05, indicating a significant difference in count, for the single and double-resistant plate counts

was at 1 and 4 h after donor inoculation, with a total experiment time of 143 and 146 h, respectively (Figure 2.26).

Figure 2.26 A comparison of the total counts on LB, R2A-G and LB-G,K for samples taken from non-selective experiment. The graph was plotted on a log₁₀ scale. For all sample times, there was a significant difference between total counts on LB and total counts on the antibiotics plates. To begin the experiment, the soil microbial consortium was inoculated into the microcosm and MMN-glucose (0.9 g/L) was ran for 142 h. At 142 h, the donor was inoculated into the microcosm and the media changed to 50% R2A for 25 h. At 167 h the media was changed back to MMN-glucose (0.9 g/L). ^a Indicates p-values of less than 0.05 between the total counts and the counts on both of the selective plates. The counts on the selective plates were not significantly different except at 143 and 146 h.

For the selective pressure experiment, there were significant differences between the total heterotrophic plate counts on R2A and the selective plate counts, with p-values of less than 0.05 for sample times taken from 96 to 167 h. There were also significant differences between the counts on the single and double-antibiotic plates, with p-values of less than 0.05 for all sample times except at 123 and 191 h, which was 4 and 98 h after donor inoculation, respectively

(Figure 2.27). There was no significant difference between the total heterotrophic plate counts on R2A and either the single or double-antibiotic plate counts at 191 h, which was 98 h after donor inoculation and 47 h after the media was switched to MMN-glucose media with 100 $\mu\text{g/mL}$ of gentamicin (Figure 2.27).

Figure 2.27 A comparison of the total counts on R2A, R2A-G and LB-G,K for samples taken from selective experiment. The graph was plotted on a log₁₀ scale. There were significant differences between the total counts on R2A to the counts on the selective plates for all sample times except at 191 h. To run the experiment, the soil microbial consortium was introduced into the microcosm and MMN-glucose (0.9 g/L) was run for 119 h. At 119 h, the donor was introduced into the microcosm and the media changed to 50% R2A. At 144 h the media was changed to MMN-glucose media (0.9 g/L) with 100 $\mu\text{g/mL}$ of gentamicin. ^a Indicates a p-value of less than 0.05 between all three plate counts on R2A, R2A-G and LB-G,K plates. ^b Indicates a p-value of less than 0.05 between the total counts and both of the selective plates.

Table 2.5: The ratios between the plate counts on R2A-gentamicin were compared to total counts for both the selective and non-selective pressure experiments. The ratio of plate counts on R2A-gentamicin (R-G) plates to the total heterotrophic plate counts on LB (L) for the non-selective pressure experiment were designated as R-G/L, while the ratio for the counts on R2A-gentamicin (R-G) to the total heterotrophic plate counts on R2A (R) for the selective pressure experiment were designated R-G/R.

Time for non-selective (h)	Ratio of R-G/L for non-selective	Time for selective pressure (h)	Ratio of R-G/R for selective
24	0.00010	96	0.05450
48	0.00122	120	0.03225 ^a
114	0.00064	123	0.05807
120	0.00011	143	0.21505 ^c
143	0.00024 ^a	167	0.39676
146	0.00021	191	1.04956
166	0.00077 ^b		
190	0.00071		
214	0.00160		
237	0.00470		

^a The donor (1×10^8 cells) was added at t=142 h for non-selective pressure and a t=119 h for selective pressure, ^b media was changed to MMN-glucose at t=167 h (0.9 g/L), ^c media was changed to MMN-glucose with 100 µg/mL of gentamicin at t=144 h

The ratio between the counts on R2A-G to the total counts for both experiments increased after donor addition (Table 2.5). The ratio continued to increase after the media was changed to either MMN-glucose or MMN-glucose with gentamicin for the non-selective and selective pressure experiments, respectively (Table 2.5).

2.4 – DISCUSSION

Selecting a Recipient for pJP4

The fact that *Pseudomonas putida* and the mixed soil community, collected from Ryerson University, made good recipients for pJP4 was not surprising. The *Pseudomonas* genus is often cited as a recipient of pJP4 (DiGiovanni *et al.* 1996). As well, the recipients of pJP4 fall within the phylum of *Proteobacteria*, where only the *Alpha-* *Beta-* and *Gamma-proteobacteria* classes have been identified as recipients of pJP4 (Don and Pemberton, 1981). *Proteobacteria* actually dominate soil environments (Fierer *et al.* 2005), thereby, with *proteobacteria* as the dominant phylum, a soil microbial community would provide a high number of recipients for pJP4. As well, many publications refer to a variety of soil microorganisms as acceptors of the pJP4 plasmid and often use soil communities to examine pJP4 transfer (Filonov *et al.* 2010, DiGiovanni *et al.* 1996 and Newby, Josephson and Pepper, 2000). The genera often cited as recipients of pJP4 come from *Pseudomonas*, *Ralstonia*, *Burkholderia* and *Stenotrophomonas*, all of which are present in the soil environment (Goris *et al.* 2002).

Preliminary Microcosm Setup for Soil Microbial Community

Comparing the total counts on LB and R2A to the thickness measured on glass beads taken from the top and bottom ports, the biofilm thickness from the top port showed a high correlation to the total counts on R2A and LB plates (Figure 2.7). A relationship between biofilm thickness and total counts may be present if, when biofilm thickness increases, the total number of microorganisms in the effluent increases as the biofilm is shed and vice versa, though similar results would then be expected in terms of biofilm thickness in the bottom port, which was not seen (Figure 2.7 and Figure 2.8). A further explanation could be that the cells shed from

the top part of the microcosm, as well as in the effluent, resettled in the bottom part of the microcosm, allowing for the discrepancy in the correlation for the biofilm thickness between the two ports and between the thickness of the biofilm in the bottom port compared to the total heterotrophic plate counts. Furthermore, the total heterotrophic plate counts on both LB and R2A plates were highly related, suggesting that cultivatable microorganisms were able to grow in a similar fashion on the two different plate types, with the nutrients not significantly affecting the total counts (Figure 2.7). Also, the *tfdB* gene was identified in the soil inoculum as well as in the effluent samples (Figure 2.9), which was unexpected but not surprising as studies have previously found that untouched soil can harbor the ability to degrade 2,4-D, with a further discovery of microorganisms that carry a form of the *tfdB* gene (Vallaes *et al.* 2006). Single and double antibiotic resistance microorganisms were also cultivated from the effluent, which was not unanticipated, as many multiple-resistant species have been previously identified in the soil, some having as many as 7 antibiotic resistances (Wright, 2010). Antibiotic resistance has also been found for the antibiotic gentamicin (Wright, 2010), which was one of the antibiotics used in this study.

Donor Persistence

The donor persistence experiment was conducted in order to examine if the donor is able to persist within microcosm. Both the plate counts and PCR (Figure 2.10 and Figure 2.11) verified that the donor was present after 3 h. With a pore volume of 27.5 mL and a flow rate of 10 mL/h, the donor should have been removed from the microcosm after 2.75 h if it was not able to persist within the microcosm. However, after 2.75 h, the donor was still detected in the effluent, indicating that it was able to persist within the microcosm (Figure 2.10 and Figure

2.11). As well, at 4 h, the donor count actually increased (Figure 2.10). With a generation time of 1.85 h at RT (Figure 2.11), and the full volume being replenished every 2.75 h, the donor may be growing within the pore volume instead of forming a biofilm on the glass bead. However, the generation time was calculated at the exponential phase and did not take into account the lag phase, which may increase the generation time of the donor once it is inoculated into the microcosm.

Donor Persistence with Soil Inoculum

Donor persistence in the microcosm was also examined with prior inoculation of the soil microbial community, which was completed in order to examine if the soil community had any impact on donor persistence. The experiment was run for 28 h, with the PCR confirming donor through amplification of *gfp* for all sample times, including at 28 h (Figure 2.12). The donor was also confirmed through its presence on LB-G,K plates by identifying green fluorescent colonies through the dissection scope. The donor count decreased from 0.5-1.5 h, and then remained stable from 1.5-4 h (Figure 2.13). The number of microorganisms growing on the double antibiotic plates (G,K) was counted in order to identify if donor cell addition had any impact on the population of antibiotic resistant species (Figure 2.13). The total count of G,K resistant microorganisms declined by almost 30 times 0.5 h after donor addition (Figure 2.13). The antibiotic resistance community stabilized 1.5 h after donor addition, but did not return to its peak at 0 h (Figure 2.13). Thereby, the total number of antibiotic resistant microorganisms in the effluent declined after donor addition. The decrease in the total number of doubly resistant organisms may have been due to donor addition, which washed out any unattached cells. The donor and total counts on the double antibiotic plates were also highly correlated, which could be

related to the fact that many of the double-resistance antibiotic cells were washed out, with most of the double-resistance cells now representing the donor cells.

Microcosm Run with Soil and Donor with or without any Selective Pressure Persistence of Donor

The donor was able to persist for 98 h for both the selective and non-selective pressure experiment when 1×10^9 cells were added into the microcosm 4-5 d after the soil microbial community was inoculated (Figure 2.14). The donor count declined at 48 and 72 h for both experiments, but then increased at 98 h for the selective pressure experiment, while the donor count for the non-selective pressure experiment continued to decline at 98 h (Figure 2.14). This result may suggest that the donor thrives better in a minimal media environment with gentamicin, as the donor count was significantly higher for the selective pressure experiment than for the non-selective experiment at 98 h (Figure 2.14). However, the persistence of the donor was evident for both selective and non-selective pressure experiments (Figure 2.14). The reason that the donor may thrive better in an environment with gentamicin could be that it gains a competitive advantage over microorganisms that do not tolerate exposure to an increased gentamicin concentration (100 $\mu\text{g/mL}$ compared to 25 $\mu\text{g/mL}$ employed for plate counts).

Even though donor colonies were evident on selective plates, donor cells in the effluent could not be confirmed with PCR for *gfp* as bands were only seen for samples taken 1 and 4 h after donor inoculation for the non-selective pressure experiment and only for one sample taken 1 h after donor inoculation for the selective pressure experiment (Figure 2.15). Thereby, in order to confirm donor presence in the effluent, colonies that were deemed the donor through dissection microscopy were removed from selective plates and their DNA extracted; this was done for the selective pressure experiment. A band for *gfp*, after PCR, was seen for all sample

times when this methodology was used (Figure 2.16A). Thereby, even though PCR should detect *gfp* in the effluent, this method may not be fully reliable. Thus, multiple methods should be used to confirm for donor presence. PCR for *gfp* for the biofilm extracted from glass beads also gave a positive result for all sample times, except for the sample extracted at 4 hours from the top port, for the selective pressure experiment (Figure. 2.16B), which again confirmed the need for multiple methods in identifying donor presence in the microcosm.

Transfer of pJP4 in Selective and Non-selective Pressure Experiments

In order to identify transconjugants, DNA was extracted from effluent samples for both selective and non-selective pressure experiments and *dsRed* was PCR-amplified. An extra band at the position for *dsRed* was seen for the selective and non-selective pressure experiments compared to *gfp*. For the non-selective pressure experiment, only 2 bands for *gfp* were seen for samples taken 1 and 4 h after donor inoculation, while 3 bands were seen for *dsRed* for samples taken 1, 4 and 24 h after donor inoculation (Figure 2.15A and Figure 2.17A). Moreover, for the selective pressure experiment, 1 band was seen for *gfp* for the sample taken 1 h after donor inoculation, while 2 bands were seen for *dsRed* for samples taken 1 and 4 h after donor inoculation (Figure 2.15B and Figure 2.17B). A band for *dsRed* in a sample where no *gfp* band is present may indicate the presence of transconjugants as this gene can be extracted from both transconjugant and donor cells, while *gfp* can only be extracted from donor cells. While standard PCR is not quantitative, one can speculate that, as *dsRed* can potentially be extracted from a greater number of cells than *gfp*, the combined effect of transconjugant and donor cells may provide enough DNA for PCR-amplification of *dsRed*, where *gfp*, originating from only donor cells, may not. The same result can be seen with the biofilm extracted for the non-selective

pressure experiment, where no bands were visualized for *gfp*, while bands were present for *dsRed* at samples taken 48 and 72 h after donor inoculation from the bottom port (Figure 2.18). Again, a band for *dsRed*, where no *gfp* band was seen, may indicate the presence of transconjugants.

In addition, the biofilm attached to glass beads was extracted from both experiments and visualized for red fluorescence using CLSM. For the non-selective pressure experiment, only a few red fluorescent cells were evident throughout the entire experiment, with less than 10 red cells identified per sample time (Figure 2.20 and Table 2.4). For the selective pressure experiment, less than 10 red cells were visualized at 24 hours (top port), which was similar to the results found for the non-selective pressure experiment. However, 48 h after donor addition, which was 23 h after gentamicin was added to the media, more than 50 red cells were visualized (Figure 2.21 and Table 2.4). Also, 10-50 red cells were visualized 72 and 98 h after donor inoculation, which was 47 and 73 h after gentamicin was added, respectively (Figure 2.21 and Table 2.4). From these values, it was clear that a greater number of red cells were visualized for the selective pressure experiment, and only after gentamicin was added into the media (Table 2.4). It has been previously noted that, in the presence of antibiotics, the transfer of a plasmid with that resistance will increase (Ma and Bryers, 2012). In the presence of an antibiotic, bacterial cells in a biofilm with resistance to that antibiotic are aware of its presence in the environment, which in turn increases the transfer of the resistance gene (Ma and Bryers, 2012). Thereby, the addition of gentamicin seemed to exert sufficient selective pressure for the transfer of pJP4. The persistence of transconjugants, though, was evident for both the selective and non-selective pressure experiments; transconjugants were still visualized 72 and 98 h after donor inoculation, when the donor counts were low (Table 2.4 and Figure 2.14).

Transconjugants were isolated for both selective and non-selective pressure experiments. However, a slightly greater variety of transconjugants was identified for the selective pressure experiment. The same strain of *Stenotrophomonas maltophilia* was identified for 7 out of 8 transconjugants isolated for the non-selective pressure experiment, where the 8th strain was a different strain of *Stenotrophomonas maltophilia* (Table 2.2). Three different strains were identified for the selective pressure experiment for 5 of the isolated transconjugants; *Pseudomonas hibiscicola* and two different strains of *Stenotrophomonas maltophilia* (Table 2.3). A greater diversity of transconjugants may be related to an increase in the number of transconjugants visualized for the selective pressure experiment (Table 2.4). An increased transfer rate may increase the detection of a variety of different recipients of pJP4, as a higher number of microorganisms would have received the plasmid.

Essentially, however, pJP4 was transferred to the same genera under both selective and non-selective pressure, as *Pseudomonas hibiscicola* belongs to the *Stenotrophomonas maltophilia* group (*Pseudomonas hibiscicola* strain ATCC 19867, NCBI). As well, when the full 16S rRNA sequence for *Pseudomonas hibiscicola* was BLASTed, *Stenotrophomonas maltophilia* IAM 12423 and *Stenotrophomonas maltophilia* ATCC 19861 were the 2nd and 3rd closest matches, with a 99% similarity. Thereby, all three strains may be very closely related, again corroborating the fact that a high diversity of transconjugants was not found for either experiment.

Other studies have also documented a lack of diversity in recipients using either selective or non-selective pressure. In a study by Filonov *et al.* (2010), only *Pseudomonas* strains were isolated after the transfer of the plasmid pNF142::TnMod-OTc, capable of degrading naphthalene, in soil plots, either with or without naphthalene. Another study by DiGiovanni *et*

al. (1996), using the strain *Alcaligenes eutrophus* with the pJP4 plasmid, which was performed in a microcosm with unsterilized soil, only isolated three different species of transconjugants in 2 genera, *Pseudomonas* and *Burkholderia*. A study by Newby, Josephson and Pepper (2000) using 100-g of unsterilized soil with *Escherichia coli* ATCC 15224 as donor of pJP4, found 199 transconjugants that belonged to only two genera, *Pseudomonas* and *Burkholderia*.

In these experiments, only a few genera were identified as recipients, with or without selective pressure. These experiments were also conducted using a microcosm filled with unsterilized soil, whereas in this experiment, 10 µL of the glycerol stock was used, which may have reduced the variety of microorganisms used. Another issue could be that selecting transconjugants through plates may not be a very sensitive technique, especially as some microorganisms may not be cultivated. Thereby, some strains may have just not been isolated.

In conclusion, similar experiments conducted in soil microcosms or soil plots saw a lack of diversity in the types of transconjugants that were isolated, with only 1 or 2 genera being identified. Thereby, even though pJP4 can be transferred to *Alpha-* *Beta-* and *Gamma-proteobacteria*, some studies only isolated a few genera as recipients of pJP4. Furthermore, the transfer of pJP4 to *Ralstonia* and *Stenotrophomonas* has also been documented (Goris *et al.* 2002), which is consistent with this study, where recipients of pJP4 were identified as *Stenotrophomonas* strains.

Plate Counts and Biofilm Thickness from Selective and Non-selective Pressure Experiments

The general trend seen after donor addition was an increase in total count 1 h after donor inoculation, a decrease in total count 4 h after donor inoculation and an increase in total count 24 h after donor inoculation, which was seen for both experiments (Figure 2.22 and Figure 2.24).

The theory behind this recurring pattern is that due to a significant addition of donor cells into the microcosm, the added donor cells, as well as any unattached cells, were washed out of the microcosm; many cells being unable to attach to glass bead surfaces. After a few hours, when a significant number of introduced cells were washed out, the number of cells removed in the effluent decreased, which was seen at 4 h (Figure 2.22 and Figure 2.24). At 24 h, as the biofilm thickness increased, more cells were shed into the effluent. This increase in biofilm thickness, leading to an increase in total cell count, was corroborated with biofilm data at 4 and 24 h, where, in both experiments, biofilm thickness increased from 4 to 24 h in both the top and bottom sections of the microcosm (Figure 2.23 and Figure 2.25).

Moreover, for the selective pressure experiment, the biofilm thickness increased in response to the addition of gentamicin (Figure 2.25). This result was not surprising as biofilm thickness increases in response to antibiotics at either sub-inhibitory or inhibitory concentrations, especially from aminoglycosides like gentamicin (Hess *et al.* 2011 and Hoffman *et al.* 2005). In fact, at 195 h, 73 h after the media was changed to MMN-glucose with gentamicin, the biofilm thickness was at an all-time high of around 33 μm and 37 μm measured on glass bead taken from the top and bottom ports, respectively (Figure 2.25). Thereby, the biofilm thickness at 195 h was actually higher than when a rich medium, 50% R2A, was used. Thus, it may be possible that gentamicin can influence biofilm thickness to a greater extent than a rich medium. This increase in biofilm thickness can be a contributing factor in the increased number of transconjugant cells seen after gentamicin addition (Table 2.4). A study by Krol *et al.* 2011 found that recipient density actually limited conjugation events. Thereby, an increase in biofilm thickness could potentially increase the number of recipients, promoting greater interactions between donor and recipient cells and resulting in a higher number of transconjugants (Table 2.24).

Moreover, average biofilm thickness after media change to either MMN-glucose or MMN-glucose with gentamicin for non-selective or selective pressure experiments, respectively, remained high. However, the total count 47 h after gentamicin addition was at an all-time low, with a total count close to 3×10^5 CFU/mL, while the total count for the non-selective pressure experiment was comparatively higher, around 9×10^7 CFU/mL 47 h after media change to MMN-glucose (Figure 2.22 and Figure 2.24). Thereby, even though the number of cultivatable CFUs from the effluent declined dramatically for the selective pressure experiment, the biofilm thickness remained very high after gentamicin addition, which was comparatively different to the non-selective pressure experiment, where the total counts and the biofilm thickness remained high throughout (Figure 2.22, Figure 2.23, Figure 2.24 and Figure 2.25). This result suggests a direct response to the addition of gentamicin, where biofilm thickness increased though the number of microorganisms in the effluent decreased due to the inhibitory effect of gentamicin.

Furthermore, a comparison was made between the total heterotrophic plate counts and the counts on the two selective plates, R2A-G and LB-G,K for both experiments (Figure 2.26 and Figure 2.27). For the non-selective pressure experiment, there was a difference between the data when comparing the total heterotrophic plate counts on LB to the plate counts on the two selective plates (Figure 2.26). There was, however, no difference between the data when comparing the counts on the two selective plates (Figure 2.26). The counts on R2A-G were similar to the counts on LB-G,K for all sample times except 1 and 4 h after donor addition (Figure 2.26). The reason that there was no difference in count between the two selective plates was related to the fact that a large number of gentamicin resistance microorganisms were also doubly resistant to kanamycin. The reason that there was a significant difference between the 2 counts at 143 and 146 h was that there was an addition of another doubly resistant

microorganism, the donor. That would have changed the ratio of the number of gentamicin resistant colonies cultivated on R2A-G plates to the number cultivated on the LB-G,K plates, which showed an increase in colony number after donor addition. Even though the donor is resistant to gentamicin, it has a difficulty growing on R2A-G plates, as R2A is a less rich medium than LB, thus the number of microorganisms growing on these plates did not see a notable increase after donor addition (Figure 2.26). Once a lot of the donor cells were flushed out, the counts between the two plates was no longer different (Figure 2.26).

As well, for the selective pressure experiment, there was a difference in count between the total heterotrophic plate counts on R2A and the plate counts on the two selective plates (Figure 2.27). Also, there was a difference in count between the two selective plates (Figure 2.27). The reason for this difference in count between the two selective plates was due to a greater number of microorganisms being singly resistant to gentamicin. The only time the counts were not significantly different was 4 h after donor addition, which would have increased the total count on the LB-G,K plates due to the growth of a large number of donor cells (Figure 2.27). Thereby, at that time point, the two selective plate counts were similar, until the donor count declined, and with it, the similar counts between the two selective plates (Figure 2.27). The general difference in the counts between the two selective plates for the selective pressure experiment and the general lack of difference in the counts for the non-selective pressure experiment may have occurred by chance. As only 10 μ L of the glycerol stock solution was used to grow the soil microbial community, some microorganisms may have been preferentially withdrawn more than others, or else certain microorganisms may have been outcompeted by others within the microcosm after inoculation.

For the selective pressure experiment, 72 h after donor inoculation and 47 h after gentamicin addition, the total heterotrophic plate counts on R2A compared to the two selective plate counts were not different (Figure 2.27). This lack of difference between the counts was related to the change in the media, where gentamicin was added. This result suggests that the total number of cultivatable microorganisms in the effluent were comprised mostly of microorganisms that were resistant to gentamicin, confirming the capability of gentamicin, at a concentration of 100 µg/mL, to select for microorganisms resistant to gentamicin. This result does not mean that the remaining microorganisms in the biofilm were resistant to gentamicin, just that most of the microorganisms being discharged in the effluent, and those cultivatable, were not that different to the number of those resistant to gentamicin.

There was also great increase in the number of gentamicin resistant species after donor addition (Figure 2.26, Figure 2.27 and Table 2.5), which could be explained as an increase in the number of transconjugants. One argument against this proposition could be that some of the gentamicin resistant species were selected for by the use of a rich medium, 50% R2A. However, even after the media was changed to MMN-glucose for the non-selective pressure experiment, the ratio of gentamicin resistant species to the total count steadily increased (Table 2.5). The same result was also noted for the selective pressure experiment, where the ratio of gentamicin resistant species to the total count also increased; a 2x from 143 to 167 h, which was 23 h after gentamicin was added (Table 2.5). An increasing ratio of gentamicin resistant species to total count, especially after media change to MMN-glucose or MMN-glucose with gentamicin for non-selective or selective pressure, respectively, may, as mentioned before, indicate an increasing number of transconjugants. For the selective pressure experiment, any previously

existing gentamicin resistant species may have also been selected for by the addition of gentamicin.

In conclusion, a higher number, as well as diversity, of transconjugants was found for the selective pressure experiment. Biofilm thickness was also highest after the addition of gentamicin, which may have increased the efficiency of plasmid transfer.

CHAPTER 3: OVERALL CONCLUSIONS AND FUTURE RECOMMENDATIONS

The specific objectives of the project were to design and construct a microcosm, identify whether donor cells can survive for a sufficient period of time for plasmid transfer to occur and to determine the effect of selective and non-selective conditions on biofilm structure and consequently plasmid transfer. In conclusion, a microcosm simulating a porous aquifer was constructed and it was discovered that the addition of a large number of donor cells (1×10^9) boosted donor persistence in a minimal medium environment, where they were able to persist for a considerable amount of time. Furthermore, using antibiotics as a selective pressure increased the transfer of pJP4. Moreover, biofilm thickness increased in response to gentamicin addition, an effect that may have contributed to the increase in the number of visualized transconjugant cells.

To continue this project, DGGE should be conducted on effluent samples from the selective and non-selective pressure experiments in order to identify the effect of gentamicin on the microbial profile. As well, the ability of the isolated transconjugants to degrade 2,4-D should be established. In order to do this, the rate of 2,4-D degradation should be examined with each of the isolated transconjugants. As well, the 16S rRNA of the isolated transconjugants should be sequenced again using different primers, which amplify a greater length of the 16S rRNA gene, in order to confirm the identity of the isolated strains.

Moreover, in order to investigate whether the donor strain is capable of persisting without the use of a rich medium, the same selective and non-selective experiments should be run without the use of 50% R2A after donor inoculation (using 1×10^9 cells). As well, a method of quantifying the number of red transconjugant cells should be put into place, as a sufficient method of quantifying transconjugants was not established. Flow cytometry could be

investigated as a potential method of separating and quantifying transconjugants in effluent as well as homogenized biofilm samples. Lastly, 2,4-D should also be used as a selective pressure to compare it to the use of antibiotics as a selective pressure.

APPENDICES

Appendix A: CLSM IMAGES

Figure 5.1 CLSM images for controls. No fluorescence was visualized for the R2A spot-plated soil control at 1260x magnification using transmission mode and the 543nm and 488 nm lasers (A). Green fluorescence was visualized for the R2A spot-plated donor *P. putida* SM1443 strain at 630x magnification using transmission mode and the 543nm and 488 nm lasers (B).

Figure 5.2 CLSM images for groundwater and peat conjugation experiments. Cells taken from mating patch where a groundwater community and *P. putida* SM1443 donor were conjugated predominantly showed green fluorescence, where only a few cells fluoresced red (A). Cells from a mating patch where a peat community and *P. putida* SM1443 donor were conjugated showed both green and red fluorescence (B). Images were taken at 630x using both 488 and 543 nm lasers.

Figure 5.3 CLSM images of biofilm on glass beads from non-selective pressure experiment. Glass beads were stained with SYTO62 and visualized by using the 633 nm laser. Images of biofilm from non-selective pressure experiment 24 h after inoculation of soil microbial community (top port) (A) and 166 h after the inoculation of soil microbial community, or 24 h after the inoculation of donor (top port) (B).

Figure 5.4 Biofilm examined under CLSM from glass beads for selective pressure experiment. The biofilm was removed from glass bead with 5 minutes of vortexing; the cells were concentrated using centrifugation. CLSM was used to visualize red or green fluorescence cells using 548 and 488nm lasers, respectively. Samples are showing CLSM images of biofilm slurry 48 h after donor inoculation, top port (A), and the biofilm slurry taken 72 hours after donor inoculation, top port (B).

Appendix B: GRAPHS

Figure 5.5 Peat growth curve and CFU/mL to OD measurements. The peat (taken from the glycerol stock) growth curve was conducted in 100% R2A at room temperature ($22 \pm 2^\circ\text{C}$) in a shaking incubator set at 290 RPM. Total counts were compared to OD measured using a spectrophotometer at 660 nm (A). The CFU/mL in relation to OD of peat sample was measured for a total of 30 h (B). Solid line represents a best fit trendline.

Figure 5.6 Soil growth curve and CFU/mL to OD measurements. Ryerson University soil culture (taken from glycerol stock) was grown in 100% R2A at room temperature ($22 \pm 2^\circ\text{C}$) in a shaking incubator at 290 RPM. Total counts were compared to OD that was measured using a spectrophotometer at 660 nm (A). CFU/mL in relation to OD for the soil microbial community was measured for a total of 6 h (B). Solid line represents a best fit trendline.

Figure 5.7 Donor growth curve and CFU/mL to OD measurements. The donor culture (taken from glycerol stock) was grown in 100% LB with gentamicin (25 $\mu\text{g/mL}$) and kanamycin (100 $\mu\text{g/mL}$) at room temperature ($22 \pm 2^\circ\text{C}$) in a shaking incubator at 290 RPM. Plate counts were compared to OD measurements at 600nm (A). CFU/mL in relation to OD of the donor sample was measured for a total of 22.5 h (B). Solid line represents a best fit trendline.

Appendix C: AGAROSE GELS

Figure 5.8 Gel for *gfp*, *dsRed* and *tfdB* for donor. A 1% gel was run at 85 volts for 45 minutes. DNA from donor cells was extracted for *gfp* PCR (A). Both DNA extraction and plasmid extraction were performed and PCR conducted for *dsred* (B), and *tfdB* (C). The 1st band originated from total DNA extraction and the 2nd band originated from plasmid extraction using donor cells and *dsRed* and *tfdB* were PCR amplified (B and C).

Appendix D: TABLES

Table 5.1 The CFU/mL on LB and selective plates for non-selective pressure. The CFU/mL on R2A plates, R2A-gentamicin and LB-gentamicin/kanamycin plates, as well as the donor CFU/mL, for effluent samples for the non-selective pressure experiment

Time (h)	CFU/mL on LB plates	CFU/mL on R2A-gentamicin plates	CFU/mL on LB-gentamicin/kanamycin plates	Donor CFU/mL
24	7.9×10^7	8.2×10^3	1.11×10^4	N/A
48	2.295×10^8	2.81×10^4	2.565×10^4	N/A
114	1.07×10^8	6.8×10^4	4.9×10^4	N/A
120	8.85×10^7	1×10^4	1.035×10^4	N/A
143	3.6×10^8	8.5×10^4	2.225×10^6	$2.1 \times 55 \times 10^6$
146	5.75×10^7	1.19×10^4	8.35×10^4	6.45×10^4
166	2.34×10^8	1.8×10^5	1.79×10^5	6.35×10^4
190	2.61×10^8	1.84×10^5	4.09×10^5	1.55×10^4
214	8.9×10^7	1.42×10^5	1.525×10^5	1.7×10^3
238	7.05×10^7	3.3×10^5	3.65×10^5	7.0×10^2

Table 5.2 The CFU/mL for R2A and selective plates for selective pressure. The CFU/mL on R2A plates, R2A-gentamicin and LB-gentamicin/kanamycin plates, as well as the donor CFU/mL, for effluent samples for the selective pressure experiment

Time (h)	CFU/mL on R2A plates	CFU/mL on R2A-gentamicin plates	CFU/mL on LB-gentamicin/kanamycin plates	Donor CFU/mL
24	N/A	3.9×10^5	7.1×10^4	N/A
96	2.055×10^8	1.12×10^7	2×10^6	N/A
120	2.76×10^8	8.9×10^6	2.55×10^6	2.6×10^5
123	7.75×10^7	4.5×10^6	1.065×10^6	2.65×10^5
143	2.33×10^8	5×10^7	2.51×10^6	6×10^3
167	1.24×10^8	4.9×10^7	1.585×10^6	4.5×10^3
191	3.43×10^5	3.6×10^5	9.1×10^5	2.75×10^4
217	N/A	1.02×10^6	9.1×10^5	2.75×10^4

Table 5.3 CFU/mL of donor colonies and total number of colonies on selective plates for the donor persistence experiment with soil inoculum

Time (h)	CFU/ml of donor colonies on LB-gentamicin/kanamycin plates	CFU/mL of total colonies on LB-gentamicin/kanamycin plates
0	0	2.79×10^6
0.5	4.8×10^4	9.5×10^4
1	3.65×10^4	1.16×10^5
1.5	8.2×10^3	3.5×10^4
2	7.25×10^3	3.65×10^4
2.5	4.55×10^3	4.5×10^4
3	3.75×10^3	3.85×10^4
4	4.65×10^3	4.4×10^4

Appendix E: SEQUENCE DATA

Table 5.4: The sequences of isolated transconjugants received by BLASTing individual sequences against similar 16S-rRNA sequences for bacteria and archaea in the nucleotide database in NCBI.

Closest match	Source	Sequence	% identity	Accession number of closest match
<i>Pseudomonas panacis</i> strain CG20106 or <i>Pseudomonas synxantha</i> strain IAM1256	Ryerson University soil and donor plate mating	gntttacaatccgaagaccttcttcacacacgcggcatggctggatcaggctttcgccattgtccaatattccccactgctgcctcccgtaggagtctggaccgtgtctcagttccagtggtgactgatcctctcagaccagttacggatcgtcgcttggtgagccattacccaccaactagctaataccgacctaggtcatctgatagcgcaaggcccgaagggtcccctgtttctccgtaggacgtatgcggtattagcgtccgtttccgaacgttatccccactaccaggcagattcctaggcattactacccgtccgccgtctcaagagaagcaagcttctctaccgctcgactgcatgtgttaggcctgccg ccagcgttcaatctgagccaggatcaaacnennngt	100	NR_043195.1 or NR_043425.1
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 4 h after donor inoculation from effluent for non-selective pressure experiment	tttctttcccaacaaaagggtttacaacccgaaggccttcttcacca cgcggatggctggatcaggcttgcgccattgtccaatattccccact gctgcctcccgtaggagtctggaccgtgtctcagttccagtggtg atcatcctctcagaccagctacggatcgtcgcttggtgggcctttacc ccgccaactagctaataccgacatcggtcattcaatcgcgcaagggtcc gaagatcccctgtttcacccgtaggctgtatgcggtattagcgttaagt ttccctacgttatccccacgacagagtagattccgatgtattcctacc cgtccgccactcgccaccagagagcaagctctctgtgtgccgtt cgacttgcatgtgttaggcctaccgccagcgttactctgagccagga tcaaacac	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> ATCC 19861	Transconjugant isolated 72 h after donor inoculation from effluent for non-selective pressure experiment	Reverse sequence: ctttacaacccgaaggccttcttcacccacgcggatggctggatcag gcttgcgccattgtccaatattccccactgctgcctcccgtaggagtc tggaccgtgtctcagttccagtggtgctgatcctctcagaccagct acggatcgtcgccentgggtgggcctttaccccgccaactagctaatacc gacatcggtcattcaatcgcgcaagggtccgaagatcccctgtttca cccgtaggctgtatgcggtattagcgttaagttccctacgttatccccca cgacagagtagattccgatgtattcctacccgtccgccactcgccac ccagagagcaagctctctgtgtgccgttcgactgcatgtgttagg cctaccgccagcgttcacnntgag Forward sequence: atcggatctactctgtcgtgggggataacgtagggaaacttacgcta ataccgatacctacggntgaagcaggggatcttcggaccttgc gcgattgaatgagccgatgtcggttagctagttggcggggtaaagg ccaccaaggcgacgatccgtagctgggtctgagaggatgatcagcc acactggaactgagacacgggtccagactcctacgggagggcagcagt ggggaatattggacaatgggcgcaagcctgatccagccataaccgcg tgggtgaagaaggccttcgggtgtaaagccctttgttgnгааагаа	99	NR_040804.1

Closest match	Source	Sequence	% identity	Accession number of closest match
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 24 h after donor inoculation from effluent for non-selective pressure experiment	agggctttacaacccgaaggcctttcaccacgcggtatg gctggatcaggcttgcgccattgtccaattccccactgct gcctcccgtaggagtctggaccgtgtctcagttccagtgtgg ctgatcatcctctcagaccagctacggatcgtcgccttggtgg gcctttaccccgccaactagctaattccgacatcggtcattca atcgcgcaaggtccgaagatcccctgctttaccgtaggtc gtatgcggtattagcgtaagtttcctacgttatccccacgac agagtagattccgatgtattcctcaccgctccgccaactcgcca cccagagagcaagctctcctgtgctgccgttcgacttgcattgt gttaggcctaccgccagcgttcactctgagccaggatcaaac nc	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 48 h after donor inoculation from effluent for non-selective pressure experiment	gggctttacaacccgaaggcctttcaccacgcggtatgg ctggatcaggcttgcgccattgtccaattccccactgctg cctcccgtaggagtctggaccgtgtctcagttccagtgtggct gatcatcctctcagaccagctacggatcgtcgccttggtggg cctttaccccgccaactagctaattccgacatcggtcattcna tcgcgcaaggtccgaagatcccctgctttaccgwgaggtc gtatgcggtattagcgtaagtttcctacgttatccccacgac agagtagattccgatgtattcctcaccgctccgccaactcgcca cccagagagcaagctctcctgtgctgccgttcgacttgcattgt gttaggcctaccgccagcgttcactctgagccaggatcaaac nc	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 48 h after donor inoculation from effluent for non-selective pressure experiment	ttgcgccattgtccaattccccactgctgcctcccgtagga gtctggaccgtgtctcagttccagtgtggctgatcatcctctca gaccagctacggatcgtcgccttggtgggcctttaccccgcc aactagctaattccgacatcggtcattcaatcgcgcaaggtc cgaagatcccctgctttaccgtaggtcgtatgcggtattag cgtaagtttcctacgttatccccacgacagagtagattccg atgtattcctcaccgctccgccaactcgccaccagagag caagctctcctgtgctgccgttcgacttgcattgtgttaggccta ccgccagcgttcactctgagccaggatcaaacnc	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 72 h after donor inoculation from effluent for non-selective pressure experiment	ctggatcaggcttgcgccattgtccaattccccactgctg cctcccgtaggagtctggaccgtgtctcagttccagtgtggct gatcatcctctcagaccagctacggatcgtcgccttggtggg cctttaccccgccaactagctaattccgacatcggtcattcaat cgcgcaaggtccgaagatcccctgctttaccgtaggtcgt atgcggtattagcgtaagtttcctacgttatccccacgaca gagtagattccgatgtattcctcaccgctccgccaactcgccac ccagagagcaagctctcctgtgctgccgttcgacttgcattgtg ttaggcctaccgccagcgttcactctgagccaggatcaaacn cnntg	99	NR_041577.1

Closest match	Source	Sequence	% identity	Accession number of closest match
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 72 h after donor inoculation from effluent for non-selective pressure experiment	ctttacaacccgaaggcctttcaccacgcggtatggctgg atcaggcttgcgcccattgtccaatattccccactgctgcctcc cgtaggagctcggaccgtgtctcagttccagtgtggctgatca tccttcagaccagctacggatcgtcgcttggtgggcc ttaccccgccaactagctaataccgacatcggtcattcaatc gcgcaagggtccgaagatccctgctttaccgtaggtcgta tgcggtattagcgtaagttccctacgttatccccacgacag agtagattccgatgtattcctacccgtccgccaactcggcacc cagagagcaagctcctctgtgctgccgttcgacttgcatgtgt taggcctaccgccagcgttactctgagccaggatcaaacn cc	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> IAM 12423	Transconjugant isolated 96 h after donor inoculation from effluent for non-selective pressure experiment	tttctttcccaaaaaagggtttacaacccgragcctttcac ccacgcggtatggctggatcaggcttgcgcccattgtccaat attccccactgctgcctcccgtaggagtctggaccgtgtctca gttcagtgtggctgatcatcctctcagaccagctacggatcg tcgcntgggtgncctttaccccgccaactagctaataccgac atcggtcattcaatcgcgcaagggtccgaagatccctgcttt caccgtaggtcgatgcggtattagcgtaagttccct acnttatcccc	98	NR_041577.1
<i>Pseudomonas hibiscicola</i> strain ATCC 19867	Transconjugant isolated 1 h after donor inoculation from effluent for selective pressure experiment	aaagggctttacaacccgaaggcctttcaccacgcggtga tggttgatcaggcttgcgcccattgtccaatattccnactg ctgcctcccgtaggagtctggaccgtgtctcagttccagtgtg gctgatcctctcagaccagctacggatcgtcgcttggtg ggcctttaccccgccaactagctaataccgacatcggtcattc aatcgcgcaaggcccgaagggtccctgctttaccgtaggt cgtatgcggtattagcgtaagttccctacgttatccccacga aaaagtagattccgatgtattcctacccgtccgccaactcgc acccaaggagcaagctcctctgtgctgccgttcgacttgcat gtgttaggcctaccgccagcgttnactctgagcc	98	NR_024709.1
<i>Pseudomonas hibiscicola</i> strain ATCC 19867	Transconjugant isolated 4 h after donor inoculation from effluent for selective pressure experiment	ctggatcaggcttgcgcccattgtccaatattccccactgctg cctcccgtaggagtctggaccgtgtctcagttccagtgtggct gatcatcctctcagaccagctacggatcgtcgcttggtggg cctttaccccgccaactagctaataccgacatcggtcattcaat cgcgcaaggcccgaagggtccctgctttaccgtaggtcgt atgcggtattagcgtaagttccctacgttatccccacgaaaa agtagattccgatgtattcctacccgtccgccaactcggcacc caaggagcaagctcctctgtgctgccgttcgacttgcatgtgt taggcctaccgccagcgttactctgagccagnntnnaaact cnnt	99	NR_024709.1

Closest match	Source	Sequence	% identity	Accession number of closest match
<i>Stenotrophomonas maltophilia</i> strain ATCC 19861	Transconjugant isolated 24 h after donor inoculation from effluent for selective pressure experiment	acaaaagggtttacaacccgaagcctttcaccacgcgg tatggctggatcaggcttgcgccattgtccaattccccact gctgctcccgtaggagtctggaccgtgtctcagttccagtgt ggctgatcctctcagaccagctacggatcgtcgccttggt gggcctttaccccgccaactagctaaccgacatcggctcatt caatcgcgcaagggtccgaagatcccctgcttcacccgtagg tcgtatgcggtattagcgtaaagttccctacgttatccccacg acagagtagattccgatgtattcctcaccgctccgcccactcgc caccannnannaagntcctcngtgcgtgnc	99	NR_040804.1
<i>Stenotrophomonas maltophilia</i> strain IAM 12423	Transconjugant isolated 48 h after donor inoculation from effluent for selective pressure experiment	tgnatttctttccaacaaaagggtttacaacccgaaggcctt cttcaccacgcgggtatggctggatcaggcttgcgccattgt ccaatattccccactgctgctcccgtaggagtctggaccgtg tctcagttccagtgtggctgatcctctcagaccagctacg gatcgtcgccttggtgggcctttaccccgccaactagctaate cgacatcggctcattcaatcgcgcaagggtccgaagatcccct gcttcacccgtagggtcgtatgcggtattagcgtaaagttccct acgttatccccacgacagagtagattccgatgtattcctcac ccgtccgcccactcggcaccagagagcaagctcctgtgct gccgttcgacttgcattgttaggcctaccgccagcggtcact ctgagccagnntcaaa	99	NR_041577.1
<i>Stenotrophomonas maltophilia</i> strain ATCC 19861	Transconjugant isolated 72 h after donor inoculation from effluent for selective pressure experiment	caaaagggtttacaacccgaagcctttcaccacgcgg tatggctggatcaggcttgcgccattgtccaattccccact gctgctcccgtaggagtctggaccgtgtctcagttccagtgt ggctgatcctctcagaccagctacggatcgtcgccttggt gggcctttaccccgccaactagctaaccgacatcggctcatt cnatcgcgcaagggtccgaagatcccctgcttcacccgtagg tcgtatgcggtattagcgtaaagttccctacgttatccccacg acagagtagattccgatgta	99	NR_040804.1

*All isolated transconjugants were from the *Gammaproteobacteria* class

APPENDIX F: METHODS FOR FUTURE WORK

DGGE

Four hundred to five hundred ng of 16S rRNA PCR samples are loaded in a 8% polyacrylamide gel with 30-70% denaturants as a gradient using the DCode Universal Mutation Detector system (BioRad Laboratories, Mississauga, ON). The gel is run at 80 volts for 16 h at 60°C and is stained in 0.5 h in a 1:10000 dilution (25µl of SYBR Gold in 250 mL of 1xTAE) of SYBR Gold (Invitrogen Cat# S11494) with shaking and de-stained for 10-15 minutes in 250 mL of 1xTAE with shaking. The individual are excised, diluted in 50µl of dH₂O and kept at 4°C for 48-72 h to prepare the eluted DNA for sequencing. PCR is used to re-amplify the DNA using the same primers (no GC-clamp this time). The amplification begins with a denaturation step at 96°C for 5 minutes, then 30 cycles beginning with a temperature of 94°C for 1 min, a temperature of 60°C for 30 sec, and a temperature of 72°C for 1 min. The products are purified using the IBI Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA) before sequencing.

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