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Transfer of the Catabolic Plasmid Pjp4 in Bacterial Biofilms in Lab-Scale Continuous Flow-Through Cells

Farhana Shamsad
Ryerson University

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by

Farhana Shamsad

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ABSTRACT:

Limited information is available on how external environmental factors (e.g., the type of carbon source) affect biofilm architecture, conjugative transfer of the plasmid pJP4 and xenobiotic degradation in biofilms. The aim of this project was to investigate the influence of glucose and 2,4-dichlorophenoxyacetic acid, two different carbon sources which represent the absence and presence of selective pressure, respectively, on the combined effect of biofilm architecture, transfer of the plasmid pJP4 in soil derived mixed culture biofilms and consequent biodegradation of 2,4-D. The pJP4 plasmid was transferred to soil-derived mixed culture recipients in plate mating experiments and isolated transconjugant colonies were characterized as *Comamonas testosteroni*. Donor and transconjugant cells were not detected microscopically in biofilms and no transconjugant colonies were isolated; however, *gfp*, *dsRed*, and *tfdB* genes were detected in biofilm effluents with and without selective pressure. Heterogeneous biofilm architecture was observed for both with and without selective pressure.

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List of Abbreviations

2,4-D : 2,4-Dichlorophenoxyacetic acid

5(6)-CFDA,SE : 5-(and-6)-Carboxyfluorescein diacetate, succinimidyl ester

CLSM : Confocal laser scanning microscopy

DGGE : Denaturing gradient gel electrophoresis

EDTA : Ethylenediaminetetraacetic acid

FAB : Fastidious anaerobe broth

GFP : Green fluorescent protein

gfp : Gene encoding green fluorescent protein

HGT : Horizontal gene transfer

HPLC : High performance liquid chromatography

IPTG : Isopropyl-β-D-thiogalactopyranosid

LB : Luria bertani

MMN : Melin norkrans medium

RFP : Red fluorescent protein

dsRed : Gene encoding red fluorescent protein

SBBR : Sequencing batch biofilm reactor

TAE : tris base, EDTA, acetic acid

CHAPTER 1. INTRODUCTION

1.1. Project significance:

Numerous synthetic products are used by modern industries and agricultural processes. These products are continuously released into the environment (Neilson et al. 1992). Some of these compounds are very resistant to microbial degradation (Neilson et al. 1985). Others are degraded and utilized as carbon sources. 2,4-dichlorophenoxyacetic acid (2,4-D) is a chlorinated compound and has been widely used as a herbicide (Bhat et al. 1993). It has become a significant environmental pollutant (Bhat et al. 1994). Many bioremediation studies have focused on chloroaromatic compounds, including the herbicide 2,4-D (Aspray et al. 2005). Pemberton and Fisher (1977) first reported the involvement of plasmids in the degradation of 2,4-D. Pemberton et al. and others worked on the detailed characterization of the 2,4-D degradative plasmid pJP4 from *Alcaligenes eutrophus* JMP134.

Some studies have concentrated on the effect of environmental conditions including flow rate (Venugopalan et al. 2005) and nutrient availability (Wolfaardt et al. 1994a) on biofilm development and on plasmid transfer (Smets et al. 1995). But limited information is available on how the external environmental factors (different carbon sources) will affect biofilm characteristics, plasmid transfer and xenobiotic degradation in biofilms. In this research project, we investigated the effects of different carbon sources (glucose or 2,4-D) on biofilm architecture, plasmid transfer and persistence in biofilms and the degradation of 2,4-D in biofilms. Glass surface was used as attachment surface of biofilm. For plasmid transfer event, *Pseudomonas putida* SM1443::*gfp*2x which carries plasmid pJP4::*dsRed* was used as donor strain. Plasmid pJP4 carries genes for the degradation of 2,4-D and 3- chlorobenzoate (Don and Pemberton

1981). Plasmid pJP4 is a broad host range conjugative plasmid, which belongs to the incompatibility group P1. It has been shown to be transferred between bacterial cells at high rates (Bathe et al. 2004a).

1.2. Objectives and hypothesis:

The overall goal of this project was to determine the effect of varying environmental conditions on biofilm architecture and subsequently the effect of varied biofilm architecture on plasmid transfer.

- To describe biofilm architecture in the absence of selective pressure (glucose) or presence of selective pressure (2,4-D).
- To determine the extent of transconjugant formation in the absence of selective pressure (glucose) or presence of selective pressure (2,4-D) and to infer possible relationships about plasmid transfer events and biofilm architecture.

It was hypothesized that the donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* could transfer the plasmid to the members of a soil-derived microbial community both in the absence and presence of selective pressure and that in the presence of selective pressure (2,4-D) plasmid transfer would occur more frequently than in the absence of 2,4-D. It was hypothesized that biofilm architecture would be different in the presence versus the absence of selective pressure.

1.3. Literature review:

1.3.1. Biofilms:

Horizontal gene transfer rates are typically higher in biofilm communities compared with those in planktonic states. Biofilms provide excellent conditions for bacterial interaction because of high cell density and well organized microbial community allowing physical cell-cell contact (Madsen et al. 2012). Microorganisms often live within surface associated multicellular communities known as biofilms (Branda et al. 2005). In biofilms, constituent cells are held together by an extracellular matrix composed of exopolysaccharides (EPS), proteins and sometimes nucleic acids of biogenic origin (Whitchurch et al. 2002, Branda et al. 2005). They protect bacteria from environmental stresses and provide spatial stability (Stoodley et al. 2004, Hausner et al. 1999). Biofilm formation is characterized by the interaction of cells with surface or interface as well as with each other. Bacterial biofilms are found in diverse environmental conditions, e.g. water pipe lines, on hospital devices as well as in natural environments.

1.3.2. Biofilm imaging:

The architecture (physical structure) of biofilms in laboratory flow cells can be monitored by confocal laser scanning microscopy (Xavier et al. 2003). Confocal laser scanning microscopy (CLSM) in conjunction with quantitative image analysis allows for examination of the three dimensional structure of biofilms. COMSTAT (Heydorn et al. 2000), ISA and PHLIP (Xavier et al. 2003) are some of the image analysis software most commonly use to quantify biofilm parameters such as mean thickness, roughness, substratum coverage and surface to volume ratio.

1.3.3. Biofilm architecture:

Biofilm architecture refers to the physical structure of biofilms. The development of different biofilm architectures depends on physical and biological factors, resulting from an interaction of mass transfer, conversion rates, or detachment forces (Loosdrecht et al. 2002). Mass transfer refers to the process by which dissolved and particulate substances are moved into and out of the biofilms by the surrounding fluid. Detachment refers to loss of single cells or aggregates of cells from the biofilm. Detachment can be an active process (dispersal), an induced mechanical process (e.g. through fluid shear) or a chemical process (by adding agents that dissolve the EPS matrix) (Stoodley et al. 2004). There are several ways in which distinct biofilms are formed. Diffusion limited biofilms have heterogeneous and porous structure. High detachment (or shear) forces create compact biofilms. Low detachment force gives rise to porous biofilm and detachment occurs mainly by sloughing (Loosdrecht et al. 2002). The physical structure of biofilm growth may play an important role in biofilm processes (Wuertz et al. 2004), including transfer of genetic information.

1.3.4. Mobile genetic elements:

Mobile genetic elements that encode catabolic genes play a major role in the adaptation of microbial populations to xenobiotic organic compounds (Top et al. 2002). Examples of mobile genetic elements include plasmids, bacteriophages, conjugative transposons and integrative conjugative elements (Slater et al. 2008).

Plasmids are extrachromosomal self-replicating elements. Self transferable plasmids are vehicles for the transmission of genetic information between species (Thomas 2000). The term plasmid was introduced by Lederberg, 1952. In bacteria essential genes (genes necessary for growth and

reproduction) are present in a large circular chromosome. Plasmids carry genes that are not normally needed by their bacterial host for growth and reproduction, but the host cells may benefited by these genes under certain conditions (Schumann, 2008). Plasmid can be classified by several criteria (e.g. conjugative or mobilizable, incompatibility groups, copy number, host range).

Plasmids are found in almost all bacterial species. Their size can range from 1kb to greater than 200 kb. Some phenotypes conferred by different plasmids are i) resistance to antibiotics, ii) production of antibiotics, iii) production of bacteriocins (proteins that can kill cells of the same or closely related species) iv) degradation of complex organic compounds or v) production of virulent factors (Schumann, 2008).

Bacteriophages are viruses that infect prokaryotes. They can integrate into the host genome and then be vehicles for horizontal gene transfer (Canchaya et al. 2003). Integrative conjugative elements are self transferable conjugative elements that integrate into the genome of new hosts (Burrus et al. 2004). Transposable elements are genetic elements that can move within or between replicons by action of their transposase (Mahillon et al. 1998).

Genes that encode the degradation enzymes for xenobiotics are often located on mobile genetic elements (MGEs) (Nojiri et al. 2004). Leisinger (1983) defined the xenobiotic compounds as guest chemicals, not natural to the environment. Industrial activity over the past century resulted in release of various quantities of xenobiotic compounds into the environments [Nojiri et al. 2003]. These compounds create a strong selective pressure for the development of bacterial strains with improved degradative capabilities. Under favourable environmental conditions, indigenous microbial populations show the ability to degrade the recalcitrant substrate.

Molecular analysis of the catabolic pathways of xenobiotic degrading bacteria indicated that they might have adapted to such compounds by expressing new functions (such as by the recruitment of enzymes involved in the degradation of other naturally occurring compounds) to resist the potential toxic effects of the molecules (Copley 2000) or microorganisms in the environment can use a number of xenobiotic compounds as sources of carbon and energy (Tsuda et al. 1999). The genetic information encoding these metabolic activities is often found on plasmids or other mobile elements (Tsuda et al. 1999).

Microbial communities can adapt to the presence of xenobiotics in the environment by several mechanisms: i) there can be an increase in population size of those organisms that tolerate or degrade the compounds by induction of appropriate genes, ii) the cells can adapt by various kinds of mutations ranging from single nucleotide insertions, deletions or substitution or DNA rearrangements. Gene conversion, gene duplication and transposition play important roles in the rearrangement of DNA fragments. iii) Horizontal gene transfer (e.g. conjugation, transformation, and transduction) among microbial communities is an important mechanism for genetic adaptation (Lie et al. 1993). Catabolic genes are often carried on plasmids (Bathe et al.2010). Major catabolic MGEs include plasmids, transposons and conjugative transposons (Nojiri et al. 2003).

1.3.4.1. Catabolic plasmids:

The first reports of plasmid associated catabolic pathways were made in early 1970's. The catabolic pathways were for complex octane, naphthalene, salicylate, toluene and these all were found in *Pseudomonas* (Williams 2004). Catabolic plasmids are accessory DNA elements present in the cytoplasm of many soil bacteria. They encode genes which confer the ability to degrade naturally occurring and synthetic molecules (Pemberton et al. 2001).

Many synthetic chemicals are resistant to degradation when released into soil, water and air. Repeated use and release of these synthetics are causing environmental pollution. The degradation of these compounds is carried out by bacteria where as some compounds are recalcitrant (Sayler et al.1990). Many enzymatic steps are needed to degrade these compounds. These degradative or catabolic activities are encoded by genes on the main chromosome or on catabolic plasmids. Sometimes these catabolic genes occur within transposable elements and they are between plasmids and the main chromosome (Pemberton et al. 2001).

Catabolic plasmids are commonly large (more than 50 kb). Degradation of naturally occurring compounds seems to be often encoded by IncP-2 and IncP-9 plasmids. On the other hand, degradation of man-made compounds often encoded by broad host range IncP-1 plasmids. IncP-1 plasmids are self-transmissible and have wider host range than IncP-2 and IncP-9 plasmids (Top et al. 2000). The degradative capability of the host can differ according to the bacterial species and conditions under which it is cultured (Shintani et al. 2010). Table 1.1 shows a list and characteristics of frequently investigated catabolic plasmids .

Table 1.1: Some examples of catabolic plasmids, substrates they can degrade, name of bacterial strain, incompatibility group and name of catabolic genes

Plasmid	Substrate	Host Strain	Size (kb)	Incompatibility group	Catabolic genes	Conjugative transferability
CAM	Camphor	*p. putida* PpG1	500	p-2	*cam*	+
NAH7	Naphthalene	*P. putida* G7	83	P-9	*nah*	+
NIC	Nicotine	*P. convexa* Pcl	NA	NA	NA	NA
OCT	Octane, Decane	*P.olevorans* PpG6	500	P-2	*alkBFGHJ KL alkST*	+
SAL1	Salicylate	*P. putida* R1	85	P-9	*Sal*	+
pAC25	3-chlorobenzoate	*P.putida* AC858	117	P-1	NA	+
pAAM10.6	Phenol	*P. fluorescens* biotype F Cb36	10.6	NA	*pheBA*	-
pBRC60	3-chlorobenzoate	*Comamonas testosteroni* BR60	88	P-1	*cbaRABC*	+
pCIT1	Aniline	*Pseudomonas* sp. CIT1	100	NA	NA	NA
pDK1	Xylene, toluene	*P. putida* HS1	180	NA	*xyl*	+
pENH91	3-chlorobenzoate	*Ralstonia eutropha* NH9	78	P-Ih	*cbnABCD*	NA

pHMT112	Benzene	*P. putida* ML2	112	NA	*bedC1C2BA, bed*	+
pJP4	2,4-D	*Ralstonia eutropha* JMP134	88	P-1β	*tfdA, tfdB, tfdCDEF*	+
pNB2	Nitrobenzene, also 3-chloroaniline	*P. putida* HS12	44	NA	*amoABC*	NA + (Bathe et al. work).
pP51	Chlorobenzene	*Pseudomonas* sp. P51	110	NA	*tcbCDEF, tcbAB*	-
pPGH1	Phenol	*P. putida* H	220	NA	*phlABCDEFGH*	NA
pTK0	Toluene	*P. putida* PPK1	150	NA	NA	-
pWW0	Xylene, Toluene	*P. putida* mt-2	117	p-9	*xyl*	+
pWW174	Benzene	*Acinetobactor calcoaceticus* RJE174	200	NA	*catA, catBCDE*	+

(Ref: Noriji et al. 2004)

1.3.4.2. Plasmid pJP4:

Plasmid pJP4 is 80 kb in size and is one of the best characterized catabolic plasmids (Don and Pemberton 1985). It was isolated by Don and Pemberton from the strain *Alcaligenes eutophus* JMP123 (Don and Pemberton 1981). Plasmid pJP4 encodes gene for mercury chloride resistance and *tfd* genes that encode degradation of 2,4 dichlorophenoxyacetic acid (2,4- D). It also encodes

resistance to phenyl mercury acetate (PMA), 2 methyl-chlorophenoxyacetic acid and 3 chlorobenzoate (3CB) (Don and Pemberton 1985). It belongs to the incompatibility group P1. Plasmid incompatibility is the inability of two plasmids to coexist stably in the same host cell. As work on plasmids progressed, it was recognized that a given pair of plasmids either could or could not coexist stably and this led to the classification of plasmids into incompatibility groups (Palchaudhuri et al. 1976). Plasmid pJP4 is a broad host range plasmid, indicating that it can replicate in a number of bacterial species. The transconjugants of plasmid pJP4 have been found to belong to the α, β, γβ, and γ classes of *Proteobacteria*, mainly to *Rhizobiaceae* and *Comamonadaceae* families and genus *Stenotrophomonas* (Bathe et al. 2004a) and *Burkholderia* and *Ralstonia* genera (Newby et al. 2000). Plasmid pJP4 has been found to contribute to horizontal gene transfer (HGT) in soil, water and engineered environments (Aspray et al. 2005, Newby et al. 2000, Bathe et al. 2004b, 2005).

1.3.5. Horizontal gene transfer:

Horizontal gene transfer refers to the acquisition of foreign genes by organisms. HGT is the transfer of genetic material between bacterial cells uncoupled with cell division. On the other hand, vertical inheritance is the transmission of genetic material from mother cell to daughter cell during cell division (Lawrence 2005). HGT occurs among organisms those share similar factors e.g. genome size, genome G/C composition, carbon utilization and oxygen tolerance (Jain et al. 2003).

The major mechanisms of horizontal gene transfer are transformation, transduction and conjugation (Frost et al. 2010). Of these three mechanisms, conjugation is likely the most powerful technique, which involves transmission of self-transmissible plasmids or conjugative

transposons from donor to recipient via direct cell to cell contact (Frost et al. 2010, Roberts et al. 2001). A donor cell can convert a population of recipient cells to donor cell status by the process of epidemic spread, which is a feature of conjugation (Frost et al. 2010). Transformation is the uptake of free DNA by competent bacteria. Transduction is the transfer of gene, mediated by bacteriophages (Heuer et al. 2007).

Transformation and transduction depend on the recipient cell to participate in the process but conjugation is the function of donor cell, which identifies a recipient and transports the DNA into it (Frost et al. 2010).

Horizontal gene transfer plays a primary role in the ecological adaptation of prokaryotes by importing new genes that encode for new traits (Dorman 2009).

1.3.6. 2,4-D:

Developments in the fields of agriculture and industry have introduced new organic pollutants which are used as paints, solvents, refrigerants, herbicides and pesticides (Bhat et al. 1994). One of the pollutants frequently found in the environment is 2,4-dichlorophenoxyacetic acid (2,4-D). Genes encoding 2,4-D degradation are often located on a conjugative plasmid and plasmid pJP4 has become a model for the 2,4-D degradation (Dejonghe et al. 2000). The synthesis of 2,4-D was first reported in 1941 and has been produced throughout the world since the 1950s (Garabrant et al. 2002). In the Second World War, it was used to destroy the Japanese rice crop (Sanborn et al. 2004). Figure 1.1 shows the chemical structure of 2,4-D. The chemical Abstracts Service (CAS) number of this organic molecule is 94-75-7, its molecular formula is $C_8H_6O_3Cl_2$ with a molecular weight of 221. 2,4-D and its alkaline derivative are highly water soluble compared with the amine and ester forms. The half-life of this chemical is from 20 to 200 days.

It is a widely used herbicide throughout the word and it is the third most widely used herbicide in Canada based on the active ingredient applied (Financial Post, June 16, 2008 by NP Editor).

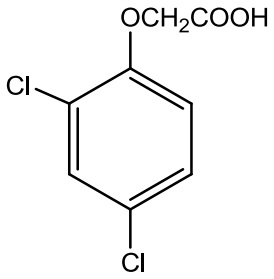


Figure 1.1: Structure of 2,4-D (Bhat et al. 1993)

The plant growth regulator indole-3-acetic acid (also known as auxin; Figure 1.2) regulates numerous aspects of plant growth and development including embryogenesis, lateralroot formation, vascularization, and tropic growth responses. While endogenous auxin plays a critical role in normal plant growth and development, the application of high doses results in phytoxicity. This observation led to the development of several synthetic auxins, including 2-4-dichlorophenoxyacetic acid (2,4-D) (Ito et al. 2006).

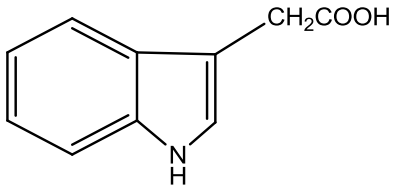


Figure 1.2: Chemical structure of Indole-3-acetic acid (Auxin) (Delker et al. 2008)

Unfortunately, the herbicide does not affect target weeds alone. The widespread and intensive uses of this successful organic herbicide cause toxicological and environmental problems (Teixeira et al. 2007). In 1982, the WHO considered it moderately toxic (class II) and

recommended a maximum concentration of 0.1 ppm in drinking water (Kundu et al. 2004). At high doses 2,4-D damages the liver and kidney and irritates mucous membrane (Garabrant 2002). It can also cause acute congestion and degenerative changes in the central nervous system (Odaci et al. 2009).

On one hand, 2,4-D helps to increase food supply and beautification, but on the other hand, it is an environmental curse causing health problems.

1.3.7. Degradation mechanism of 2,4-D:

Some synthetic organic compounds used as herbicides have substantial toxic effect for the environment. Some of these, such as 2,4-dichlorophenoxyacetic acid (2,4-D) are readily degraded by microorganisms present in soil and water (Don and Pemberton 1981). Most of these microorganisms, carry genes encoding 2,4-D degradation are often located on plasmids (Top et al. 1995). Plasmid pJP4 is 80 kb in size and contains genes whose products degrade 2,4-D to 2-chloromaleylacetate (Figure 1.3) (Newby et al. 2000). The enzymes used for the degradation pathway are 2,4-D monooxygenase, 2,4-dichorophenol hydroxylase, 3,5-dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, chlorodienelactone cycloisomerase and chlorodienelactone hydrolase. The final product succinic acid is produced which is metabolized by all microorganism to generate energy (Glick et al. 2003).

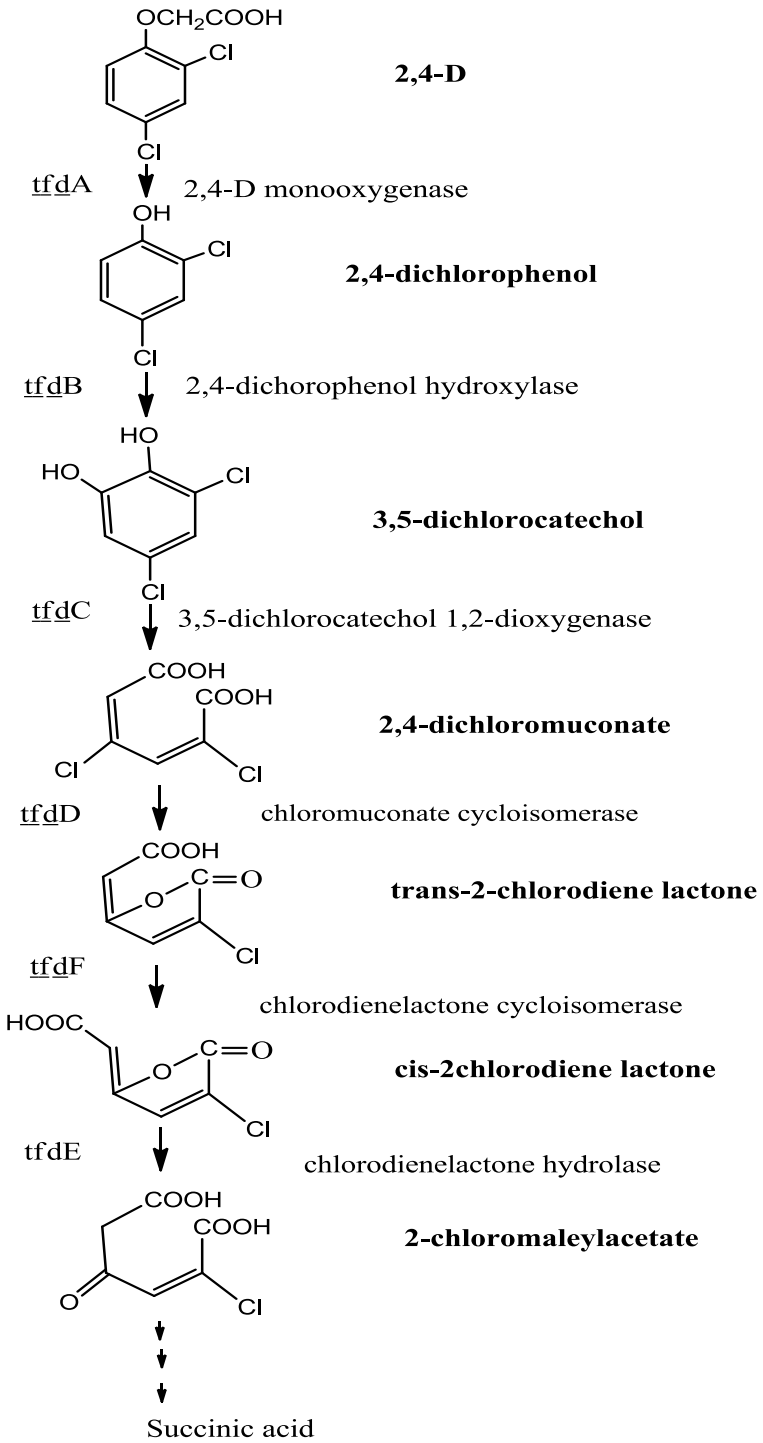


Figure 1.3: The proposed degradative pathway of 2,4-D, with structural genes coding for the respective enzymes (Bhat et al. 1993)

1.3.8. The green fluorescent protein (GFP):

The expression of reporter proteins (GFP and DsRed) has been widely used in gene transfer studies as this allows monitoring donor cells and plasmid transfer to the recipient cells using confocal laser scanning microscopy (Nancharaiah et al. 2007). Fluorescent based methods permits the detection of donor and transconjugants entering the environment. The green fluorescent protein is a valuable marker for monitoring gene expression and protein localization in living cells (Jakobs et al. 2000). Proskar et al. (1992) cloned *gfp* complementary DNA from the bioluminescent jellyfish *Aequoria victoria* and Chalfie at al. (1994) showed the expression of cloned *gfp* gene produces green fluorescence in various prokaryotic (*Escherichia coli)* and eukaryotic (*Caenorhabdites elegans*) cells (Chalfie, et al. 1994, Errampalli et al. 1999). GFP is composed of 238 amino acids and molecular mass is 27 kDa (Chalfie, et al. 1994; Errampalli, 1999). GFP absorbs blue light at the maximum peak of 375 nm and a minor peak of 470 nm and emits light at 509 nm (Ward et al. 1980). Detection of GFP requires irradiation by the blue light or near ultraviolet light. GFP is resistant to denaturants and proteases and stable at high temperature (65°C), pH values 6 to 12 (Ward et al. 1980). The GFP system is ideal for studying of multiple species bacterial biofilms and adhesion of bacteria to flocks in activated sludge. (Errampalli et al. 1999).Three dimensional profiles of *gfp* -labelled bacteria can be obtained from confocal laser scanning microscopy. The GFP system is useful for studying horizontal gene transfer between microorganisms (Errampalli et al. 1999). Christensen et al. (1998) investigated *gfp* -tagged TOL plasmid transfer between two *Pseudomonas* strains in a biofilm community. Aspray et al. (2005) confirmed *gfp* -tagged pJP4 plasmid transfer from donor to a soil derived bacterial community.

1.3.9. The red fluorescent protein (RFP):

DsRed has attracted interest as a complementary partner to GFP that would allow simultaneous multicolor imaging of at least two different proteins in living cells (Nancharaiah et al. 2003). Matz et al. (1999) showed that cloning of the gene that encodes red fluorescent protein from the Indo pacific sea anemone *Discosoma striata* results in the formation of a red fluorescent protein (RFP), commercially available under the trade name DsRed (Jacobs et al. 2000). The red fluorescent protein shares only 25% sequence similarity with *Aequoria* GFP (Patterson et al. 2001). Red fluorescent protein matures slowly and it has strong tendency to form tetramers (Baird et al. 2000). Its excitation wavelength is 558 nm and it emits red light with the maximum emission at 583 nm (Baird et al. 2000). Figure 1.4 shows the GFP and RFP spectra.

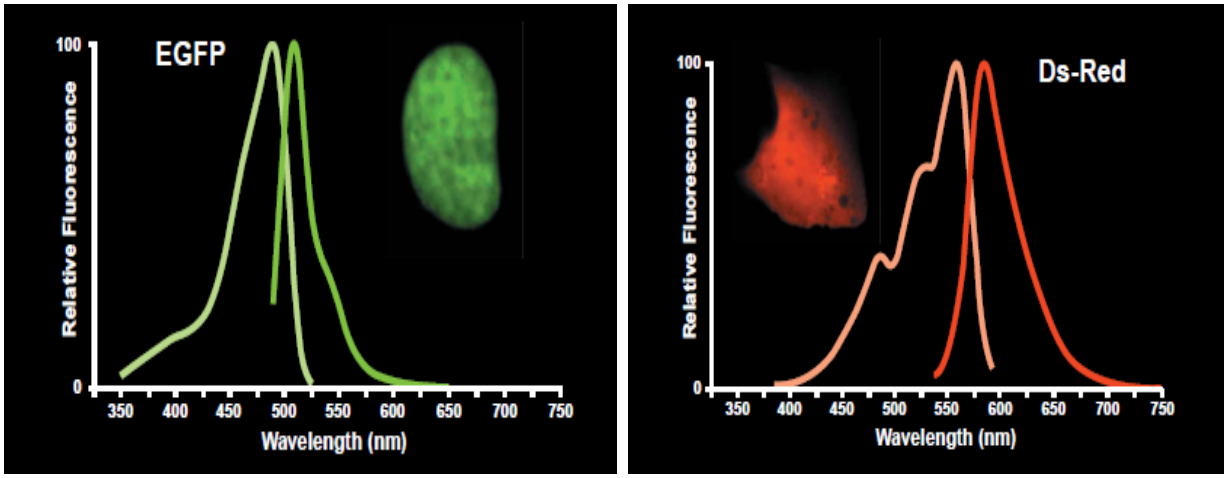


Figure 1.4: GFP and RFP spectra. GFP maximum absorbance is at 488 nm and RFP maximum absorbance is at 543 nm wavelength (Patterson et al. 2001).

RFP is also a very useful tool for gene transfer studies because it can be used to detect transconjugants when the plasmid is tagged with RFP protein. For example, the red fluorescent protein or its gene (*dsRed*) was used to detect and isolate pJP4 transconjugants from a sequencing batch biofilm reactor (Bathe et al. 2004a, b).

Application of reporter genes for monitoring gene transfer allowed the quantification of gene transfer frequencies in different environments e.g. agar surface, biofilms (Nancharaiah et al 2003).

1.3.10. The role of catabolic plasmid transfer in bioremediation:

Bioaugmentation plays an important role in the remediation of contaminated environments. Bioremediation is a process by which microorganisms degrade or detoxify contaminants (Bathe et al. 2010). Bioaugmentation is a bioremediation strategy which relies on the introduction of xenobiotic degrading bacteria or their genes into an existing the microbial community (Tsutsui et al. 2010). The introduced bacteria usually cannot adapt to the new environment and decline in numbers in many cases (Limbergen et al. 1998). To solve this problem plasmid mediated bioaugmentation has been proposed. This process introduces and propagates transmittable plasmids encoding genes for the degradation of xenobiotics in the microbial community (Top et al. 2002). Horizontal gene transfer is a tool for bioaugmentation. The introduced plasmids are transferred to indigenous bacteria via horizontal gene transfer like conjugation which plays a major role in spreading genetic information encoded on plasmids and can be exploited in bioaugmentation (Tsutsui et al. 2010)..Plasmid transfer via conjugation has been frequently detected in many environments, including soils, activated sludge, sediments, the rhizosphere and phyllosphere of plants, and model, engineered and natural biofilms (Hausner et al. 2011).

Bioaugmentation introduces new metabolic capacities, such as the ability to degrade recalcitrant compounds or to inactivate heavy metals, into a microbial community (van Limbergen et al. 1998). Gene bioaugmentation is a bioremediation strategy that enhances biodegradative potential via dissemination of degradative genes from introduced microorganisms to indigenous microorganisms (Inoue et al., 2011). To achieve successful bioaugmentation, it is necessary to select suitable microorganisms and adopt a proper strategy. The combination of different techniques e.g. denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE) and in situ fluorescent protein detection can provide fate of the added microorganisms and evaluate effectiveness of bioaugmentation (Yu et al. 2009). Plasmid pJP4 was previously used in bioaugmentation experiments to treat contaminated soil and waste water system.

Newby et al. (2000) used two pJP4 hosts as inocula for field soil bioreactors: the natural host, *Ralstonia eutropha* JMP134, and a laboratory-generated strain amenable to donor counter selection, *Escherichia coli* D11. Inoculation with *R. eutropha* was considered to be both cell bioaugmentation and gene augmentation since this organism can both utilize the degradative genes on the plasmid to degrade 2,4-D and transfer the plasmid. In contrast, inoculation with *E. coli* D11 was considered to be only gene augmentation since the host itself could not degrade 2,4-D but could transfer the plasmid. The soil system was contaminated with 2,4-D alone or was contaminated with 2,4-D and cadmium (Cd). Plasmid transfer to indigenous populations, plasmid persistence in soil, and degradation of 2,4-D were monitored over a 63-day period in the bioreactors. Different treatments were used for this study, treatment 1 (2,4-D); (B) treatment 2 (2,4-D and *E. coli*); (C) treatment 3 (2,4-D and *R. eutropha*); (D) treatment 4 (2,4-D and Cd); (E) treatment 5 (2,4-D, Cd, and *E. coli*); (F) treatment 6 (2,4-D, Cd, and *R. eutropha*). To assess the

impact of contaminant re-exposure, aliquots of bioreactor soil were re-amended with additional 2,4-D. Both introduced donors remained culturable and transferred plasmid pJP4 to indigenous recipients, although to different extents. Isolated transconjugants were members of the *Burkholderia* and *Ralstonia* genera. Upon a second exposure to 2,4-D, enhanced degradation was observed for all treatments, suggesting microbial adaptation to 2,4-D. Upon re-exposure, degradation was most rapid for the *E. coli* D11-inoculated treatments. This study revealed that plasmid transfer occurred from both of the introduced donors to indigenous recipients. This study demonstrated the potential for increased rates of 2,4-D degradation following bioaugmentation of bioreactors and of microcosms with pJP4-harboring microorganisms.

Dejonghe et al. (2000) monitored transfer of 2,4-D degradation plasmids pEMT1 and pJP4 from an introduced donor strain, *Pseudomonas putida* UWC3, to the indigenous bacteria of two different horizons (A horizon, depth of 0 to 30 cm; B horizon, depth of 30 to 60 cm) of a 2,4-D-contaminated soil as a means of bioaugmentation. When the soil was amended with nutrients, plasmid transfer and enhanced degradation of 2,4-D was observed. In the B horizon, indigenous bacteria were unable to degrade any of the 2,4-D (100 mg/kg of soil) during at least 22 days but inoculation with either of the two plasmid donors resulted in complete 2,4-D degradation within 14 days.

Newby et al (2002) used column studies to evaluate dissemination of plasmid pJP4 under unsaturated or saturated flow conditions in a 2,4-D contaminated soil. Columns were destructively sampled following 1 week of percolation to assess the vertical distribution of donors, transconjugants, and 2,4-D concentrations within the soil. In unsaturated soil, pJP4 was detected in both culturable donor and transconjugant cells within soil 10.5 cm from the inoculated end of the column. In saturated soil, no transconjugants were detected; however,

donors were found throughout the entire length of the column (30.5 cm). These results suggest that donor transport, particularly in conjunction with plasmid transfer to indigenous recipients, allows for significant dispersal of introduced genes through contaminated soil.

Bathe et al. (2004b) investigated the possibility to enhance degradation of 2, 4-dichlorophenoxy acetic acid in a sequencing batch biofilm reactor (SBBR) by using the plasmid pJP4 carrying genes for 2, 4-D degradation. The SBBR was a bench-scale reactor filled with glass beads that acted as a biofilm carrier. Donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* was introduced in the lab scale SBBR, which was inoculated with activated sludge. The SBBR contained artificial wastewater to supply nutrients to biofilms within the system. Several carbon sources were supplied to the biofilms – sodium acetate, citric acid, sodium gluconate, D (+)-glucose and model pollutant 2,4-D. When 2,4-D was supplied to microorganisms all other carbon sources were removed, thus, when present, 2,4-D was the sole carbon source. The 90% 2,4-D was degraded in the bioaugmented reactor within 40h, a control reactor which did not receive the plasmid contained 60% of the initial 2, 4-D concentration after 90h.

Aspray et al. (2005) introduced a 2,4-D degrading donor strain, *Pseudomonas putida* SM1443 (pJP4::gfp) into flow cell chambers containing 2-day old biofilm communities. They identified pJP4::*gfp* transconjugants by identifying GFP fluorescing cells. A 2,4-D degrading transconjugant strain was isolated from the flow cell system belonging to the genus *Burkholderia*.

Tsutsui et al. (2010) examined plasmid mediated bioaugmentation in activated sludge process. They used a filter mating method for the conjugative transfer of plasmid pJP4 from *Escherichia coli* HB101 and *Pseudomonas putida* KT2440 to activated sludge bacteria.

Quan et al. (2010) showed that aerobic sludge granules pregrown on glucose were bioaugmented with a plasmid pJP4 carrying strain *Pseudomonas putida* SM1443 in a fed batch microcosm and a laboratory scale sequencing batch reactor (SBR) to enhance their degradation capacity to 2, 4-dichlorophenoxyacetic (2,4-D). Aerobic sludge granulation is an innovative cell immobilization technology in biological wastewater treatment. Aerobic granules are formed through the process of microbial self-aggregation under specific selective pressures such as hydraulic shearing forces, short settling time (Quan et al. 2011). They established that 2,4-D at the initial concentration of 160 mg/L was removed by the bioaugmented granules system within 62 h, while the control removed 26% within 66h.

Inoue et al. (2012) used 2,4-D contaminated soil slurry and strains of *Pseudomonas putida* KT2440 or *Escherichia coli* HB101 harboring plasmid pJP4 in microcosms to assess possible effects of gene bioaugmentation on the overall microbial community structure and ecological functions (carbon source utilization and nitrogen transformation potentials). In microcosms which were inoculated with *P. putida* KT2440 (pJP4) and *E. coli* HB101(pJP4), 2,4-D was completely degraded within 6 days after a lag phase of 3 days. In uninoculated microcosm a lag period of 6 days was observed prior to the onset of 2,4-D degradation, and 2,4-D was below the detection limit on day 20. This study suggested that gene bioaugmentation with *P. putida* and *E. coli* strains harboring pJP4 is effective for the degradation of 2,4-D in soil without large impacts on the indigenous microbial community.

Quan et al. (2011) developed 2,4-D degrading aerobic granular sludge in two sequencing batch reactors (SBR), one reactor was bioaugmented with a plasmid pJP4 donor strain *Pseudomonas putida* SM1443 and the other was control. Granules capable of utilizing 2,4-D (about 500 mg/L) as the sole carbon source was successfully cultivated in both reactors. The granules in the bioaugmented reactor demonstrated higher 2,4-D degradation rates than that in the control reactor. The difference in degradation rates was more significant for the granules sampled on day 18 than that on day 105, indicating that bioaugmentation exhibited more benefits for the granules at the initial operation stage. Transconjugants receiving plasmid pJP4 were established in the granule microbial community after bioaugmentation and persisted till the end of operation. Compared with the control granules, the granules in the bioaugmented reactor demonstrated a better settling ability, larger size, more abundant microbial diversity and stronger tolerance to 2,4-D.

Transfer of some other catabolic plasmids e.g., plasmid pNB2 which encodes genes necessary for 3-chloroaniline (3-CA) degradation and the TOL plasmid pWWO which carries genes encoding enzymes for toluene degradation also plays important role in bioaugmentation.

Bathe et al. (2005, 2009) showed plasmid mediated bioaugmentation of wastewater microbial communities in laboratory scale bioreactor. They established that, the catabolic plasmid pNB2 can facilitate the degradation of a xenobiotic compound, 3-chloroaniline and the applicability of this plasmid to accomplish 3-chloroaniline degradation in a bioreactor after in situ transfer to recipient strains.

Bathe et al. (2005) showed the application of plasmid mediated bioaugmentation of model wastewater in laboratory scale sequencing batch moving bed reactors receiving 3-chloroaniline

(3-CA). They used the donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying a *dsRed* tagged pNB2 derivative. They used three reactors inoculated with bacteria from activated sludge. Reactor PB received the donor strain not able to degrade 3-CA. Positive control reactor P received a 3-CA degrading *Comamonas testosteroni* pNB2 transconjugant. The negative control reactor N was unchanged. Reactor P inoculated with activated sludge and the 3-CA degrading pNB2 transconjugant (*C. testosteroni* SB3) showed a rapid degradation of 3-CA and in reactor PB, degradation started slowly. No degradation was observed in reactor N. PCR reaction showed that donor abundance was dropped in reactor PB but the plasmid abundance did not decrease which indicates the transfer of the plasmid pNB2 to other bacteria.

Nancharaiah et al. (2008) showed successful bioaugmentation of aerobic granular sludge using *P. putida* KT2442 cells carrying plasmid pWWO. They monitored incorporation of the added donor cells into pre-existing microbial granules and the transfer of plasmid to the microbial community using shake flask microcosms.

Pei and Gunsch (2009) confirmed the transfer of plasmid pWWO from *Pseudomonas putida* BBC443 to microbial communities in batch reactors inoculated with activated sludge from a municipal wastewater treatment plant.

In summary, HGT has been successfully demonstrated in bioaugmentation experiments mostly at the lab scale.

1.4: Scope of work and thesis outline

The scope of work was designed to address one of the key gaps in the literature, how the different carbon sources affect biofilm architecture, plasmid transfer and xenobiotic degradation in biofilms. Research carried out within the frame of this thesis to satisfy objectives listed in

section 1.2. is described in the following chapters. Specifically, chapter 2 is a formal manuscript with this study's results. Chapter 3 will outline the conclusions and suggest future work as a follow up of this study.

CHAPTER 2: BIOFILM ARCHITECTURE ANALYSIS AND PLASMID TRANSFER UNDER NON SELECTIVE AND SELECTIVE PRESSURE

2.1. Introduction:

Catabolic genes or those coding for antibiotic or metal resistance are often carried on plasmids. Plasmid conjugation is the most common mechanism of horizontal gene transfer used as a tool for bioaugmentation, which is a process of introduction of desired metabolic functions into an existing microbial community. Biofilms are believed to be a hot spot of horizontal gene transfer due to the dense microbial population and the close contact among microorganisms in biofilms (Bathe et al. 2010, Quan et al. 2011). Plasmid transfer via conjugation has been detected in many environments including soils, activated sludge, sediments, engineered and natural biofilms (Bathe et al 2010). The flow cell system is an *in vitro* analytic tool, which in conjunction with CLSM, enables the detailed study of the growth of live, fully hydrated biofilms, with the ability to control different parameters such as growth medium composition, flow rate and incubation temperature (Crusz et al. 2012). Flow cells were used previously to study biofilm architecture. Moller et al. (1997) cultivated a microbial community in flow cells with 2,4,6-trichlorobenzoic acid as sole carbon source. They found a characteristic architecture of biofilm, including a basal cell layer and conspicuous mounds of bacterial cells under this condition. But when they changed carbon source to trypticase soy broth which was nonchlorinated, biofilm architecture was changed, including loss of mound structures.

Flow cell systems play an important role in gene transfer studies as plasmid transfer at single cell level can be monitored and donor cells and plasmid carrying cells can be screened rapidly (Aspray et al.2005). Christensen et al. (1998) successfully showed conjugational transfer of the

TOL plasmid (pWWO) in a flow chamber biofilm community engaged in benzyl alcohol degradation. They used GFP fluorescence based detection to detect the transconjugants. Aspray et al. (2005) also used a flow cell to study a soil microbial community irrigated with 2,4-dichlorophenoxyacetic acid (2,4-D). They detected the transfer of pJP4::*gfp* from the donor to the bacterial community by GFP fluorescence in the transconjugant cells.

In this study, a flow cell was used to obtain information about biofilm formation under continuous flow conditions and plasmid transfer events. The aim of this project was to investigate the influence of two different carbon sources, glucose and 2,4-dichlorophenoxyacetic acid (2,4-D), which represent the absence and presence of selective pressure, respectively, on biofilm architecture, transfer of the degradative plasmid pJP4 in soil derived mixed culture biofilms and consequent changes in biodegradation of 2,4-D. 2,4-D has been widely used as a herbicide and has become a significant environmental pollutant. In this research project, 2,4-D is being used as a model pollutant as well as selective pressure. A modified *Pseudomonas putida* SM1443 strain (Bathe et al 2004a, b) carrying a red fluorescent protein (DsRed) tagged plasmid pJP4 and a chromosomal *gfp* gene label was used as the donor strain and a soil-derived mixed culture biofilm was used as the recipient community for plasmid transfer. DsRed fluorescence was suppressed in the donor, but was detected in the transconjugant cells (Bathe et al 2004a, b). Previously, DsRed was used to isolate pJP4 transconjugants from a wastewater microbial community in a sequence batch biofilm reactor (Bathe et al. 2004a, b). Plasmid pJP4 is one of the well characterized plasmids which is a broad-host-range, self-transmissible, IncP group plasmid carrying the genes *tfdA-tfdF* for the conversion of 2,4-D to 2-chloromaleylacetate (Don and Pemberton 1981). In spite of past studies which explored plasmid mediated bioaugmentation in lab scale bioreactors (Bathe et al. 2004b, 2005), there exists and experimental need to

investigate the fundamentals of horizontal gene transfer in biofilms and other bioaggregates. Questions such as how external parameters and biofilm architecture influence the spread of genetic information within biofilms should be answered in order to design more efficient bioaugmentation strategies. The specific aims of this work were (1) to describe biofilm architecture in the absence of selective pressure (glucose) or presence of selective pressure (2,4-D); (2) to determine the extent of transconjugant formation in the absence of selective pressure (glucose) or presence of selective pressure (2,4-D) and to infer possible relationships about plasmid transfer events and biofilm architecture.

2.2. Materials and Methods:

2.2.1 Strains, sources of inoculum, media:

The donor strain used in this study was *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* (Bathe et al. 2004a, b). The donor strain showed constitutive GFP fluorescence but DsRed fluorescence was repressed due to the action of a chromosomally encoded Lac-repressor on the *lac*-promoter-controlled *dsRed* gene as described by Bathe et al. (2004b). DsRed was only expressed in transconjugants after the transfer of plasmid. The donor strain is kanamycin resistant and plasmid pJP4 encodes genes for resistance to mercury chloride and gentamycin and *tfd* genes for the initial steps in 2,4-D degradation.

A Soil sample was collected from Ryerson University, Toronto, Ontario. The 15% glycerol stocks of these soil cultures were used as sources of recipient community inoculum. The glycerol stocks were stored at -80°C.

The donor was maintained on Luria Bertani (LB) agar (35g of LB agar powder was dissolved in 1 liter of distilled water, Becton Dickinson and company, Sparks, MD, USA, lot. 2010802)

amended with kanamycin (100 µg/mL) and gentamycin (25 µg/mL). Minimal mineral medium (MMN) (Boon et al. 2001) with 5mM glucose (Alfa Aesar, MA, USA, lot. L14W023) as carbon source or with 5mM 2,4-dichlorophenoxyacetic acid (2,4-D) (Himedia Laboratories Pvt. Ltd. India, lot. 0000113766) as carbon source and selective pressure was used for flow cell experiments (composition of MMN is provided in Appendix A). In 5 mM glucose the amount of carbon was 0.36 g/L and in 5 mM 2,4-D amount of carbon was 0.48 g/L. For flow cell experiments, LB broth (25g/L, Becton Dickinson and company, Sparks, MD, USA) with kanamycin (100 µg/mL) and gentamycin (25 µg/mL) was used to pre grow donor strain and R_2A broth (3.12g/L Himedia Laboratories Pvt. Ltd. India) was used to pre grow soil derived mixed pJP4 recipient microbial community. Donor strain and soil cultures were pre-grown on a 320 rpm shaker at room temperature for 48 hrs.

2.2.2 Flow cell preparation and inoculation:

A four channel continuous flow-through system (flow cell) was used to cultivate biofilms and obtain information about biofilm formation under continuous flow condition and plasmid transfer events. Figure 2.1 shows the experimental set up. The flow cell (Figure 2.2) was constructed from stainless steel and consisted of four separate flow channels (40 mm long, 4 mm wide, 4 mm in height), sealed with 0.17-mm-thick cover slides (24×50 mm; VWR International, Mississauga, ON, Catalogue No. 16004-098) (Wuertz et al., 2001). The flow cell inlets were connected with silicone tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 0.81 mm wall thickness; VWR International, Mississauga, ON, Catalogue No. 6098 5-714) to the medium reservoir vessel. Flow cell channel outlets were connected to a waste receptacle using silicone tubing.

Prior to use, the flow cell was autoclaved at 121°C for 1 hr and then the entire flow cell set-up, including tubing and bubble traps were additionally disinfected by rinsing with 10% (v v^{-1}) bleach in distilled water, followed by a rinse with autoclaved distilled water for 12-24 hrs. MMN medium with 5mM glucose was pumped through the flow cell channels for 1 hour prior to inoculation. Next, the medium flow was stopped, and 1 mL overnight liquid culture of soil derived mixed pJP4 recipient microbial community (2.5×10^8 CFU/mL) was injected into each flow cell channel using a sterile syringe and needle through the inflow side, such that each channel was completely filled (Wuertz et al. 2001). Prior to inoculation, the injection site was surface-sterilized with a 70% ethanol solution. After inoculation the inflow side was reattached with silicon tubing. The flow cell was flipped (upside down) for 1 hour so that the injected mixed culture was allowed to adhere to the glass cover slide before being subjected to MMN with glucose medium using a multi channel peristaltic pump (Watson Marlow model 205S, Wilmington, MA). The flow rate was set to 0.5 rpm, which corresponded to a volumetric flow rate of 3.2 ml/h. All experiments were performed at room temperature.

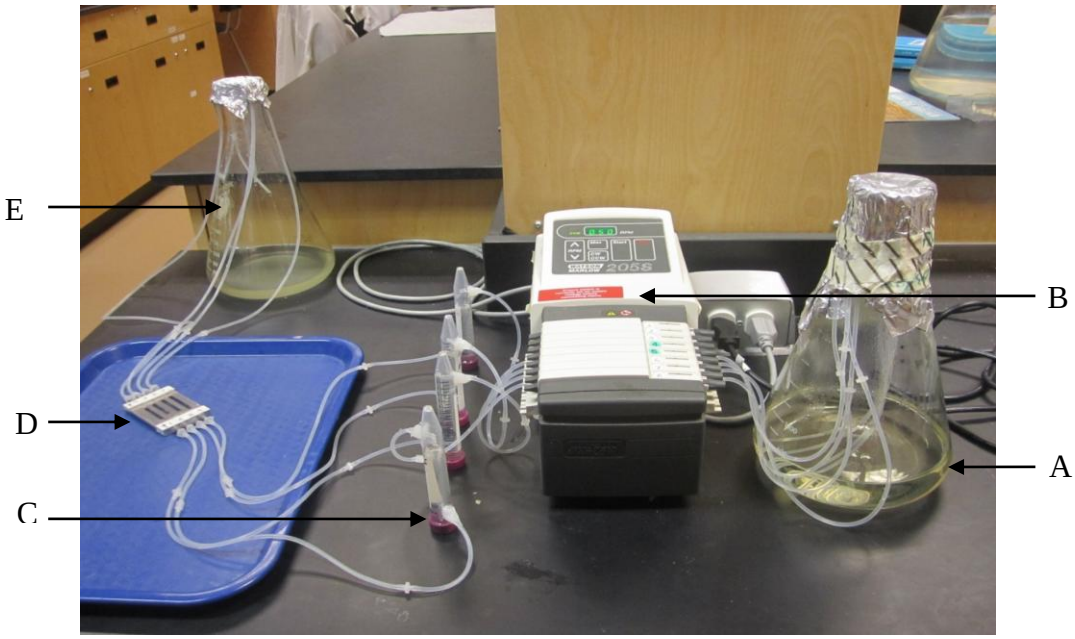


Figure 2.1: Experimental set up contained A) media reservoir, B) peristaltic pump, C) bubble trap, D) flow cell with four channels, E) effluent reservoir

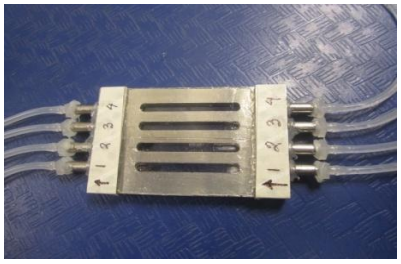


Figure 2.2: Flow cell detail with four channels for biofilm cultivation

2.2.3. Plate mating experiment:

Plate mating experiments were done to select a pJP4 recipient community. The donor strain and soil derived mixed culture were separately grown for 48 hrs with LB + kanamycin (100 µg/mL) + gentamycin (25 µg/mL) and R_2A medium respectively. One mL cultures of donor strain and soil were centrifuged separately in 1.5 mL centrifuge tubes at 16000 g for 8 minutes.

Supernatants were discarded and cell pellets were washed twice with 0.9% saline. Finally 500 µL 0.9% saline was used to resuspend the cell pallets. 400 µL of each of the cultures were mixed in a separate 1.5 mL centrifuge tube and 100 µL of this mixture was added on LB and R_2A plates in one droplet (Bathe et al. 2004a) (i.e. cells were not spread). Plate matings were left at room temperature for 4 days and then transferred to refrigerator set to 4°C for 10 days. The two week incubation period was used in order to allow for accumulation of the red fluorescent protein. During this two week incubation period mating patches were sampled twice and examined using confocal laser scanning microscope Zeiss LSM510 (Zeiss, Jena, Germany) to detect GFP and RFP fluorescence. To detect GFP and RFP fluorescence, 488 nm (argon laser) and 543 nm (HeNe laser) wave length lasers, long pass (LP) filter 560 and band pass filter 500-550 were used.

2.2.4. 2,4-D degradation in batch culture experiment:

Batch cultures were prepared in sterile, 250 mL conical flask. Batch culture experiments were done to compare the degradation rate of 2,4-D by only soil-derived mixed cultures and soil-derived mixed cultures amended with the donor strain. Eight mL culture of the donor strain and 16 mL culture of recipient community were prepared as described in section 2.2.1. Each culture was washed with MMN + 2,4-D and finally cells were resuspended in MMN + 2,4-D. One conical flask was inoculated with 8 mL soil culture and another flask was inoculated with 8 mL soil culture and 8 mL donor strain culture. 80 mL MMN medium with 5 mM 2,4-D was used as feed. The conical flasks were kept on a 320 rpm shaker at room temperature. Samples collected on different days during the experimental period, were filtered (0.45 µm) and measured the concentration of 2,4-D by spectrophotometer (section 2.2.7.).

2.2.5. Biofilm growth and plasmid transfer under non selective pressure (Minimal mineral medium with 5 mM glucose as carbon source):

After inoculation of soil mixed culture in the flow cell channels, MMN (Boon et al. 2001) medium with glucose was used continuously as feed. Figure 2.3 shows the flow diagram of the experiments with non selective pressure and selective pressure. Flow cell channels were observed within the transmission mode of Zeiss LSM510 CLSM (Zeiss, Jena, Germany) to confirm the growth of biofilms. After the confirmation of biofilm formation in flow cell channels, the donor strain was inoculated into duplicate channels of the flow cell (9.8×10^7 CFU/mL) and the medium was switched to R_2A for better growth of donor and soil community for 24 hrs.

Prior to inoculation, donor liquid culture was centrifuged for 8 minutes at 5000 g and washed twice with R_2A medium. After 24 hrs medium was switched to MMN + glucose. Effluents were collected from all 4 channels, spread plating for effluent samples was done on R_2A agar, R_2A agar + gentamycin (25 µg/mL), R_2A agar + kanamycin (100 µg/mL) + gentamycin (25 µg/mL), LB agar + kanamycin (100 µg/mL) + gentamycin (25 µg/mL) plates to isolate donor and transconjugants. DNA extraction was done for effluent samples and PCR reactions were performed to amplify 16s rRNA, *gfp, dsRed, tfdB.* Confocal laser scanning microscope was used to detect GFP and RFP emission in biofilms.

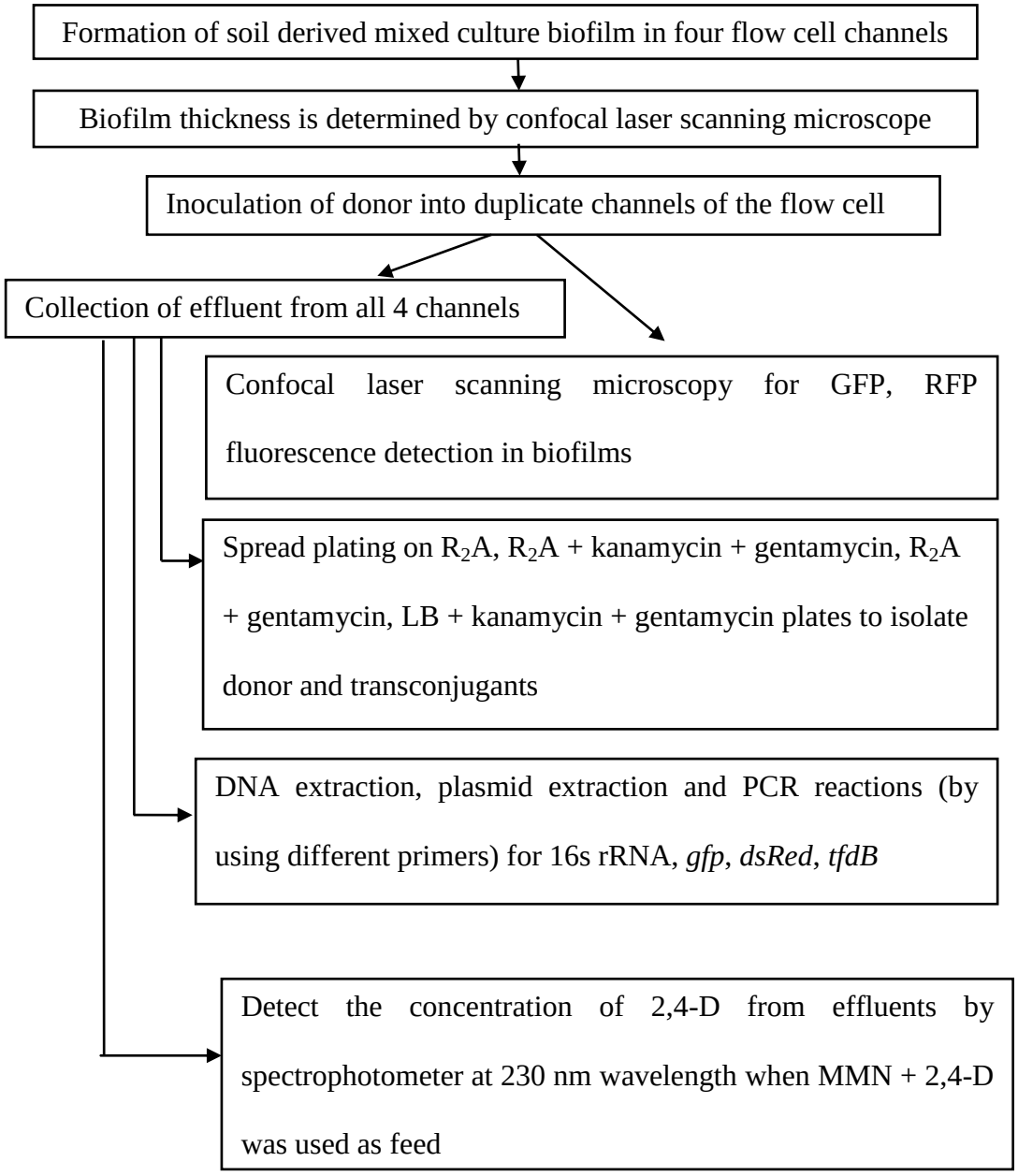


Figure 2.3: Flow diagram of the experimental design with non selective pressure (glucose) and selective pressure (2,4-D)

2.2.6. Biofilm growth and plasmid transfer under selective pressure (Minimal mineral medium with 5 mM 2,4-D as carbon source and selective pressure):

After inoculation of the soil mixed culture in the flow cell channels MMN medium with glucose was initially used as feed instead of MMN + 2,4-D to prevent the soil derived mixed culture to be adapted with 2,4-D. Flow cell channels were observed with the transmission light of Zeiss LSM510 CLSM (Zeiss, Jena, Germany) to confirm the growth of biofilms. After 48 hrs the donor strain was inoculated into duplicate channels of flow cell (7.5×10^7 CFU/mL) and the medium was switched to R_2A for 2 hrs. After 2 hrs medium was switched to MMN + 2,4-D. Effluents were collected from all 4 channels, spread plating for effluent samples was done on agar plates with or without antibiotics as mentioned in section 2.2.5 to isolate donor strain and transconjugants. DNA extraction was done for effluent samples and PCR reactions were performed to amplify 16s rRNA, *gfp*, *dsRed*, *tfdB.* Confocal laser scanning microscope was used to detect GFP, RFP fluorescence in biofilms. Concentration of 2,4-D from effluent samples was measured by spectrophotometer (Lambda 20, Perkin Elmer, USA).

2.2.7. Measurement of 2,4-D concentration from effluent samples:

Effluent samples from all four channels were collected and diluted by adding 100 µL effluent to 4.9 mL MMN medium. Samples were filtered (0.45 µm) and the 2,4-D concentration was determined by measuring absorbance by spectrophotometer (Lambda 20, Perkin Elmer, USA) at 230 nm (Bathe et al. 2004b).

2.2.8. Biofilm staining and microscopy:

For MMN + glucose experiments, biofilms of the mixed soil culture in the flow cell channels were stained with vital stain (5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE) – mixed isomers, Invitrogen, Burlington, ON, Canada, Catalog No. C1157, lot. 847176) to measure the thickness on different days during the experimental period. One µL of stain was diluted with 999 µL medium and 1mL of diluted stain was injected into each flow cell channel (final concentration of 50 µM). After injection medium flow was stopped for 15 minutes and inlet tubes were clamped. Medium was run for additional 15 minutes to remove unbound stain before visualizing under the confocal laser scanning microscope. Ten microscopic locations were investigated along the length of each channel using the z-stack function of the Zeiss LSM510 CLSM (Zeiss, Jena, Germany) with a 10× or 20× magnification lens. The distance between optical sections within image stacks was 10 µm. 488 nm wave length laser (argon laser) was used.

For MMN + glucose experiments, biofilms of the mixed soil culture in the flow cell channels were stained with Syto 62 stain (Invitrogen, Burlington, ON, Canada) on the last day of experiment to measure the biofilm thickness. Syto 62 was diluted and stained the biofilms on the same way as described above. Ten microscopic locations were investigated along the length of each channel using the z-stack function of the Zeiss LSM510 CLSM (Zeiss, Jena, Germany) with a 20× magnification lens. 633 nm wavelength laser was used and the distance between optical sections within image stacks was 10 µm.

To detect GFP and RFP fluorescence in the biofilms 488 nm and a band pass filter of 500-550 nm and a 543 nm lasers and a long pass (LP) filter of 560 nm were used, respectively.

Colonies grew from effluent samples on agar plates with or without antibiotics were checked for GFP and RFP fluorescence by using Leica fluorescence dissection microscope (Leica Microsystems, Concord, ON, Canada), Nikon eclipse 80i_C1 microscope (Nikon, Ontario, Canada) was used to detect DsRed fluorescing cells from transconjugant colonies.

2.2.9. DNA extraction and PCR:

DNA extraction from all effluent samples was performed using a GeneElute Bacterial Genomic DNA kit (#NA2110, Sigma-Aldrich, Oakville, ON, Canada), following the manufacturer's instructions.

PCR was used to amplify portions of the 16s rRNA, *dsRed, gfp* and *tfdB* genes. The 50 µL PCR reaction mixture contained 2 µL of template DNA, ddH20, 1µL of both the forward and reverse primer (primers working concentration to amplify *gfp* and *tfdB* was 50 µM and to amplify *dsRed* and 16s rRNA was 25 µM), 10x BSA (New England BioLabs, Pickering, ON), 200 µM of each dNTP (New England BioLabs, Pickering, ON), 2.5 units of Taq polymerase (New England BioLabs, Pickering, ON) in 10X Taq buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl2) (New England BioLabs, Pickering, ON). *dsRed* PCR protocol employed 35 cycles of amplification, other protocols employed 30 cycles of amplification. Table 2.1 shows the composition of primers and cycle parameters.

Table 2.1 Polymerase chain reaction primers and parameters for *gfp*, *dsRed*, *tfdB*, 16S rRNA

Primer target	Sequence	Initial Denature	Denature	Primer Annealing	Extension	Final Extension	Ref.
dsRed	Forward 5′ - TGCCATTTGCTTGGGATA-3′ Reverse5′ CTCGGTTCTTT CATACTGCTC-3′	95°C, 1 min	92°C, 45 sec	52 ºC, 45 sec	72°C 45 sec	72°C, 10 min	Ma and Bryers 2012
tfdB	Forward 5'-GCTGACGGCG TGTCGTCGCG-3' Reverse 5'-GATGGACGA TGCGCGACGTA-3'	94°C, 15 min	94°C, 30 sec	62 ºC, 1 min	72°C, 2 min	72°C, 2 min	Neilson et al. (1992)
gfp	Forward 5'-TTTCAAGAGTGCCA TGCCCGAAGG-3' Reverse 5'-CTGGTAAAAGGACAGGGCC ATCGC-3'	94°C, 2 min	94°C, 30 sec	60 ºC, 45 sec	72°C, 1 min	72°C, 2 min	Bathe et al. (2004)
16S r RNA	Forward 5'-CGCCCGCCGCGCGCGGCGG GCGGGGCGGGGGCACGGGG GGCCTACGGGAGGCAGGCA GCAG-3' Reverse 5'-ATTACCGCGGCTGCTGG-3'	94°C, 2 min	94°C, 30s	65°C, 45s	72°C, 1min	72°C, 2 min	Muyzer et al. (1993)

PCR products were loaded into a 1% agarose gel with SYBR Safe DNA gel stain (Molecular Probes, USA) with a 100-bp ladder (Fermentas, USA), ran for 45 minutes at 85 V, followed by visualization using the Invitrogen Safe Imager 2.0 (Invitrogen, Burlington, Ontario, Canada) to determine the presence of the PCR products.

2.2.10. Denaturing Gradient Gel Electrophoresis (DGGE):

All DGGE gels were 8% polyacrylamide with a denaturing gradient of 30-70% (7 M urea and 40% deionized formamide was considered to be 100% denaturant) and were cast using a gradient former (BioRad Laboratories, Mississauga, ON). For mixed cultures approximately 400 ng and for single cultures approximately 200 ng of the 16S rRNA product was loaded into each well of the DGGE gel. The gel was run in a DCode Universal Mutation Detection System (BioRad Laboratories, Mississauga, ON). Electrophoresis was run at a constant voltage of 80 V for 16 hrs at 60°C.

All gels were stained for 30 min in SYBR Gold (Invitrogen, Burlington, ON) with gentle agitation followed by brief de-staining in 1X TAE. The gel was imaged using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY). Bands of interest were excised and placed into 25 μL of sterile ddH20, followed by incubation at 4ºC fridge for 48 hrs before re-amplification.

2.2.11. Reamplification of DNA from Excised bands:

Samples were re-amplified with the same set of primers used in original PCR for the DGGE gel but without the GC-clamp. One micro litre of eluted DNA was re-amplified with the bacterial primers as follows: an initial denaturation of 5 min at 96°C, followed by 30 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min (Yeung *et al.*, 2011).

2.2.12. DNA Sequencing:

Reamplified PCR products were purified using the IBI Gel/ PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA), quantified and sent to The SickKids Center for Applied Genomics

(TCAG),for sequencing. DNA sequences were checked for similarity found in the NCBI Database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.13. Biofilm architecture analysis:

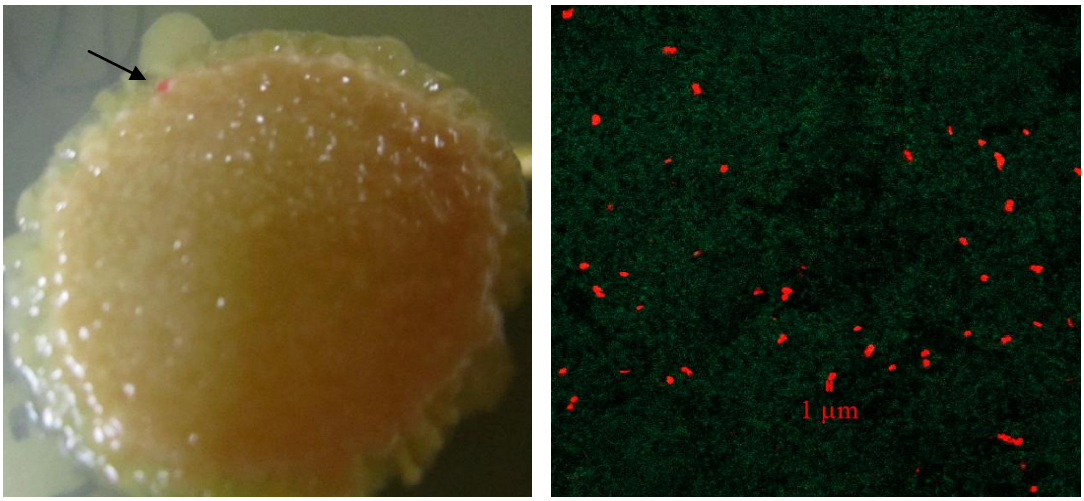
Image stacks were analysed from MMN + glucose and MMN + 2,4-D treated microbial cultures using the COMSTAT 2 program (Heydorn et al. 2000) for the quantification of biofilm biomass, average thickness, roughness coefficient, maximum thickness and surface to volume ratio. COMSTAT 2 is an image analysis script that runs in Image J (rsb.info.nih.gov/ij/). The biofilm biomass was quantified as the volume of biomass per substratum area (μm3/μm2). Thickness was measured as the maximum thickness (μm) over a given location.

2.3 Results:

2.3.1 Plate mating experiment:

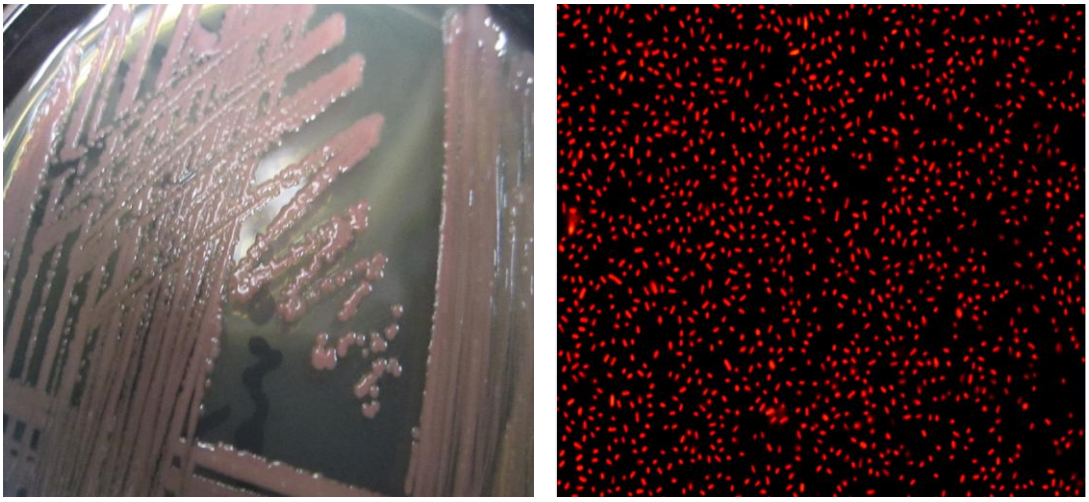
A plate mating experiment between soil-derived mixed microbial community and donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* was performed to evaluate the donor strain's ability to transfer plasmid pJP4 and to select a suitable recipient community for pJP4. Red fluorescent cells collected from the mating patch on days 10 and 14 were visualized using CLSM (Figure 2.4b). After two weeks of incubation, pink colonies appeared on the mating patch (Figure 2.4a). Pink transconjugants colonies isolated on LB and R_2A + gentamycin plates and showed red fluorescence under a dissection microscope (Figure

2.5a). Cream color colonies were also isolated from mating patch (image not shown) which showed red fluorescence under a dissection microscope. Image b in figure 2.4 shows a CLSM image of mating patch where pJP4 transconjugants exhibit red fluorescence and donor strain exhibits expected green fluorescence. Image a in figure 2.5 shows isolated transconjugant colonies and image b in figure 2.5 shows RFP expressing cells from isolated transconjugant colonies. Both colonies which demonstrated red fluorescence of individual cells were cultured as pure cultures. The 16s rRNA amplified products of these colonies were sequenced and after blast of the sequence data showed that the pink colony had a 98% similarity with *Comamonas testosteroni* strain Y10-2 16S and the cream colony showed a 100% similarity with *Comamonas testosteroni* strain Dc1 16S. *Comamonas testosteroni* belongs to the phylum *Proteobacteria*, class β-*Proteobacteria*, order *Burkholderiales* and family *Comamonadaceae.*



a b

Figure 2.4: Donor mating with soil-derived mixed community a. Soil + donor mating patch on LB plate, arrow indicates pink color transconjugant colonies b. CLSM image of soil + donor mating patch, 488 and 543 nm wavelength excitation. Red cells represent transconjugants and green cells represent donor strain



a b

Figure 2.5: a. Isolated pink transconjugant colonies from plate mating b. RFP expressing cells from isolated transconjugant colonies (60× magnification)

2.3.2 Experiment with non selective pressure (MMN + glucose):

2.3.2.1 PCR based detection of donor and transconjugants in effluent samples:

The occurrence of donor cells and cells carrying the 2,4-D degradative plasmid pJP4 was evaluated by a qualitative PCR approach targeting the *gfp* gene as a marker for donor cells, and the *dsRed* gene residing on the plasmid. The results of this analysis are shown in figures 2.6, 2.7 and 2.8. Channel 1 and 4 received the donor strain (while channels 2 and 3 were "no donor" controls), the effluent samples collected from channels 1 and 4 after days 1 and 7 of donor inoculation showed the presence of a 341 bp *gfp* product (figure 2.6, lanes 3-6 and figure 2.7 lane 4). The amplified *gfp* product was not present in samples collected on days11 and 14. Amplified *gfp* product was not present in the control channels (Figure 2.6: lanes 11-14). Figure 2.8 shows the presence of 480 bp *dsRed* products in effluent samples collected from channel 1 on day 1 after donor inoculation (lane 3) and effluent samples collected from channel 1 and 4 on day 7

after donor inoculation (lanes 5,6). Effluents from control channels did not show presence of *dsRed* gene (lanes 11-14).

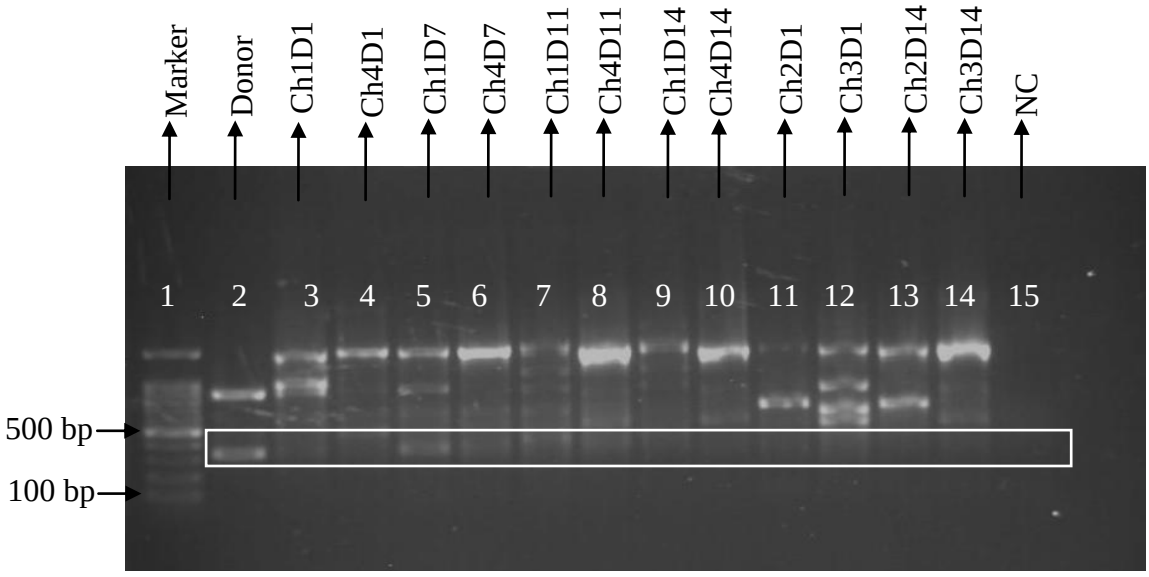


Figure 2.6: Agarose-gel showing PCR-products of the *gfp* gene (341 bp) (white box) in the chromosome of the donor cells. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lanes 3-14: effluent samples collected on different sampling days from donor inoculated and control channels. Channel 1 and 4 received the donor, channel 2 and 3 served as control (no donor), lane 15: NC, negative control (no template DNA)

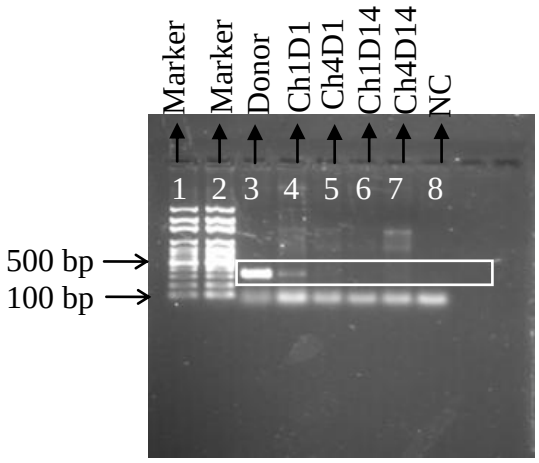


Figure 2.7: Agarose-gel showing PCR-products of the *gfp* gene (341 bp) (white box) in the chromosome of the donor cells. Lanes 1, 2: 100 bp marker, lane 3: donor strain as positive control, lane 4-7: effluent samples collected on different sampling days from channel 1 and 4 (donor inoculated channels), lane 8: NC, negative control (no template DNA)

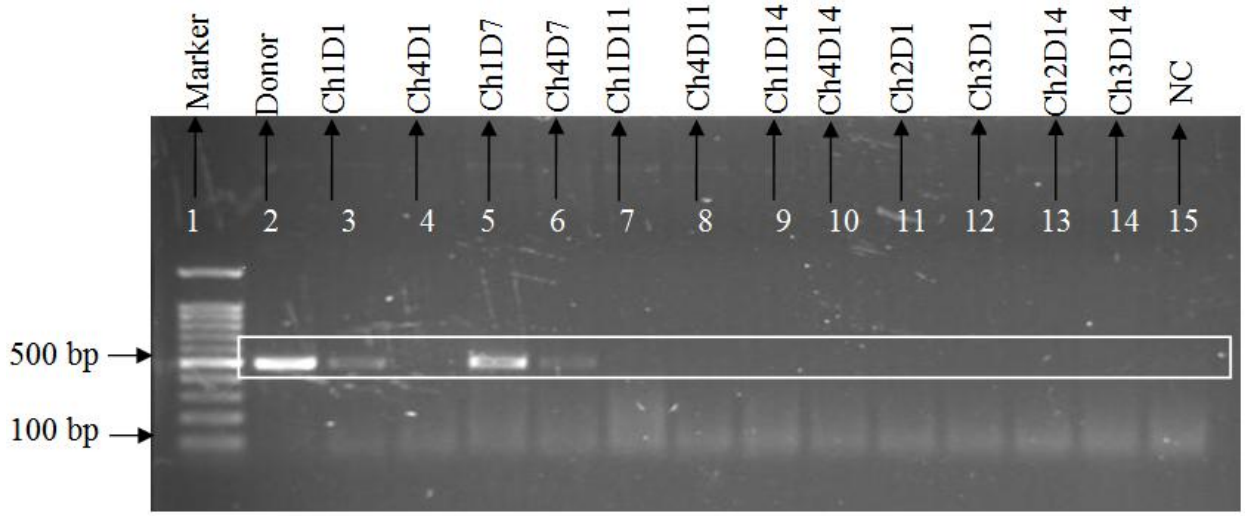


Figure 2.8: Agarose-gel showing PCR-products of the *dsRed* gene (480 bp) (white box) residing in the pJP4 plasmid. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lane 3-14: effluent samples collected on different sampling days from donor inoculated and control channels. Channel 1 and 4 received the donor, channel 2 and 3 served as control (no donor), lane 15: NC, negative control (no template DNA)

2.3.2.2 Plate count results for MMN + glucose experiment:

Isolation of donor and plasmid recipients was attempted by plating effluent samples onto R_2A, R_2A + gentamycin, R_2A + gentamycin + kanamycin and LB + gentamycin + kanamycin plates. Colonies grew on these plates but after two weeks of incubation to allow for RFP production pink colonies which are typical of colonies expressing DsRed were not observed. Cells from colonies that grew on LB + gentamycin + kanamycin plates were checked by CLSM but they did not show green fluorescence typical of donor cells. Table 2.2 shows the quantitative results for effluent samples on different medium plates. There was no significant difference in cell counts between day 1 and 7. R_2A plates without antibiotics had the highest counts for both day 1 and

day 7. The CFU/mL counts on day 7 was slightly lower than day 1. The total range of CFU/mL was 2.1×10^6 - 3.3×10^8. The range of CFU/mL count on R_2A plate was 1.7×10^8 - 3.3×10^8. On R_2A + gentamycin plates the average CFU/mL count for donor inoculated channels on day 1 was 6.1×10^7 which was slightly lower than control channels and on day 7 the count was 1.1×10^7 CFU/mL which was slightly higher than control channels. There was no observable difference in CFU/mL counts on R_2A + gentamycin + kanamycin plates on day 1 between donor inoculated and control channels, on day 7 the value was 2.1×10^6 CFU/mL which was slightly lower than control channels. On LB + gentamycin + kanamycin plates average CFU/mL count for donor inoculated channels were 1.2×10^7 on day 1 and 4.0×10^6 on day 7 which were less than control channels.

The range of CFU/mL count on R_2A plate without antibiotic was 1.7×10^8 - 3.3×10^8 and the range of CFU/mL on antibiotic plates was 2.1×10^6- 8.2×10^7. The ranges of CFU/mL were 6.0×10^6- 1.9×10^8 and 2.1×10^6- 3.3×10^8 for control (no donor) and donor inoculated channels, respective

Table 2.2: Quantitative results for effluent samples collected from control and donor inoculated channels on day 1 and day 7 after donor inoculation

Plate	**Treatment**	Day 1	Day 7
		CFU/mL	**CFU/mL**
R_2A	Glucose+ Donor-	2.8×10^8	1.9×10^8
	Glucose +, Donor +	3.3×10^8	1.7×10^8
R_2A + gentamycin	Glucose+ Donor-	8.2×10^7	6.0×10^6
	Glucose +, Donor +	6.1×10^7	1.1×10^7
R_2A + gentamycin + kanamycin	Glucose+ Donor-	4.3×10^7	6.2×10^6
	Glucose +, Donor +	4.5×10^7	2.1×10^6
LB + gentamycin + kanamycin	Glucose+ Donor-	4.9×10^7	7.7×10^6
	Glucose +, Donor +	1.2×10^7	4.0×10^6

2.3.2.3. Confocal microscopy images of biofilm exposed to MMN + glucose:

The biofilms of all four channels were stained with 5 (6) - CFDA, SE. This is a cell permeant, fluorescein based stain, which passively diffuse into the cells and remains nonfluorescent until it's acetate groups are cleaved by intracellular esterases to yield highly fluorescent fluorophores. For each channel seven stacks were collected in *Z* direction with the Zeiss LSM 510. Thick biofilm was observed in all four channels Figure 2.9 shows a z-stack image of biofilm with added donor strain fed with MMN + glucose. 488 and 543 nm wavelength lasers were used. Cluster of organisms forming the biofilm can be seen. Red fluorescing transconjugants were not

detected in the biofilms and as the biofilms were stained with 5 (6) - CFDA, SE which is a green fluorescent dye donor cells could not be differentiated in the biofilms.

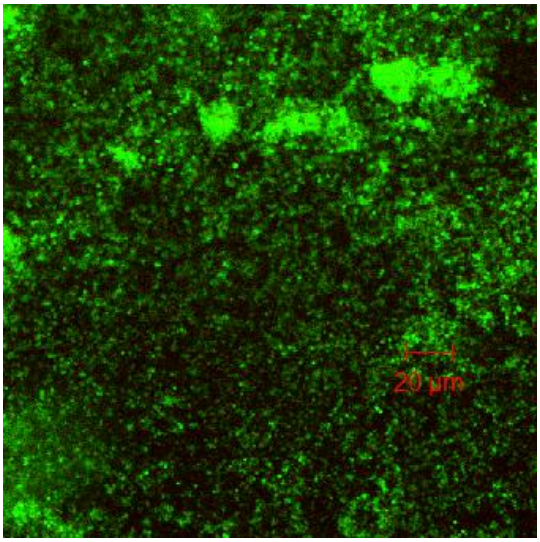


Figure: 2.9: A z-stack representing CLSM image of biofilm formed with added donor after 10 days of donor inoculation. MMN + glucose was used as feed. Biofilm was stained with 5 (6) - CFDA, SE. (10× magnification). 488 and 543 nm wavelength lasers were used.

2.3.2.4. Biofilm quantitative analysis by COMSTAT software:

Biofilm development at the glass surface of replicate flowcell chambers was investigated with CLSM and COMSTAT image analysis. Biofilm average thickness, maximum thickness, roughness coefficient, biomass, surface to volume ratio were measured by analysis of confocal microscopy images with COMSTAT software. Table 2.3 shows the mean of each architectural parameter when glucose was used as carbon source in MMN medium. The mean average thickness was lower for control (no donor) than the donor inoculated channels. The highest mean maximum thickness was obtained for channel 2 (control) which was 95.71 (µm) and lowest mean maximum thickness was obtained for channel 3 (control) which was 48.57 (µm). The

range of mean of roughness coefficient was 1.24 – 1.93. The mean biomass was highest for channel 4 which received the donor and the value was 6.09 (µm^3/µm^2) and the lowest value was for channel 2 (control) which was 0.51(µm^3/µm^2). The range for surface to volume ratio was 0.17- 0.85 **(**µm^2/µm^3). Table 2.3 shows that standard deviation values were high for average thickness and maximum thickness which represents heterogeneous biofilm architecture. Due to the high standard deviation values no statistical analyses were carried out.

Table 2.3: COMSTAT analysis of various architectural parameters of biofilms namely average thickness (µm), maximum thickness (µm), biomass (µm^3/µm^2), roughness coefficient, surface to volume ratio (µm^2/µm^3). The medium was MMN + glucose.

Channel	**Treatment**	**Mean of Average Thickness (µm)**	**Stdev of Average thickness**	**Mean of Maximum Thickness (µm)**	**Stdev of Maximum thickness**	**Mean of Roughness coefficient**	**Stdev of Roughness Stdev of**	**Mean of Biomass (µm^3/µm^2)**	**Stdev of biomass**	**Mean of Surface to Volume Ratio (µm^2/µm^3)**	**Stdev of S/V ratio**
Channel 2	Glucose+, Donor -	1.21	1.3	95.71	19.9	1.93	0.1	0.51	0.4	0.65	0.4
Channel 3	Glucose+, Donor -	1.14	0.9	48.57	9.0	1.91	0.1	0.56	0.5	0.85	0.6
Channel 1	Glucose+, Donor +	4.07	6.3	57.14	26.9	1.67	0.5	3.06	4.0	0.45	0.5
Channel 4	Glucose+, Donor+	17.12	24.8	61.43	67.7	1.24	0.5	6.09	5.6	0.17	0.1

2.3.3 Experiment with selective pressure (MMN + 2,4-D):

2.3.3.1 PCR based detection of donor and transconjugants in effluent samples:

Qualitative PCR approach targeting the *gfp* gene as a marker for donor cells, and the *dsRed and tfdB* genes residing on the plasmid pJP4 was also used to evaluate the presence of donor cells and cells carrying the plasmid pJP4 when MMN + 2,4-D was used as feed. The results of this analysis are shown in the agarose gel in figure 2.10, 2.11, 2.12 and 2.13. Channel 2 and 4 received the donor strain and channel 1 and 3 were control. The effluent samples collected from channel 2 and 4 after day 1 and day 4 of donor inoculation showed the presence of 341 bp *gfp* product (figure 2.10, lanes 3-6). After effluent plating (day 1 after donor inoculation) one type of colony grew on LB + kanamycin + gentamycin plates were confirmed as donor strain as the desired size of amplified *gfp* product was present (figure 2.11, lanes 3, 4). In figure 2.12, lanes 3-6 show the presence of amplified product of *dsRed* gene on day 1 and 4 in the effluents from channel 2 and 4 which received the donor strain. Very faint bands were present on day 7. But there was no amplified product for *dsRed* gene in the effluents from control channels. In figure 2.13 lanes 3-12 show the presence of amplified product of *tfdB* gene in the effluents of both control and donor inoculated channels. In this study, the presence of *tfdB* gene was detected in the soil-derived inoculum as well.

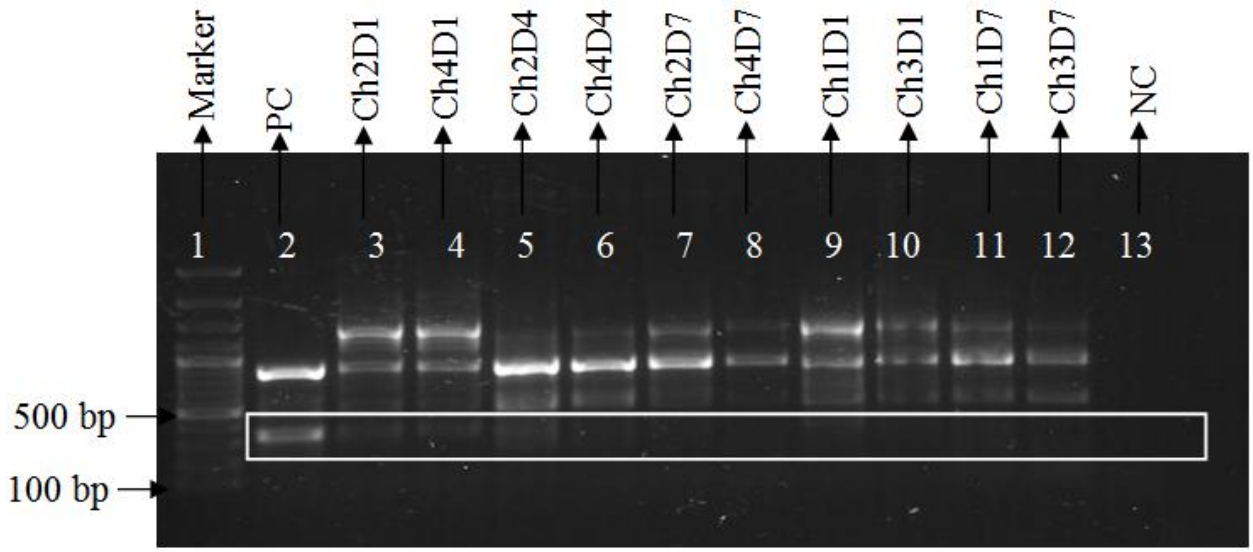


Figure 2.10: Agarose-gel showing PCR-products of the *gfp* gene (341 bp) (white box) in the chromosome of the donor strain. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lane 3-12: effluent samples collected on different sampling days from donor inoculated and control channels. Channel 2 and 4 received the donor, channel 1 and 3 served as control (no donor), lane 13: NC, negative control (no template DNA).

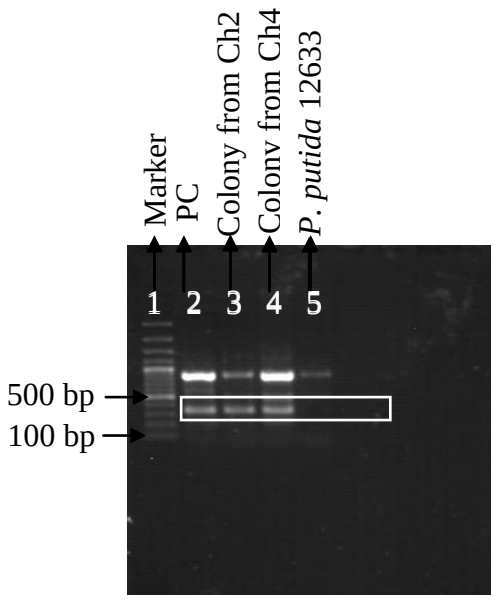


Figure 2.11: Agarose-gel showing *gfp* PCR-products (341 bp) (white box) of the donor strain grown on LB + kanamycin + gentamycin plates after effluent plating. Lane 1: 100 bp marker, lane 2: donor as positive control, lane 3 and 4: colonies grew on LB + kanamycin + gentamycin plate from donor inoculated channels after day 1. Lane 5: *Pseudomonas putida* ATCC 12633 as negative control

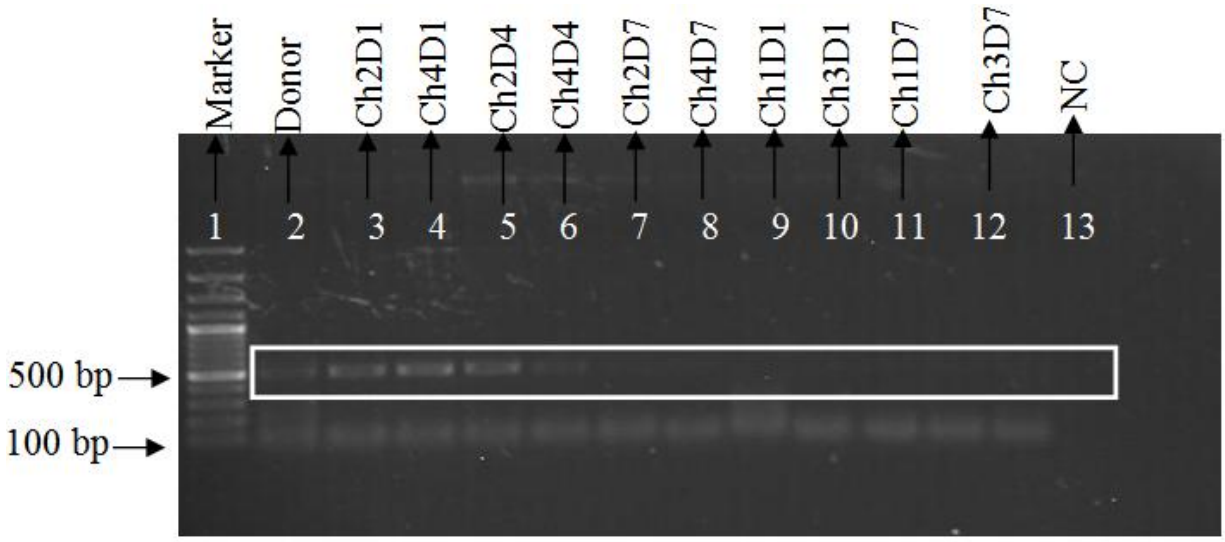


Figure 2.12: Agarose-gel showing PCR-products of the *dsRed* gene (480 bp) (white box) residing in the pJP4 plasmid. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lane 3-12: effluent samples collected on different sampling days from donor inoculated and control channels. Channel 2 and 4 received the donor strain, channel 1 and 3 served as control (no donor), lane 13: NC, negative control (no template DNA)

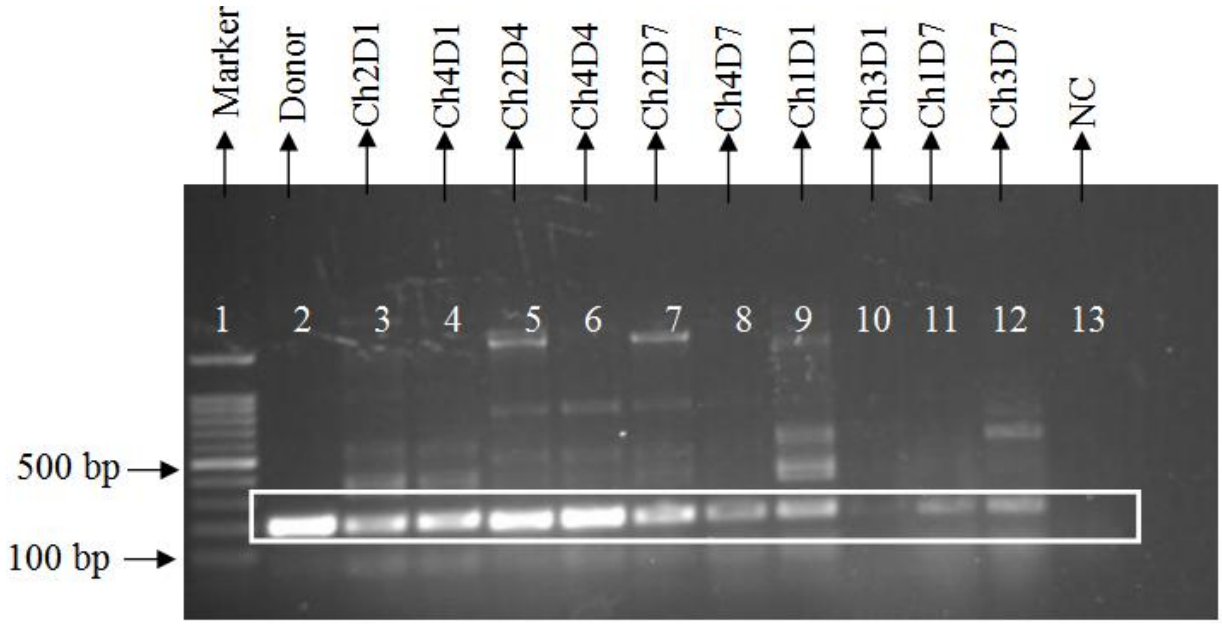


Figure 2.13: Agarose-gel showing PCR-products of the *tfdB* gene (205 bp) (white box) residing in the pJP4 plasmid. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lane 3-12: effluent samples collected on different sampling days from donor inoculated and control channels. Channel 2 and 4 received the donor strain, channel 1 and 3 served as control (no donor), NC: negative control (no template DNA)

2.3.3.2. Plate count results for MMN + 2,4-D experiment:

Isolation of donor and plasmid recipients was attempted by plating effluent samples onto plates with or without antibiotics. Colonies grew on these plates but after two weeks of incubation to allow for RFP production pink colonies which are typical of *dsRed* expressing transconjugant colonies were not observed. One type of colony grew on R_2A and LB + gentamycin + kanamycin plates on day 1 showed green fluorescence under dissection microscope typical of donor colonies. The CFU/mL count for green fluorescence colonies on R_2A plate was 4.1×10^6 and on LB + gentamycin + kanamycin plate the value was 1.8×10^6 CFU/mL (Table 2.4). There was no

significant difference in cell counts between day 1 and day 7 (Table 2.4). The total range for CFU/mL count was 7.7×10^5 to 1.9×10^9. R_2A plate without antibiotics had the highest count and CFU/mL range was 1.3×10^8- 1.9×10^9. On R_2A + gentamycin plate the average CFU/mL count for donor inoculated channels on day 1was 4.0×10^6 which was slightly lower than control channels and on day 7 the count was 1.1×10^6 CFU/mL which was also slightly lower than control channels. On R_2A + gentamycin + kanamycin plates the average CFU/mL count for donor inoculated channels on day 1 was 1.3×10^6 which is higher than control channel, on day 7 the value is 9.5×10^5 CFU/mL which is slightly lower than control channels. On LB + gentamycin + kanamycin plates average CFU/mL count for donor inoculated channels was 3.5×10^6 which was slightly higher than control channels on day 1 and on day 7 value was 1.1×10^6 CFU/mL which was lower than the control channels.

The range of CFU/mL count on R_2A plate without antibiotic was 1.3×10^8 - 1.9×10^9 and the range of CFU/mL on antibiotic plates was 7.7×10^5 - 6.9×10^6. The ranges of CFU/mL were 7.7×10^5 -1.9×10^9 and 9.5×10^5 - 2.8×10^8 for control (no donor) and donor inoculated channels, respectively.

Table 2.4: Quantitative results for effluent samples collected from control and donor inoculated channels on day 1 and day 7 after donor inoculation

Plate	**Treatment**	**Day 1**			**Day 7**		
		CFU/mL	**GFP***	**RFP***	**CFU/mL**	**GFP***	**RFP***
R_2A	2,4-D+ Donor-	1.9×10^9	ND	ND	2.6×10^8	ND	ND
	2,4-D + Donor +	2.8×10^8	4.1×10^6	ND	1.3×10^8	ND	ND
R_2A + gentamycin	2,4-D+ Donor-	6.5×10^6	ND	ND	5.4×10^6	ND	ND
	2,4-D + Donor +	4.0×10^6	ND	ND	1.1×10^6	ND	ND
R_2A + gentamycin + kanamycin	2,4-D+ Donor-	7.7×10^5	ND	ND	5.9×10^6	ND	ND
	2,4-D + Donor +	1.3×10^6	ND	ND	9.5×10^5	ND	ND
LB + gentamycin + kanamycin	2,4-D+ Donor-	2.7×10^6	ND	ND	6.9×10^6	ND	ND
	2,4-D + Donor +	3.5×10^6	1.8×10^6	ND	1.1×10^6	ND	ND

*Potential colony fluorescence was checked using a dissection microscope. ND: Not detected

2.3.3.3 Confocal microscopy images of biofilm exposed to MMN + 2,4-D:

The biofilms of all four channels were stained with Syto 62 on day 7. Syto 62 was used because it does not interfere with GFP fluorescence. However, because it is not a vital stain, it was applied only once, at the end of the experiment. For each channel ten stacks were collected in *Z* direction with the Zeiss LSM 510. Thick biofilm (42 -62 µm) was observed in all four channels.

Figure 2.14 shows a z-stack image of biofilm with added donor strain after 7 days of donor addition. MMN + 2,4-D was used as feed. 633 nm wavelength laser was used. Clusters of organisms forming the biofilm can be seen. RFP fluorescing transconjugants and green fluorescing donor cells were not detected in the biofilms.

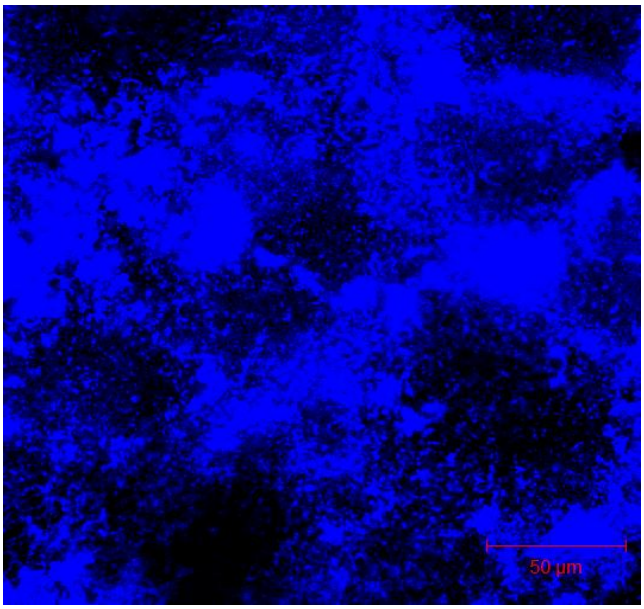


Figure 2.14: A z-stack representing CLSM image of biofilm formed with added donor after 7 days of donor inoculation. MMN + 2,4-D was used as feed. Biofilm was stained with Syto62 and 633 nm wavelength laser was used (20× magnification).

2.3.3.4. Biofilm quantitative analysis by COMSTAT software:

Biofilm average thickness, maximum thickness, roughness coefficient, biomass, surface to volume ratio were also measured by analysis of confocal microscopy images with COMSTAT software when 2,4-D was used as carbon source in MMN medium. Table 2.5 shows the mean of each parameter. The highest mean average thickness was obtained for channel 2 (donor inoculated) which was 31.0 µm and lowest value was for channel 4 (donor inoculated) which

was 12.66 µm. The highest mean maximum thickness was obtained for channel 2 (donor inoculated) which was 62.0 µm and lowest value was for channel 3 (control) which was 42.0 µm. The range for mean roughness coefficient was 0.56 -1.12. The highest mean biomass was highest for channel 2 (donor inoculated) which was 21.82 (µm^3/µm^2) and lowest value was for channel 4 (donor inoculated) which was 11.73 (µm^3/µm^2). The range for mean surface to volume ration was 0.07-0.11(µm^2/µm^3). The standard deviation values for average thickness, maximum thickness, biomass were high (same as experiment with MMN + glucose) which indicates heterogeneous biofilm architecture. Table 2.3 and 2.5 also show that values for mean average thickness, mean maximum thickness, mean biomass were higher when 2,4-D was used instead of glucose as carbon source.

Table 2.5: COMSTAT analysis of various architectural parameters of biofilms namely average thickness (µm), maximum thickness (µm), biomass (µm^3/µm^2), roughness coefficient, surface to volume ratio (µm^2/µm^3). The medium was MMN + 2,4-D.

Channel	**Treatment**	**Mean of Average Thickness (µm)**	**Stdev of Average thickness**	**Mean of Maximum Thickness (µm)**	**Stdev of Maximum thickness**	**Mean of Roughness coefficient**	**Stdev of Roughness Stdev of**	**Mean of Biomass (µm^3/µm^2)**	**Stdev of biomass**	**Mean of Surface to Volume Ratio (µm^2/µm^3)**	**Stdev of S/V ratio**
Channel 1	2,4-D +, Donor -	17.14	13.6	46.00	24.1	0.91	0.5	13.89	6.5	0.08	0.0
Channel 3	2,4-D +, Donor -	12.67	12.2	42.00	29.4	1.12	0.6	12.91	10.1	0.11	0.1
Channel 2	2,4-D +, Donor +	31.00	13.3	62.00	20.4	0.56	0.4	21.82	7.6	0.07	0.0
Channel 4	2,4-D +, Donor +	12.66	12.6	46.00	14.3	1.02	0.6	11.73	7.3	0.10	0.0

2.3.3.5 Spectrophotometric measurement of 2,4-D concentration:

Spectrophotometric measurement was done to detect the concentration of 2,4-D in the MMN medium reservoir and effluents from control and donor inoculated channels on different sampling days. Table 2.6 shows 2,4-D concentration for effluents from both control and donor inoculated channels very slightly decreased after day 1 of donor inoculation. After days 4, 5 and 7 concentration of 2,4-D was higher for effluents from both control and donor inoculated channels than the initial MMN reservoir. Table 2.7 shows UV absorbance of effluents from channels with or without donor inoculation at 230 nm and medium was MMN + glucose or MMN + 2,4-D. On day 1 the UV absorbance values for effluents from MMN + 2,4-D experiments were 0.7718 and 0.7891 for control and donor inoculated channels, respectively. On day 7 the values were 1.0417 and 0.9572. The UV absorbance range was 0.0085-0.1429 for the effluents from MMN + glucose experiment.

Table 2.6: Measurements of 2,4-D concentration in the initial MMN medium and effluents from control and donor inoculated channels

Time after donor inoculation	**Initial 2,4-D concentration (mM) in MMN medium**	**Treatment**	**2,4-D concentration (mM) in effluents**
Day 1	1.2031	Donor -	1.1349
		Donor +	1.0955
Day 4	1.0622	Donor -	1.3082
		Donor +	1.3316
Day 5	1.0622	Donor -	1.6246
		Donor +	1.5119
Day 7	1.2031	Donor -	1.4549
		Donor +	1.3346

Table 2.7: UV absorbance of effluents from channels with or without donor inoculation at 230 nm

Time after donor inoculation	**Treatment**	**Absorbance**	**Treatment**	**Absorbance**
Day 1	2,4-D + Donor -	0.7718	Glucose + Donor -	0.0738
	2,4-D + Donor +	0.7891	Glucose + Donor +	0.1429
Day 7	2,4-D + Donor -	1.0417	Glucose + Donor -	0.0346
	2,4-D + Donor +	0.9572	Glucose + Donor+	0.0085

2.3.4 DGGE analysis for donor, inoculum, plate mating transconjugants, effluents from the last day of the experiments:

PCR-DGGE analysis of donor, inoculum, plate mating transconjugants, effluents from the last days of MMN + glucose (day 14) and MMN + 2,4-D (day 7) experiments were performed to analyze composition of inoculum and composition of the microbial communities on the last day of experiments. Figure 2.15 shows an image of DGGE-gel. Arrowhead indicates prominent bands and these bands were excised for sequencing. DGGE profiles for inoculum (lane 2) and last day effluents (lanes 7-10) are different. DGGE profiles for last day effluent samples for MMN + glucose (lanes 7, 8) and MMN + 2,4-D (lane 9,10) are also different. Transconjugants isolated from plate mating (lanes 3-6) and last day effluent samples for MMN + glucose experiment (lanes 7, 8) share a common band (as indicated by a white box). DGGE profiles also show that the donor (profile shown in lane 1) was not present in the effluents on the last day of both experiments and plate mating transconjugants were not present on the last day of MMN + 2,4-D experiment.

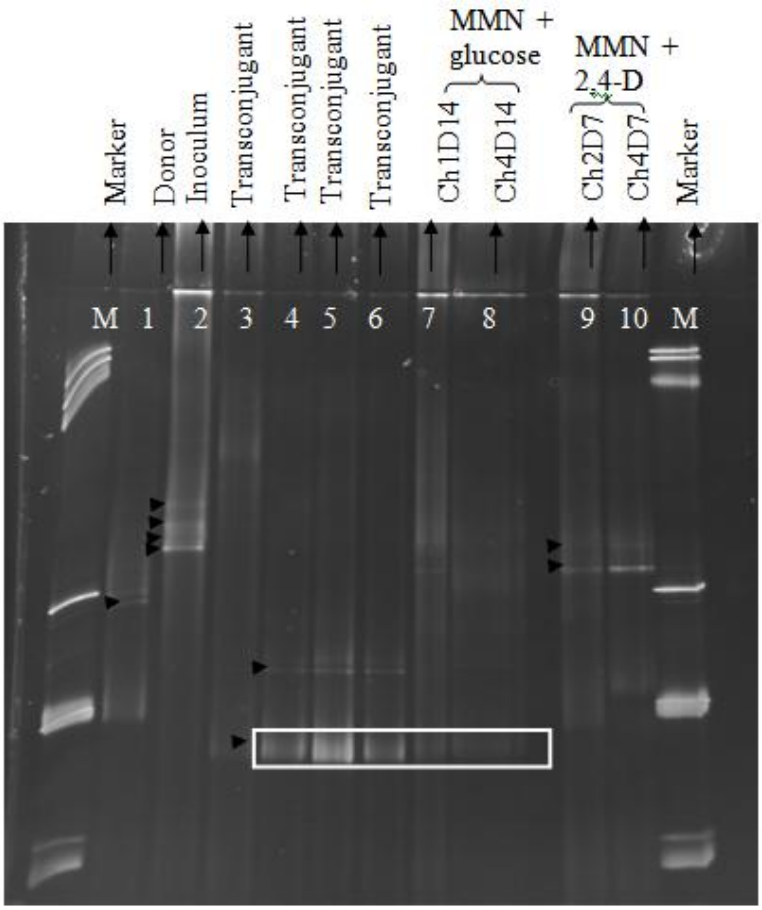


Figure 2.15: DGGE profiles of donor, plate mating transconjugants, effluent samples from the last day of the experiments. Lane 1 represents DGGE profile for donor strain, lane 2 represents DGGE profile for inoculum, lanes 3-6 represent DGGE profiles for plate mating transconjugants, lanes 7, 8 represent DGGE profiles for last day effluent samples from channel 1 and channel 4 respectively which received the donor strain for MMN + glucose experiment, lanes 9,10 represent DGGE profiles for the last day effluent samples from channel 2 and channel 4 respectively which received the donor strain for MMN + 2,4-D experiment. Arrowhead indicates prominent bands. The white box indicates transconjugants isolated from plate mating (lanes 3-6) and last day effluent samples for MMN + glucose experiment (lanes 7, 8) share a common band.

2.4 Discussion:

2.4.1 Plate mating experiments:

An initial plate mating study was conducted to ensure that the donor strain *Pseudomonas putida* SM1443::*gfp*2x-pJP4::*dsRed* had the ability to transfer the plasmid pJP4 to potential recipients within a soil-derived microbial consortium. The mating experiment allows close spatial arrangement of bacteria cells for genetic transfer to occur through sex pili. The mating experiment allowed us to keep donor strain and soil-derived microbes in close contact in a static condition and nutrient rich environment.

Confocal microscopy was used to detect red fluorescent transconjugant cells. Pink colonies were observed within the mating patch (Figure 2.4a), and red fluorescence was maintained in cells of isolated pink colonies (Figure 2.5a). Red fluorescence was also confirmed using a dissection microscope. These observations confirmed that the donor strain was able to transfer the plasmid pJP4 to the members of soil-derived mixed community. As the donor strain was capable of transferring the plasmid to soil-derived mixed community we can conclude that the cellular machinery necessary for plasmid transfer was functional in the donor strain.

Sequencing of an isolated pink colony showed a 98% 16S rRNA sequence similarity to *Comamonas testosteroni* strain Y10-2 16S. Sequencing of an amplified 16S rRNA fragment from a cream colony, which upon microscopy revealed red fluorescence, showed 100% similarity to *Comamonas testosteroni* strain Dc1 16S. *Comamonas testosterones are* microorganisms belonging to the phylum *Proteobacteria*, class β-*Proteobacteria* and family *Comamonadaceae*. Bathe et al. (2004a) isolated and identified transconjugants of plasmid pJP4, originating from an agar plate mating of a *Pseudomonas putida* SM1443::*gfp*2x-pJP4::*dsRed*

donor with an activated sludge-derived microbial community. They found the transconjugant strains belonged to a variety of genera of the α-, β-, γβ- and γ classes of the *Proteobacteria,* mostly to the families *Rhizobiaceae* and *Comamonadaceae*. They also isolated a transconjugant which showed 98.5% sequence similarity to *Comamonas* sp. 12022. Bathe et al. (2004a) also found that α-proteobacterial isolates displayed intense red fluorescence upon microscopic observation and formed brightly pink colonies on R_2A agar. Bathe et al. (2004a) also mentioned that this methodology relies on growth of the transconjugants on solid media and on expression of DsRed within a transconjugant cell; non-culturable cells and strains which do not express DsRed can be missed by this approach.

From the plate mating experiments, potential pJP4 recipients in the soil-derived mixed culture were identified. This soil derived mixed culture was used as recipient community inoculum for flow cell experiments with non selective pressure and selective pressure.

2.4.2 Flow cell experiments with non selective and selective pressure:

The genetic contents of the donor strain and pJP4 plasmid used in this study gave us three particular avenues for the evaluation of gene transfer; fluorescent proteins (chromosomally-encoded GFP, plasmid-encoded RFP), antibiotic resistance (chromosomally-encoded kanamycin resistance, plasmid-encoded gentamycin resistance), and the *tfdB* gene in the pJP4 plasmid which encodes the degradation of 2,4-D.

2.4.2.1 Biofilm growth and plasmid transfer under non selective pressure (MMN + glucose) and selective pressure (MMN + 2,4-D):

PCR based detection of donor and transconjugants in effluent samples for both non selective pressure and selective pressure:

For non selective pressure experiment (MMN + glucose) figure 2.6 and 2.7 show the bands resulting from the amplified *gfp* product were present on days 1 and 7 in effluents collected from channels 1 and 4 which received the donor strain. Although the bands were faint, their presence indicates the occurrence of donor until day 7 after inoculation into flow cell channels. Bathe et al. (2004b) detected the presence of bands for *gfp* amplified product until day 6 from a sequencing batch biofilm reactor (SBBR) with added donor when they used synthetic wastewater with easily degradable carbon sources e.g. sodium acetate, citric acid, sodium gluconate, and D(+)-glucose as feed. This indicated the presence of donor. One of our initial experiments also confirmed that donor could form dense biofilm when MMN + glucose was used as feed (Appendix D). In our initial experiment (Appendix D) donor was present in the flow cell channels over the entire experimental time (day 8). Figure 2.8 shows the presence of bands resulting from the amplified *dsRed* product in the effluent samples. The presence of bands on days 1 and 7 from donor inoculated channels indicates that cells carrying plasmid pJP4 were present until day 7 after donor inoculation as the *dsRed* gene is residing on the plasmid pJP4. Although the band intensity for *dsRed* gene is greater than that for the *gfp* gene, this PCR based technique is not quantitative so we cannot confirm bands for *dsRed* gene originated from donor or transconjugant cells. If the quantitative PCR results can indicate abundance of donor cells decreases (amplified PCR product for *gfp* decreases) and the abundance of transconjugant cells increases (if the amount of amplified product for *dsRed* increases with a concurrent decrease in

the amount of *gfp*) we can assume that plasmid was transfer from donor strain to recipients as the *gfp* gene is encoded on the donor chromosome and the *dsRed* gene resides on the plasmid.

Presence of *tfdB* gene was detected in the biofilm effluents from our initial experiments until day 14 after donor inoculation when glucose was used as carbon source and an alternative soil (peat)-derived mixed community was used as inoculum (Appendix E, figure 4.8). Bathe et al. (2004b) also detected bands for *tfdB* gene until day 6 after donor inoculation from SBBR when they used synthetic wastewater I with easily degradable carbon sources including D (+)-glucose as feed. In our experiment, the band for amplified *tfdB* product was not present in the soil (peat)-derived inoculum (Appendix E, figure 4.8, lane 3) but bands for amplified *tfdB* product were present until day 14 after donor inoculation (Appendix E, figure 4.8, lane 4-11) which indicates the presence of pJP4 carrying cells. Here we also cannot confirm bands for *tfdB* gene originated from donor or transconjugant cells as bands for *gfp* gene were also present until day 14 (Appendix E, figure 4.7).

For selective pressure experiment (MMN + 2,4-D) figure 2.10 and 2.11 show that bands resulting from the amplified *gfp* product were present on days 1 and 4 in effluents collected from channels which received the donor. This indicates the donor persisted for four days after it was inoculated. It was previously tested that the donor strain was able to grow in MMN medium containing 5 mM 2,4-D and 1 mM NH4Cl (Bathe et al. 2004b). Figure 2.12 shows the presence of bands resulting from the amplified *dsRed* product on days 1 and 4 in effluents from donor-inoculated channels. On day 7 bands were very faint. This result indicates that cells carrying plasmid pJP4 were prominently present until day 4. As this PCR technique is not quantitative we cannot confirm bands for *dsRed* gene originated from donor or transconjugant cells. If the quantitative PCR results can indicate amplified PCR product for *gfp* decreases and the amount of

amplified product for *dsRed* increases with a concurrent decrease in the amount of *gfp* we can assume that plasmid was transfer from donor strain to recipients as the *gfp* gene is encoded on the donor chromosome and the *dsRed* gene resides on the plasmid.

For MMN + 2,4-D experiment figure 2.13 shows the presence of *tfdB* gene in the effluents from control and donor inoculated channels. Previously we detected the presence of *tfdB* gene in the soil-derived inoculum (image not shown). So, *tfdB* could not be used as an indicator of the presence of the plasmid in the biofilm effluents. Bathe et al. (2004b) detected the presence of transconjugants in SBBR on the last day of their experiment (day 32) as they detected band for *tfdB* gene but not for *gfp* when they used synthetic wastewater II with 2 mM of sodium dichlorophenoxyacetate (2,4-D) as sole carbon source.

For non selective pressure experiment (MMN + glucose) both donor and cells carrying plasmid pJP4 persisted in the effluents until day 7. For selective pressure experiment (MMN + 2,4-D) donor persisted in the effluents until day 4 and cells carrying plasmid pJP4 persisted in the effluents until day 7.

Culturable cell counts for non selective pressure (MMN + glucose) and selective pressure (MMN + 2,4-D) :

For MMN + glucose experiment there was no significant difference in the cell counts between day 1 and day 7 and also between donor inoculated and non-inoculated control channels. Table 2.2 shows 10^6-10^7 CFU/mL count for control channels on the antibiotic plates those did not receive the donor strain which indicates presence of kanamycin and gentamycin antibiotic

resistance in the soil-derived mixed inoculum. After 14 days of incubation (to allow for the expression and folding of RFP) no RFP fluorescing transconjugant colonies were detected upon visual inspection. One type of colony that grew on LB + kanamycin + gentamycin plates had the appearance similar to a donor colony (beige, slightly raised, round, entire margin) but green fluorescence in cells was not detected microscopically. These results likely indicate that colonies that grew on antibiotic plates were neither donor cells nor transconjugants as they did not display the expected fluorescence. In our initial experiments, we detected antibiotic resistant colonies from inoculum those grew on R_2A + gentamycin, R_2A + kanamycin + gentamycin, LB + kanamycin + gentamycin plates. Many antibiotic resistant colonies originating from the soil inoculum and no donor and transconjugant colonies were detected on antibiotic plates. The PCR results showed the presence of amplified product for both *dsRed* and *gfp* in effluents. Some colonies might arise from cells which received pJP4 plasmid but were not able to express the *dsRed* gene. But there is no evidence to support this possibility.

For MMN + 2,4-D experiment there was no significant difference in cell counts between day 1 and day 7 (Table 2.4). There was also no significant difference in cell counts for donor inoculated and non-inoculated channels. RFP expressing transconjugant colonies were not detected after plating the effluents. GFP fluorescing donor colonies were detected from R_2A and LB + kanamycin + gentamycin plates after day 1 of donor inoculation but not on day 7. Bathe et al. (2004b) plated cell samples from a donor- bioaugmented reactor (day 32) on MNN medium containing 2,4-D as the sole carbon source and isolated red fluorescent transconjugants. In our experiments, we used medium plates with gentamycin (25 µg/mL) to isolate pJP4 transconjugants, but we did not use medium plates with 2,4-D to isolate transconjugants. It is possible that some cells received plasmid pJP4 but the concentration of gentamycin was not

enough to create sufficient selective pressure to maintain the plasmid in transconjugants. Alternatively, transconjugant cell density was too low to be detected on plates. Additionally, some transconjugants may have been viable but not culturable.

There was no significant difference in cell counts between day 1 and day 7 for both with non selective pressure (MMN + glucose) and selective pressure (MMN + 2,4-D) experiments. For both experiments many antibiotic resistant colonies originated from the soil inoculum but no transconjugant colonies were detected on antibiotic plates. For MMN + 2,4-D experiment donor colonies were detected on R2A and LB + kanamycin + gentamycin plates on day 1 but for MMN + glucose experiment donor colony was not detected.

Confocal microscopy images of biofilm exposed to non selective pressure (MMN + glucose) and selective pressure (MMN + 2,4-D) and COMSTAT analysis of biofilm architecture:

For non selective pressure experiment (MMN + glucose) an image of the biofilm exposed to MMN + glucose is shown in figure 2.9 Clusters of cells forming the biofilms were observed. RFP expressing transconjugants were not detected in the biofilms from confocal images. 5(6) – CFDA, SE was used to stain the biofilms. 5(6) – CFDA, SE is an amine- reactive reagent which can passively diffuse into the cells. The dye remains colourless and nonfluorescent until it molecules' acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine reactive fluorophores. Upon reaction with amine-containing residues of intracellular proteins, these probes form dye-protein adducts that are well retained in the cells (Molecular probes, Invitrogen). This dye was used because it has been shown not to interfere with cell viability (Bester et al. 2010). We needed to use the dye to monitor the biofilm architecture at two

different time points days 10 and 13. As 5(6) – CFDA, SE is a green fluorescent dye, donor strain could not be detected in stained biofilms.

The reason why we could not detect RFP fluorescing cells might be the formation of thick biofilms in the flow cell channels. According to Ma and Bryers (2012) thick and compact biofilm and biofilm clusters may impede penetration of donor cells from outer to inner regions. Thus, plasmid transfer creates a thin layer of transconjugants on the outer surfaces of the biofilm microcolonies, resulting in lower efficiencies of plasmid transfer. But from our study we could not detect transconjugants on the outer surface of the thick biofilm. Biofilms that are less dense, more porous enable more donor cell access and deeper penetration of donor cells throughout the cluster leading to a faster spread of genes by conjugative plasmid transfer (Ma and Bryers 2012).

The architecture of the biofilms was evaluated by COMSTAT, an image analysis tool which can quantify parameters such as average thickness, maximum thickness, biomass, roughness coefficient or surface to volume ratio. The roughness coefficient parameter reflects biofilm heterogeneity and measures the differences of local biofilm thickness relative to the average thickness. Surface area to volume ratio indicates the fraction of the biofilm exposed to the nutrient flow. Surface to volume ratio is the surface area divided by the biovolume. Thickness is defined as the maximum thickness over a given location ignoring pores and voids inside the biofilm, (Heydorn et al. 2000). Biomass is the amount of biological materials in a given area or sample. This is an expression of how much of the image stack is covered by bacteria. Average thickness tells us about the general shape of the biofilm.

The COMSTAT analysis results (Table 2.3) show that the average thickness of control channels was lower than that of donor inoculated channels. It is possible that the added donor cells

contributed to a greater average thickness in the test (donor-amended) channels. The highest mean maximum thickness was obtained for channel 2 (95.71µm) which did not receive the donor strain. The mean biomass was highest for channel 4 which received the donor and the value was 6.09 (µm^3/µm^2). Ma and Bryers (2012) found that microcolonies obtained average thicknesses of 90–100 µm when they used FAB medium which is a chemically defined medium supplemented with 200 mg/L of glucose after 8 days of cultivation, whereas biofilms reached only 50–60 µm at 20mg/L glucose. For our experiment 0.9 g/L glucose (5 mM) was used in MMN medium. Thick biofilms were observed in flow cell channels with transmission light, from COMSTAT analysis the range of maximum thickness of biofilm was 48.57- 95.71 (µm) but the average thickness of biofilms was 1.14 - 17.12 µm and range for biomass was only 0.51- 6.09 (µm^3/µm^2). The 5(6) – CFDA, SE dye fluoresces in metabolically active cells and therefore there is a possibility that the entire biofilm may not be visualized with this particular dye. Alternatively, since the biofilm was stained with 5(6) – CFDA, SE only for fifteen minutes, this time may not have been sufficient for penetration of the dye throughout the entire biofilm in the flow cell channels. Bester et al. (2010) tested the staining efficiency of 5(6) – CFDA, SE. They injected 5(6) – CFDA, SE into flow chambers and stained for 0.5 hr. From their study they obtained average biomass 70 ± 14% and maximum thickness 98 ± 4% for *Pseudomonas* sp. strain CT07 .biofilms. They used a minimal growth medium with or without 1 mM/L citrate as carbon source.

For our experiment with MMN + glucose average thickness and maximum thickness values were highly variable, indicating that the biofilms possessed highly heterogeneous biofilm architecture. Ma and Bryers (2012) found that mature biofilms cultivated under a hydrodynamic environment developed heterogeneous architectures comprising numerous microcolonies or clusters secured

by cell–cell binding interactions and the surrounding extracellular polymeric matrix .They also found that roughness and surface to volume ratio increased significantly with decreasing glucose concentrations. They determined values for roughness coefficient of 1.78±0.09 and 0.74±0.06 at 20 mg/mL and 200 mg/mL glucose respectively and for surface to volume ratio of 7.37±0.73 **(**µm^2/µm^3**)** and 2.57±0.38 **(**µm^2/µm^3**)** at 20 mg/mL and 200 mg/mL glucose respectively. For our experiment the range of mean roughness coefficient was 1.24 – 1.93 and the range for mean surface to volume ratio was 0.17- 0.85 **(**µm^2/µm^3). The increased value for biofilm roughness coefficient and surface to volume ratio indicates heterogeneous biofilm architecture (Bester et al. 2010).

Ma and Bryers (2012) also found that low nutrient concentrations resulted in heterogeneous and porous biofilm structures. Thick, more uniform and compact biofilms were developed under increasing nutrient concentrations.

For selective pressure experiment (MMN + 2,4-D) COMSTAT analysis shows that the highest mean average thickness was measured for channel 2 (31µm) and lowest mean average thickness was measured for channel 4 (12.7 µm). Both channels received the donor strain. Similarly, the highest biomass was detected in channel 2 (21.82 µm^3/µm^2); for channel 4 the value was 11.73 µm^3/µm^2). However, the total cell count on R_2A plate on day 7 was 1.3×10^8 CFU/mL (Table 2.4) for donor inoculated channels which was less than the control channels. The range for mean roughness coefficient was 0.56 -1.12 and the range for mean surface to volume ratio was 0.07-0.11(µm^2/µm^3). The standard deviation values for average thickness, maximum thickness, biomass was high, indicating greatly heterogeneous biofilm architecture. The values for mean average thickness, mean maximum thickness, and mean biomass were higher when 2,4-D was used instead of glucose as carbon source. The 5(6) – CFDA, SE dye fluoresces in

metabolically active cells and there is a possibility that the entire biofilm may not be visualized with this particular dye, for this reason for 2,4-D experiment Syto 62 (which is a nucleic acid binding dye) was used instead of 5(6) – CFDA, SE. However, since two different stains (5 (6) – CFDA, SE and Syto 62) with two different modes of staining were used, the experiments will need to be repeated using standardized staining techniques.

The biofilms treated with MMN + 2,4-D in flow cell channels were investigated to detect RFP and GFP for transconjugant and donor cells but none could be detected microscopically. However, it was observed that the presence of 2,4-D within biofilms gave rise to autofluorescence upon excitation with the 488 nm laser in the donor non-inoculated channel. Appendix H shows the autofluorescence from accumulated 2,4-D in the biofilm EPS. According to Wolfaardt et al. (2004), many of the common organic contaminants fluorescence when excited with light of the appropriate wavelength, due to the presence of aromatic rings in their chemical structures and this property can be exploited to locate the accumulation of aromatic contaminants in biofilms Wolfaardt et al. (2005) used a continuous-flow cell systems to cultivate a degradative biofilm community with the herbicide diclofop methyl as the sole carbon and energy source. On the basis of confocal laser scanning microscopy (CLSM) and mass-spectral analyses they suggested that the accumulation of the herbicide diclofop methyl, a chlorinated two-ring compound, and its aromatic breakdown products occurred in cell capsules and the EPS matrix of a degradative biofilm community.

Bathe et al. (2004b) observed clusters of red-fluorescent cells in the bioaugmented reactor sample using epifluorescence microscopy when they used synthetic waste water with 2,4-D as a sole carbon source as feed. Aspray et al. (2005) used donor strain *P. putida* SM1443 (pJP4::*gfp*), with a chromosomal *lacI*^{q} gene tag which repressed *gfp* expression on the plasmid and, therefore,

GFP fluorescence. Following horizontal gene transfer of the plasmid to recipient populations, the absence of chromosomal *lacI*^{q} gene tag resulted in *gfp* expression in transconjugants. They observed approximately 5–10 transconjugant micro colonies, 20–40 μm in diameter, in each chamber of flow cell when they used FAB medium supplemented with 2,4-D as sole carbon source at the flow rate of 3mL/hr.

For both MMN + glucose and MMN + 2,4-D experiments highly heterogeneous biofilm architecture was obtained as the standard deviations of the parameters were high. Thick biofilms were obtained from both experiments. For MMN + glucose experiment the maximum thickness was 48.57-95.71 μm on day 10 and for MMN + 2,4-D experiment maximum thickness was 42-62 μm on day 7. RFP expressing transconjugant cells could not be observed microscopically in biofilms for both experiments.

Spectrophotometric measurement of 2,4-D concentration:

Spectrophotometric measurement was done to describe degradation of 2,4-D in the effluents from control and donor inoculated channels on different sampling days. 2,4-D concentration was determined by spectrophotometrically measuring absorbance at 230 nm (Bathe et al. 2004b). 2,4-D concentration in effluents from both control and donor inoculated channels very slightly decreased in comparison to the initial concentration in MMN after day 1 of donor inoculation. After days 4, 5 and 7 the measured concentration of 2,4-D was higher in effluents from both control and donor inoculated channels in comparison to the concentration in the MMN medium reservoir. The range of UV absorbance readings (Table 2.7) for the effluents from MMN + 2,4-D experiment was 0.7718-1.0417 on days 1 and 7. As no significant UV absorbance for the effluents from MMN + glucose experiment was obtained (0.0085-0.1429) the increased in 2,4-D

concentration in the effluents was not due to the metabolites from microbial metabolism. One possible reason for increased 2,4-D concentration might be the presence of other intermediates originating from 2,4-D degradation also absorbing light at 230 nm. 2,4-dichlorophenol which is the first intermediate in 2,4-D degradation pathway absorbs UV-light at 222-282 nm wavelength at pH 5-11 (Matafonova et al. 2011). The maximum UV-light absorption of 3,5-dichorocatechol is at 267 nm (Kuhm et al. 1990). 3,5-dichorocatechol is the second intermediated in 2,4-D degradation pathway. These intermediates may also have some UV-light absorbance at 230 nm.

In a batch culture experiment, (Appendix G) slightly enhanced degradation of 2,4-D was observed in soil-derived mixed cultures amended with the donor strain than the control.

Bathe et al. (2004b) showed 90% degradation of 2,4-D in the bioaugmented reactor which received the donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* within 40 h, a control reactor which had not received the plasmid contained 60% of the initial 2,4-D concentration after 90 h.

Quan et al. (2010) used the same donor strain for bioaugmentation experiment. They found 2,4-D at the initial concentration of about 160 mg/L was completely removed in the bioaugmented system within 62 h, while the control system that did not receive donor strain only removed 26% within 66 h.

For our future experiment we can use lower concentration of 2,4-D as selective pressure to describe biofilm architecture and detect plasmid transfer into the biofilm.

2.4.3 DGGE analysis for donor, inoculum, plate mating transconjugants, effluents from the last day of the experiments:

DGGE profiles show that the donor (Figure 2.15, lane 1) was not present in the effluents on the last day of both experiments. This support the PCR based detection for *gfp* as we could not detect a band for the amplified *gfp* product in the effluents on the last day (day 14) of the experiment when MMN + glucose was used as feed. This also supports the PCR based results and plate count results when MMN + 2,4-D was used as feed as we could detect neither a band for the amplified *gfp* product nor GFP fluorescing colonies cultured from effluents on day 7. DGGE profiles show that bands corresponding to transconjugants isolated from plate matings (lanes 3-6) were not present in the inoculums DGGE profile. The possible reason might be extraction of DNA from the inoculum led to a low concentration of the pJP4 recipient DNA and therefore no band was visible on the DGGE. DGGE profiles for last day effluent samples for MMN + glucose (lanes 7, 8) and MMN + 2,4-D (lanes 9, 10) are different, indicating that different microorganisms were present on the last days of the MMN + glucose and MMN + 2,4-D experiments. A common band was seen for plate mating transconjugants and effluent samples from the last day of MMN + glucose experiment, indicating that some potential pJP4 recipients were present. Bands corresponding to plate mating transconjugants were not present in the last day effluent samples from MMN + 2,4-D experiment which indicates that they were not present in the last day (day 7) of the MMN + 2,4-D experiment. The reason might be extraction of DNA from the effluents led to low concentration of the pJP4 recipient DNA and therefore no band was visible on the DGGE. Some sequencing results from DGGE (Appendix F) show that amplified 16S rRNA sequences from transconjugants obtained from plate mating (Figure 2.15, lanes 4,5) and from bands present in effluent samples obtained on day 7 (last day) of the MMN + 2,4-D

experiment (lane 9) show a high similarity to the classes of β-*Proteobacteria* and γ-*Proteobacteria*.

2.5 Conclusions:

The pJP4 plasmid was transferred to soil-derived mixed culture recipients in plate mating experiments and isolated transconjugant colonies were characterized as *Comamonas testosteroni*. A soil-derived mixed microbial culture which was used as a recipient community for plasmid pJP4 formed thick (42 µm – 95 µm) biofilms when glucose or 2,4-D was used as carbon source, representing the absence and presence of selective pressure, respectively. Green fluorescent protein (*gfp*) gene, red fluorescent protein (*dsRed*) gene and the *tfdB* gene were detected in biofilm effluents both with and without selective pressure. Detection of *dsRed* gene in the effluents indicated the presence of plasmid; however, it could not be incurred if the plasmid was associated with donor cells only or also with transconjugant cells. Donor and transconjugant cells could not be detected microscopically in biofilms and transconjugant colonies expressing DsRed could not be isolated with or without selective pressure. In batch culture experiment, slightly enhanced degradation of 2,4-D was observed in soil-derived mixed cultures amended with the donor strain but from the flow cell experiment degradation of 2,4-D was not detected. Heterogeneous biofilm architecture was obtained for both with and without selective pressure. Biofilms exposed to 2,4-D had higher average thickness, maximum thickness, biomass than the biofilms exposed to glucose. Follow up experiments should be done with a lower concentration of glucose and 2,4-D to reduce biofilm thickness in flow cell channels.

CHAPTER 3: CONCLUSIONS AND FUTURE RECOMMENDATIONS

The pJP4 plasmid was transferred to soil-derived mixed culture recipients in plate mating experiments and isolated transconjugant colonies were characterized as *Comamonas testosteroni*. Green fluorescent protein (*gfp*) gene, red fluorescent protein (*dsRed*) gene and *tfdB* gene were detected in biofilm effluents both with and without selective pressure. Slightly enhanced degradation of 2,4-D was observed in soil-derived mixed cultures amended with the donor strain. Heterogeneous biofilm architecture was obtained for both with and without selective pressure. Biofilms exposed to 2,4-D had higher average and maximum thickness as well as total biomass than the biofilms receiving glucose.

Although we couldn't detect transfer of plasmid pJP4 into biofilm community microscopically, flow chambers in conjunction with fluorescent protein tags can be used to observe plasmid transfer at single cell level. The continuation of this specific project may include:

- Repetition of the experiments with a lower concentration of glucose and 2,4-D to reduce biofilm thickness in flow cell channels. The biofilms which were formed in this study exceeded maximum thickness 95.71 µm and as a result detection of donor and transconjugant cells may have been impeded.

- To detect the pJP4 transconjugants with MMN + 2,4-D as feed cell samples from effluent can be plated on MMN medium containing gentamycin and 2,4-D as the sole carbon source. Both gentamycin and 2,4-D will create selective pressure to maintain the plasmid in the transconjugant cells. Bathe et al. (2004b) used medium plate with 2,4-D as sole carbon source to isolate transconjugants.

- The complexity of the experiments can be reduced by using a single strain (e.g. *Pseudomonas putida* ATCC12633) as the recipient for plasmid pJP4. In initial plate mating experiments we confirmed that *P. putida* ATCC12633 as a pJP4 recipient. To use a single strain as a recipient simplifies experimental design and allows better control of the experimental system, to provide information about the fundamentals (or " a base line") for gene transfer.

- Concentration of 2,4-D can be measured by high performance liquid chromatography (HPLC). HPLC column will separate 2,4-D and other intermediates in the degradative pathway. Identification and measurement of 2,4-D can be done more accurately by HPLC method. Flanagan et al. (1989) used HPLC method to measure chlorophenoxy herbicide including 2,4-D.

- For flow cell experiment with MMN + 2,4-D a decreased flow rate can be used to monitor significant degradation of 2,4-D. High flow rate (3 mL/hr) probably prevented breakdown of 2,4-D.

- Quantitative PCR can be used to quantify the amplified gene product. Since the *gfp* gene is encoded on the donor chromosome and the *dsRed* gene resides on the plasmid, quantitative PCR results can indicate if the abundance of donor cells decreases (amplified decrease in a PCR product for *gfp*) and the abundance of transconjugant cells increases (if the amount of amplified product for *dsRed* increases with a concurrent decrease in the amount of *gfp*). A donor strain that contains a *gfp*-tagged plasmid and a DsRed tag in the chromosome can be used *f*or this experiment. GFP is relatively persistent in the cells as it is resistant to denaturants and proteases (Ward et al. 1980). DsRed has an extended maturation time and

the lifetime of DsRed decreases with bacterial cell age due to precipitation of the protein within the cytoplasm (Jacobs et al. 2000).

APPENDICES

Appendix A: Composition of MMN medium:

MMN – phosphate buffer 100× (Boon et al. 2001)	
Na_2HPO_4	141.96 g/L
KH_2PO_4	136.09 g/L

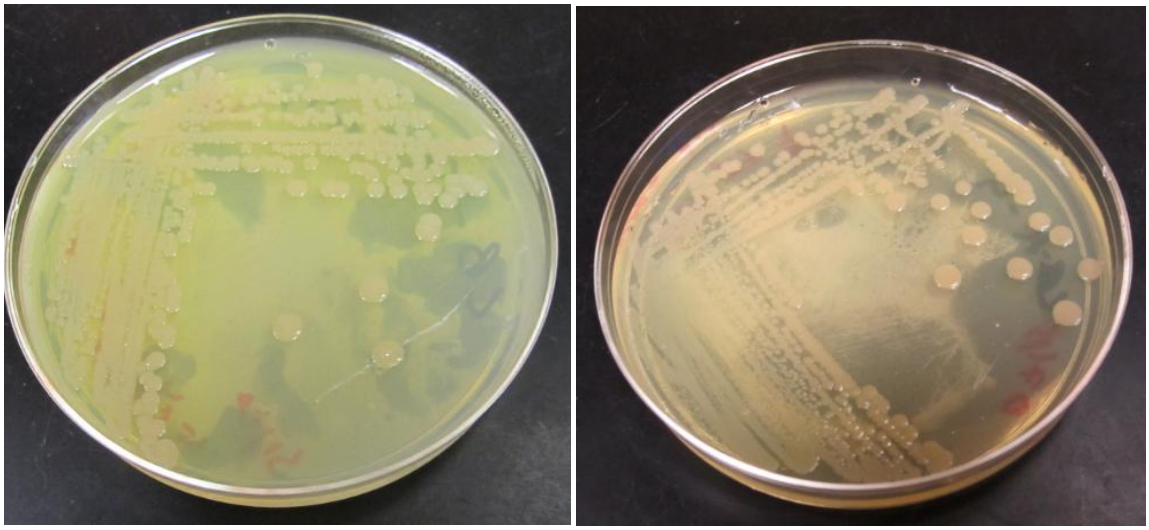
MMN salt solution 200× (Boon et al. 2001)	
$MgSO_4$	19.70 g/L
$CaCl_2.2H_2O$	1.18 g/L
H_3BO_3	0.23 g/L
$FeSO_4.7H_2O$	0.56 g/L
$ZnSO_4.7H_2O$	0.23 g/L
$MnSO_4.7H_2O$	0.34 g/L
$CuSO_4.5H_2O$	0.08 g/L
$CoCl_2.6H_2O$	0.05 g/L
$Na_2MoO_4.2H_2O$	0.02 g/L
Na_2EDTA	0.64 g/L

1× phosphate buffer was made with dH_2O and was autoclaved at 121°C. 200× MMN salt solution was filter sterilized with 0.45 µm sterile filter. 1 mM concentration of NH_4Cl was used as nitrogen source. 5 mM final concentration of glucose or 2,4-D was used as carbon source.

Appendix B: Confirmation of plasmid presence in the donor:

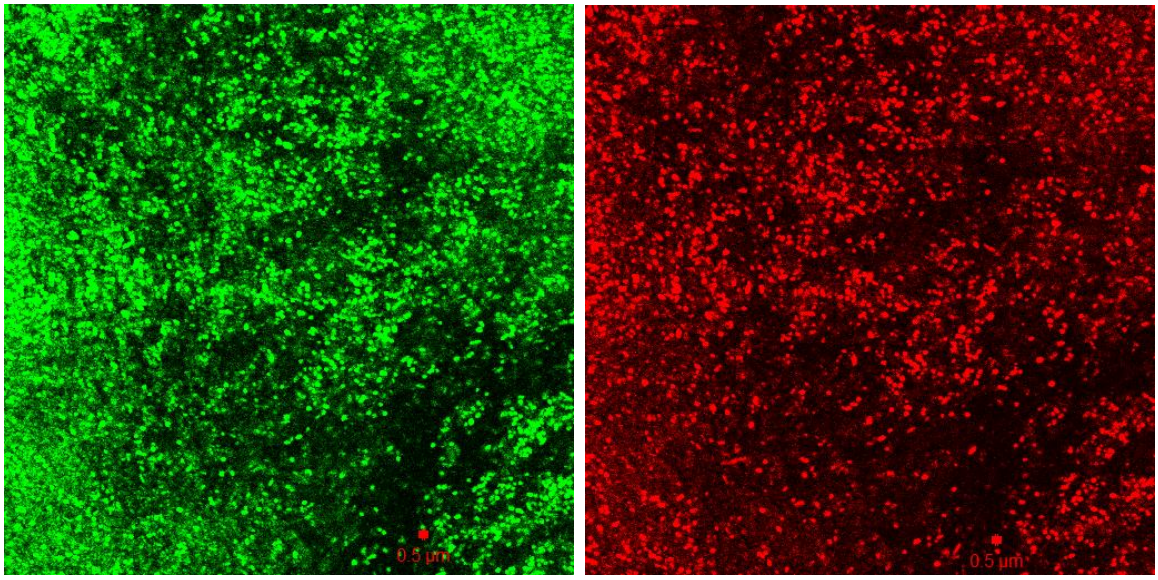
Repress*i*on of *dsRed* expression in the donor cell is achieved through a lac-operon related mechanism (Bathe et al. 2004b). The presence of Isopropyl β-D-1 thiogalactopyranoside (IPTG) interferes with this mechanism and allows for the expression of the *dsRed* gene in donor cells.

Colonies of the donor strain *P. putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed* are normally beige in colour when cultured on LB+ kanamycin + gentamycin plates (Figure 4.1a). When cultured on LB + kanamycin + gentamycin with topically applied IPTG, donor colonies appear light pink (Figure 4.1b) due to the formation of red fluorescent protein within cells. This is because IPTG induces red fluorescence. Figure 4.2 shows the CLSM image of donor cells on LB + kanamycin + gentamycin with topically applied IPTG. Green fluorescence was detected in image 4.2a and red fluorescence was detected in image 4.2b. Presence of *tfdB* gene was detected in donor strain with a PCR based technique (Figure 4.3)



a b

Figure 4.1: Confirmation of RFP formation on IPTG plates as a result of the presence of a *dsRed*-tagged plasmid pJP4 in the donor strain *Pseudomonas putida* SM1443::*gfp*2x carrying the plasmid pJP4::*dsRed*. Streaked on (a) LB+ kanamycin + gentamycin plate (b) LB + kanamycin +gentamycin topically applied IPTG



a b

Figure 4.2: CLSM image of donor cells from (a) LB + kanamycin + gentamycin + IPTG (Isopropyl β-D-1 thiogalactopyranoside) plate (488 nm wavelength excitation), 63× magnification (b) LB + kanamycin+ gentamycin + IPTG (Isopropyl β-D-1 thiogalactopyranoside) plate (543 nm wavelength excitation), 63× magnification

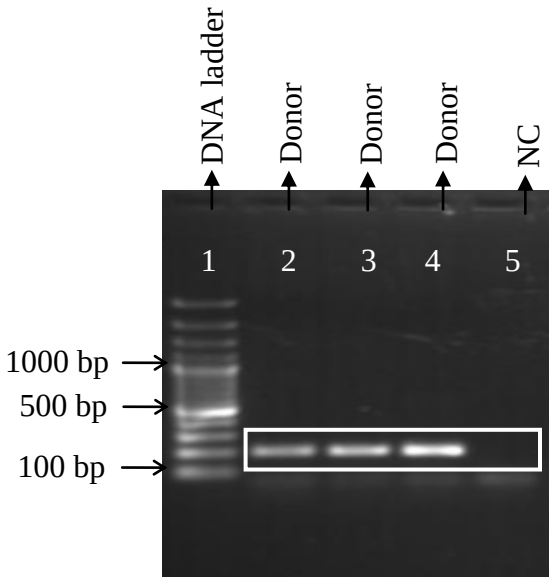


Figure 4.3: Agarose gel showing the amplified product of *tfdB* gene (205 bp) (white box) in donor strain. Lane 2: donor DNA, lane 3, 4: donor plasmid, lane 5: NC, negative control

Appendix C: Confirmation of *gfp* in donor:

The donor strain contains chromosomally encoded *gfp* gene. This was confirmed by PCR directed at the *gfp* gene (Figure. 4.4), which yielded the expected product of 341 base pairs (lane 2).

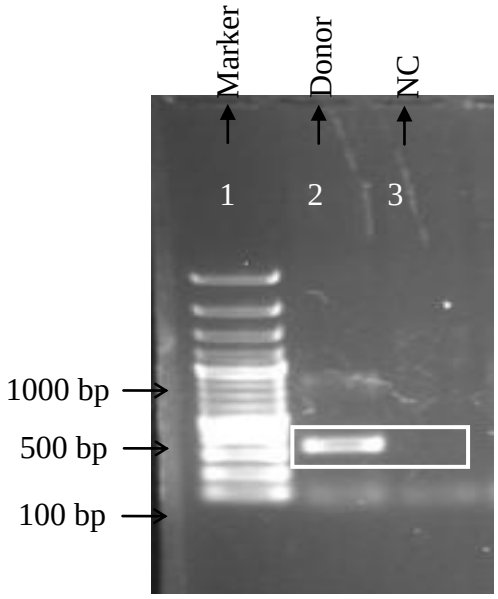


Figure 4.4: Agarose-gel showing PCR-products of the *gfp* gene (341 bp) (white box) in the chromosome of the donor cells. Lane 1: Marker, lane 2: donor strain, lane 3: NC, negative control

Appendix D: Confirmation of the donor strain capacity to form a biofilm in flowcell channels:

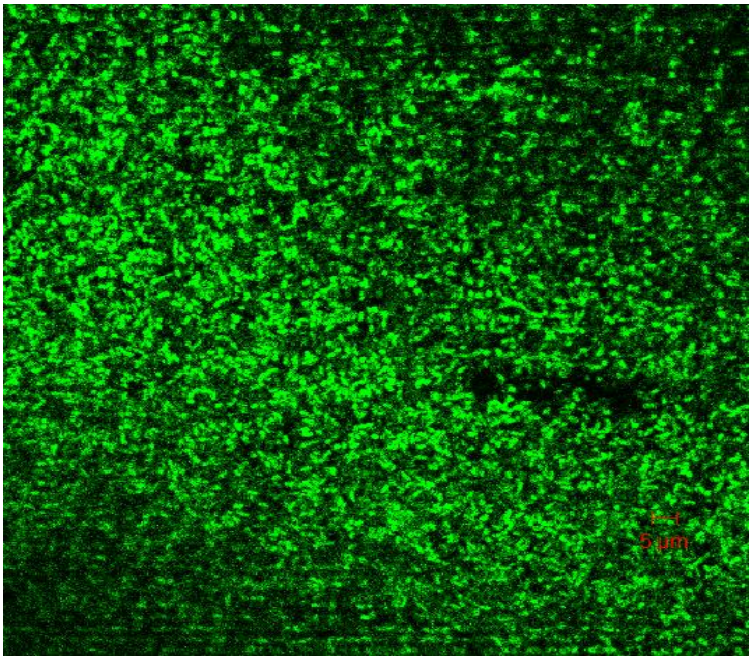


Figure 4.5: CLSM image of 5 days old donor biofilm. Confocal microscopy shows that donor strain forms dense biofilms in flow cells and remains fluorescent over a period of 5 days. GFP fluorescence was detected using a 488 laser.

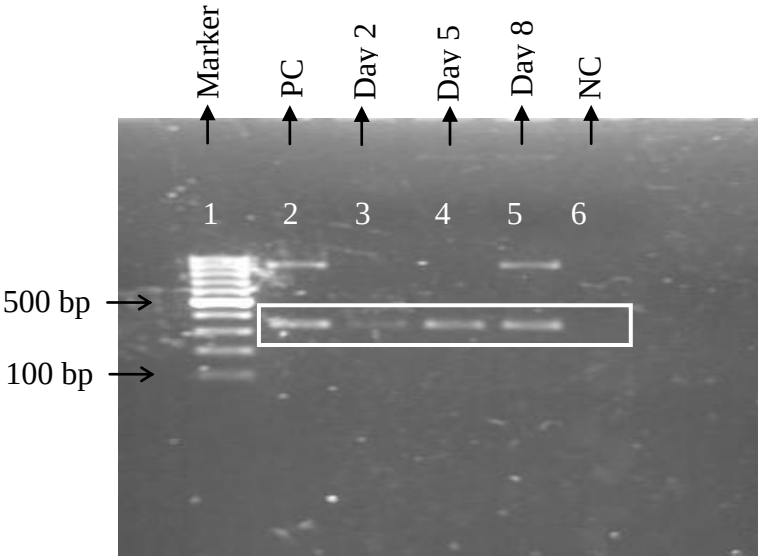


Figure 4.6: PCR-based confirmation of the persistence of the donor cells in the effluents when MMN + glucose was used as feed. It shows that donor cells persist in biofilm effluents over the experimental period (day 8)

Appendix E: Experiment with an alternative soil (peat) recipient community + donor, MMN + glucose medium was used as feed:

PCR based detection of *gfp* and *tfdB* genes in effluent samples:

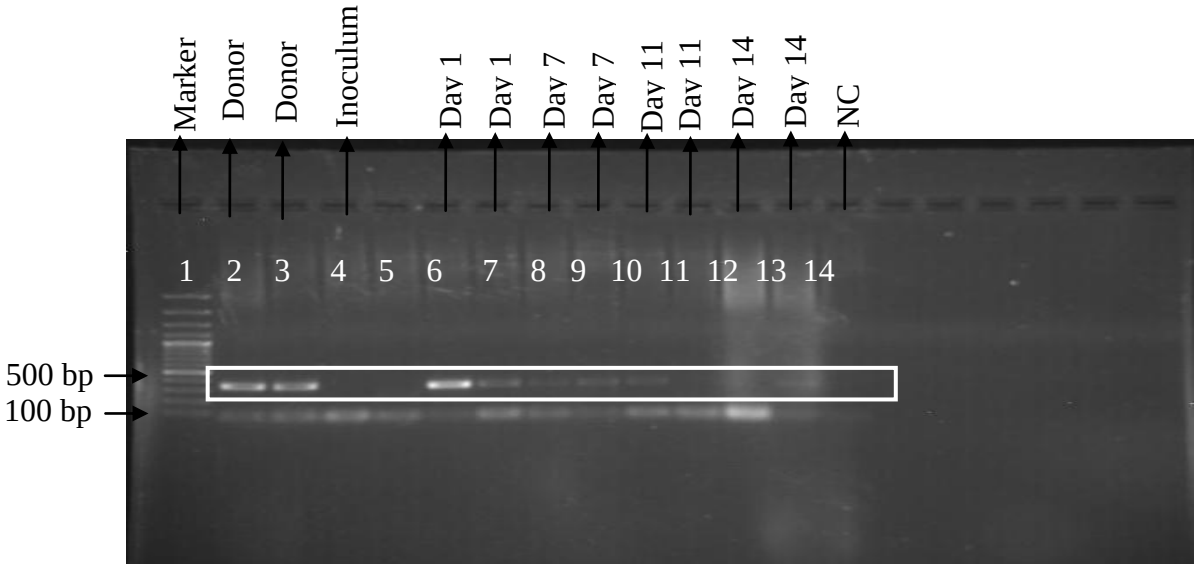


Figure 4.7: Agarose-gel showing PCR-products of the *gfp* gene (341 bp) (white box) in the chromosome of the donor cells. Lane 1: 100 bp marker, lane 2, 3: donor strain as positive control, lane 4: Inoculum, lane 6-13: effluent samples collected on different sampling days from donor inoculated channels. Channel 2 and 4 received the donor, channel 1 and 3 were control, lane 14: NC, negative control (no template DNA). This image shows that donor was detected in biofilm effluents until day 14 after inoculation.

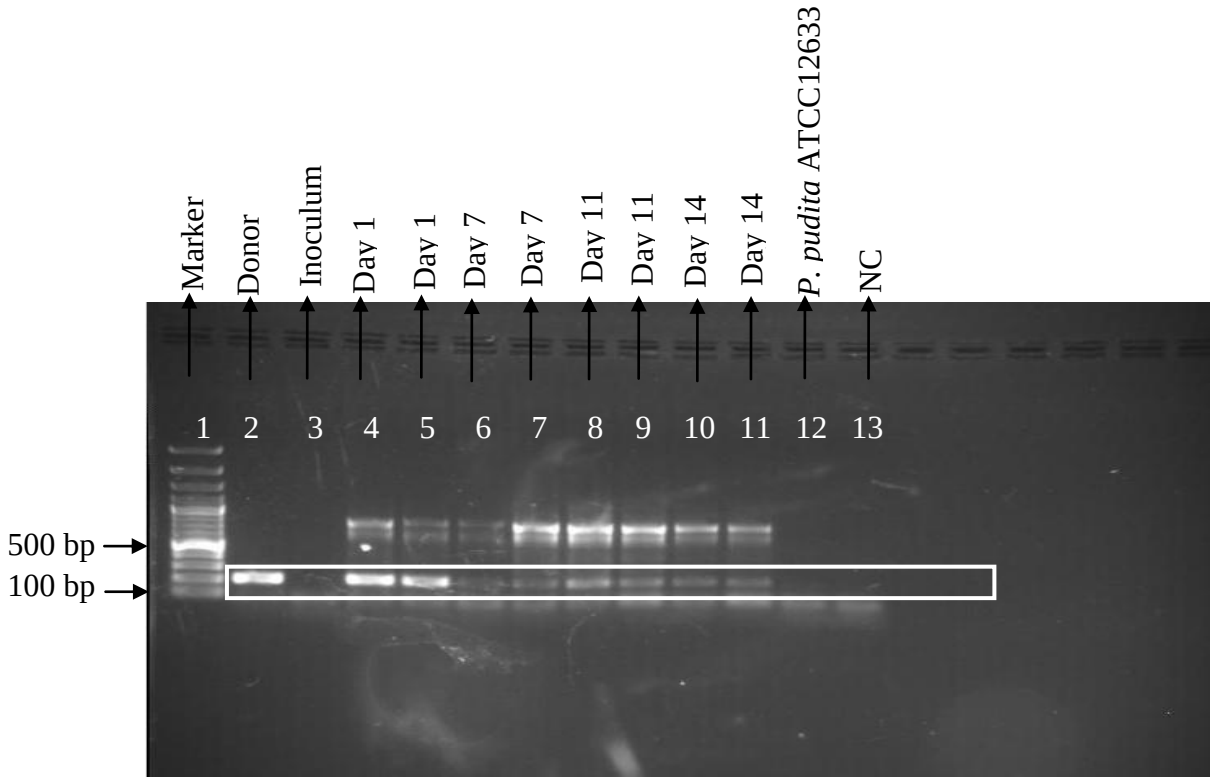


Figure 4.8: Agarose-gel showing PCR-products of the *tfdB* gene (205 bp) (white box) residing in the pJP4 plasmid. Lane 1: 100 bp marker, lane 2: donor strain as positive control, lane 3: Inoculum, lane4-11: effluent samples collected on different sampling days from donor inoculated channels. Channel 2 and 4 received the donor strain, channel 1 and 3 were control, Lane 12, 13: NC, negative controls. This image shows that *tfdB* gene was detected in biofilm effluents until day 14.

Confocal microscopy images of biofilm exposed to MMN + glucose:

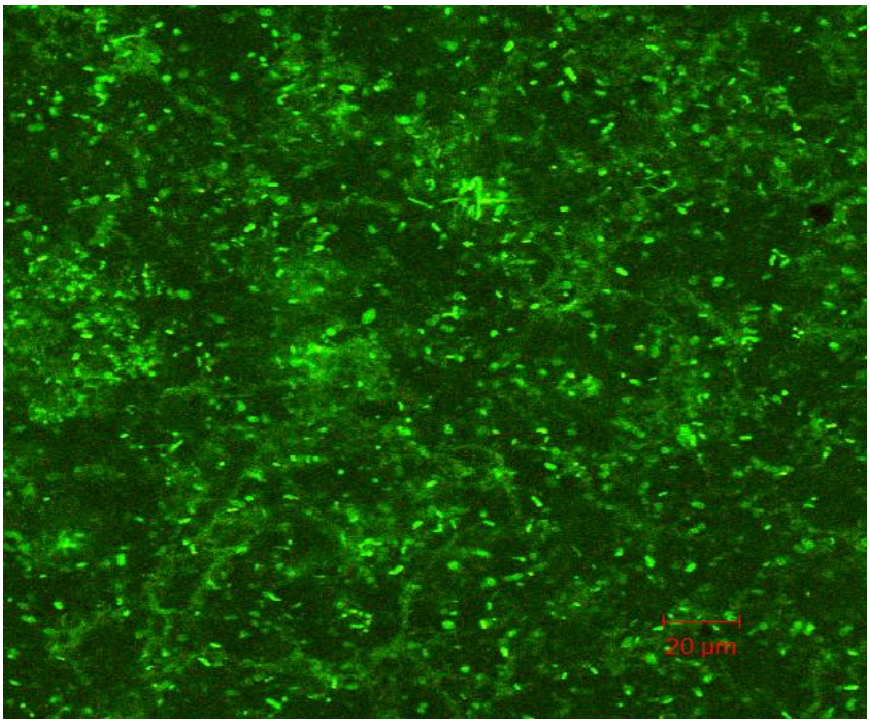


Figure 4.9: A z-stack representing CLSM image of soil (peat) biofilm formed with added donor after 7 days of donor inoculation. MMN + glucose was used as feed. Biofilm was stained with 5 (6) – CFDA, SE. (20× magnification). 488 and 543 nm wavelength lasers were used. Cluster of organisms forming the biofilm can be seen. Donor and transconjugant cells were not detected in the biofilms.

Appendix F: Sequencing results:

Table 4.1 Closest matches phylum and class from DGGE bands sequencing data (Figure 2.15).

Name	**Phylum & Class**
2d	*Proteobacteria*, γ-*Proteobacteria*
4a	*Proteobacteria*, β-*Proteobacteria*
4b	*Proteobacteria*, β-*Proteobacteria*
5a	*Proteobacteria*, β-*Proteobacteria*
5b	*Proteobacteria*, β-*Proteobacteria*
9a	*Proteobacteria*, γ-*Proteobacteria*
9b	*Proteobacteria*, γ-*Proteobacteria*

Appendix G: Batch culture experiment:

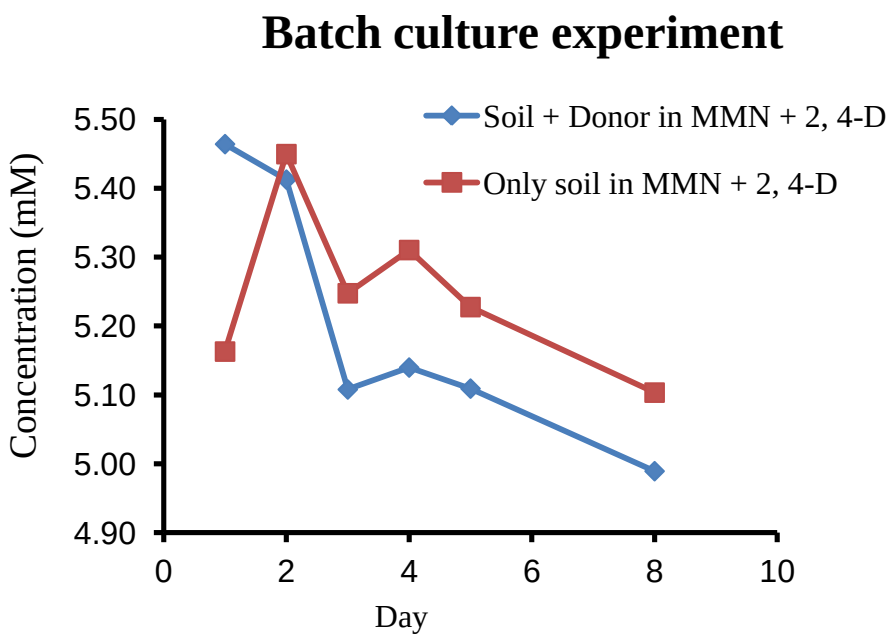
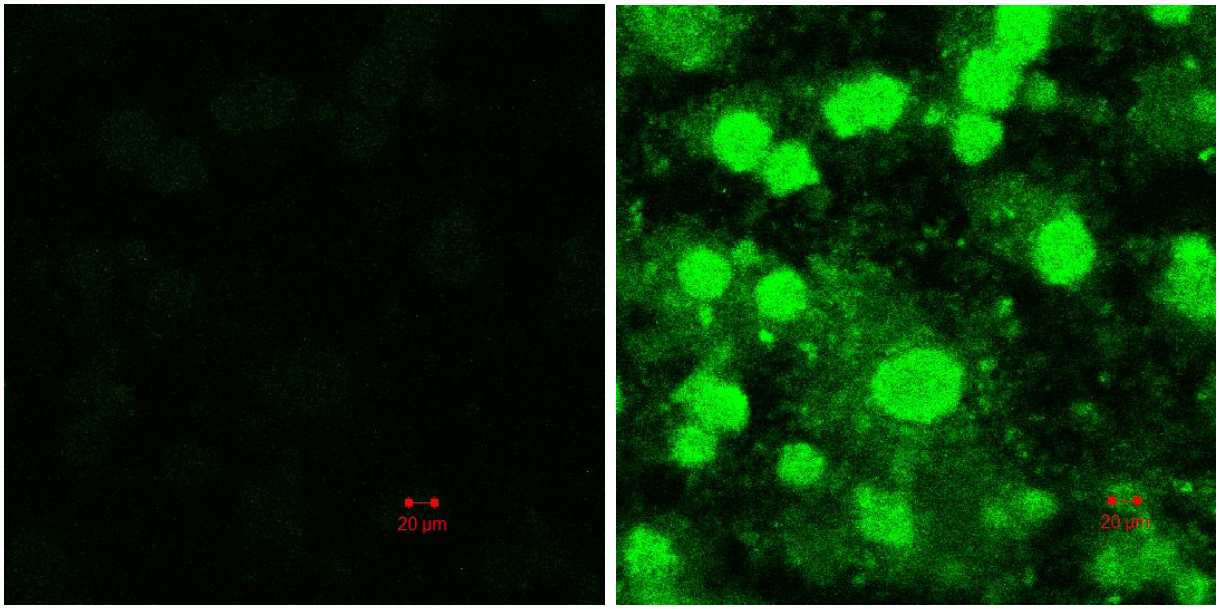


Figure 4.10: 2, 4-D degradation in batch culture experiments during days 1- 8. Blue line represents degradation by soil-derived mixed cultures amended with donor strain, red line represents degradation by only soil-derived mixed cultures (control). Slightly enhanced degradation of 2,4-D was observed in soil-derived mixed cultures amended with the donor strain.

Appendix H: Biofilm autofluorescence following 2,4-D treatment:



a **b**

Figure 4.11: Biofilm autofluorescence following 2,4-D treatment. Image a and b were obtained for soil biofilm treated with 2,4-D , without added donor strain. Two pinhole settings were used, image a was taken with pinhole size 1 and image b was taken with pinhole size 4 which indicates increased pinhole gave more green signal. For next experimental setup control channels will be needed to compare biofilms treated with 2,4-D and not treated with 2,4-D at the same time.

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