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EVALUATION OF THE TRANSFER OF THE TOL PLASMID FROM *Pseudomonas putida* TO GROUNDWATER-DERIVED BIOFILMS IN A MODEL ROCK-FRACTURE AQUIFER

by

Matthew Starek, B.Sc McGill 2006

A Thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the Program of

Molecular Science

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Abstract

Evaluation of the Transfer of the TOL plasmid from *Pseudomonas putida* to Groundwater-Derived Biofilms in a Model Rock-Fracture Aquifer

Matthew Starek

Master of Science

Molecular Science

Ryerson, 2009

Non-aqueous phase liquids (NAPLs) can become entrapped in subsurface rock fractures and become a long-term groundwater contaminant source. While remediation technologies exist, they can be expensive. Subsurface microorganisms can also degrade NAPLs trapped in the subsurface; however, this is a slow process. The possibility of enhancing microbial degradation of NAPLs via a plasmid transfer mechanism in a model rock fracture aquifer was explored. There was no indication that introduction of donor strain *Pseudomonas putida* SM1443::gfp2x-pWW0::dsRed into the model system led to transfer of the degradative TOL plasmid pWWO, or led to increased degradation of model NAPL toluene. Plate matings with the donor strain and a groundwater-derived microbial consortium indicated that few potential recipients existed in the community. Nutrient concentration was ruled out as a limiting factor of plasmid transfer.

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List of Abbreviations

BA – Benzyl alcohol

BTEX – Benzene, Toluene, Ethylbenzene, Xylene

CLSM – Confocal Laser Scanning Microscope

DGGE – Denaturing Gradient Gel Electrophoresis

EDTA - Ethylenediaminetetraacetic acid

GC/FID – Gas Chromatography/ Flame Ionization Detector

GFP – Green Fluorescent Protein

gfp – gene encoding green fluorescent protein

HGT **–** Horizontal Gene Transfer

IPTG - Isopropyl-ß-D-thiogalactopyranosid

LB – Luria Bertani

NAPL – Non-Aqueous Phase Liquid

RFP – Red Fluorescent Protein

rfp – gene encoding red fluorescent protein

SBR – Sequencing Batch Reactor

SBBR – Sequencing Batch Biofilm Reactor

TAE – Tris base, EDTA, Acetic Acid

TE – Tris base, EDTA

Tol – Toluene

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

USEPA – United States Environmental Protection Agency

Project Significance and Motivation

Anthropogenic activity has led to the introduction and persistence of non-aqueous phase liquids (NAPL) into subsurface environments (Abriola and Bradford, 1998; Collins et al, 2002; USEPA, 2004). In addition to anthropogenic remediation technologies and techniques, the breakdown of NAPLs can be accomplished by subsurface microorganisms (Environment Canada b, 2002). In response to the introduction of contaminants in the subsurface, resident microbial populations can, i) undergo natural selection for degraders of the particular compound, ii) incur genetic mutations over time such that a population of degraders is produced or iii) transfer the ability to degrade the particular compound amongst the population by horizontal gene transfer (Top et al, 2002; Top et al, 2003).

Horizontal gene transfer (HGT) has been shown to be a successful mechanism to spread a plasmid harbouring genes encoding degradative enzymes in model wastewater and model soil systems, where a lab-designed donor strain was introduced into the model system (Normander et al, 1998, Molbeck et al, 2003, Bathe et al, 2004a; Bathe et al, 2004; Bathe and Hausner, 2005; Taghavi et al, 2005; Nancharaiah et al, 2007, Pei and Gunsch, 2009; Venkata Mohan et al, 2009). There is not; however, a significant amount of information with respect to transfer of degradative plasmids between bacteria in model groundwater systems; more specifically in rock-fracture apertures which are common in Canada. The central question addressed in this study was: can the bioremediation strategy of plasmid transfer be applied in a model of NAPL-contaminated groundwater in a rock-fracture aperture? As a model NAPL we used the BTEX (benzene, toluene, ethylbenzene and xylene) compound toluene, as it is a common component in petroleum

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products and relevant to contaminated aquifers in Canada (Environment Canada, 2009).

Furthermore, we had a donor strain which contained the TOL plasmid, which encodes a

family of enzymes that are capable of degrading toluene (Greated et al, 2002).

Hypothesis

It was hypothesized that a donor strain harbouring the TOL plasmid could transfer that plasmid to members of a groundwater-derived microbial consortium residing in a model NAPL-contaminated rock-fracture aquifer, resulting in enhancement of toluene degradation.

In order to test this hypothesis, the project had the following objectives:

Objectives

1) Assemble a model rock-fracture aquifer, which is amenable to confocal microscopy

2) Characterize the biofilms in our model system in terms of architecture and diversity

3) Detect recipients of the TOL plasmid in out model rock-fracture aquifer

4) Determine what effect the addition of our donor strain had on the degradation of toluene and benzyl alcohol in our model rock-fracture aquifer

1. Introduction

1.1 Biofilms

Biofilms can be described as assemblages of microbes that "represent an interdependent community-based existence" as opposed to microbes which exist in a unicellular, free-floating, planktonic phase (Davey and O'Toole, 2000). In many cases the formation of a biofilm takes place on a surface. Residence in a biofilm, as opposed to existing in a planktonic state, can be advantageous to a microbe. Biofilms can provide protection from physical stress; individual cells may have enhanced access to nutrients and metabolic cooperation; and genetic traits can transfer more easily among microbes in a biofilm (Davey and O'Toole, 2000). The close apposition of cells within the biofilm matrix, as opposed to planktonic cells, provides an opportunity for the horizontal transfer of genetic material as cell-cell contact is required for this process (Ghigo, 2001; Thomas and Nielsen, 2005).

1.2 Groundwater

Water flowing in the subsurface through porous media or rock fractures in the water saturated zone is termed groundwater (Fig. 1). Groundwater flows directionally ,due to gravity, from recharge zones to discharge zones (Fig. 1)   Groundwater environments tend to be heterogeneous; though they are generally defined by low dissolved organic carbon, low dissolved oxygen and slow flow rates (Malard and Hervant, 1999;Coombs, 2009;). As a result, microorganisms often display reduced cell size and slow growth rates (Amy et al., 1998). It has been estimated that 90% of

subsurface microorganism exist in biofilms rather than as planktonic cells (Cozzarelli and Weiss, 2007).

Dissolved oxygen tends to be at its highest level nearest the recharge zones, and is particularly high when the water table is shallow (Malard and Hervant, 1999). Malard and Hervant (1999) compiled data from several studies which measured dissolved oxygen content in groundwater at the highest point in the water table and values ranged between 1 and 10 mg L $^{-1}$. A survey of select aquifers in North America revealed dissolved organic carbon at ranges between .001 and 1.1mM, with a median value of .05mM (Leenheer et al., 1974). Average flow rates of groundwater have been estimated at 15m per day; however the flow rate can be highly variable and depends on the permeability of the subsurface and the hydraulic gradient (Ritter, 2006).
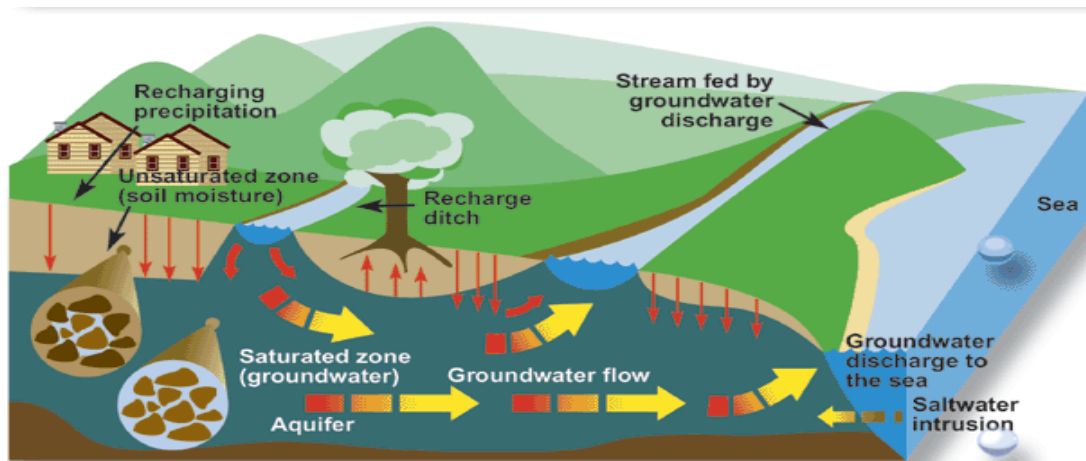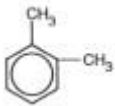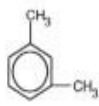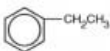
Figure 1.   Diagram Depicting Groundwater Flow.   Adapted from Environment Canada (www.ec.gc.ca/water (2009)

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1.3 BTEX

BTEX is an acronym which stands for benzene, toluene, ethylbenzene and xylene. They are characterized as "volatile organic compounds" in that they readily enter into a gaseous state from a liquid state (US EPA 2004). They are also characterized as non-aqueous phase liquids (NAPLs) in that they have a relatively low solubility in water (US EPA, 2004) (Table 1)

Table 1 - **Basic Chemical and Physical Properties of BTEX Compounds**

	benzene	toluene	o-xylene	m-xylene	p-xylene	ethylbenzene
Chemical Structure						
Chemical Formula	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}	C_8H_{10}	C_8H_{10}
Molecular Mass (g/mol)	78	92	106	106	106	106
Aqueous Solubility (mg/L)	1700	515	175	-	192	158

1.4 NAPL and BTEX as contaminants

The main mode of entry of BTEX, as well as other NAPLs, into the subsurface and groundwater is through industrial spills of petroleum products and leakage from underground storage tanks (Collins et al.,2002; US EPA, 2004). Gravitational forces cause NAPLs to sink into the subsurface until the water table is reached (Abriola and

Bradford, 1998). A balance of gravity, pressure and capillary forces in the water-saturated zone of the subsurface can cause NAPLs to become trapped or stationary and become long-term contaminant reservoirs for groundwater supplies (Abriola and Bradford, 1998).

Human exposure to BTEX contaminated groundwater can occur when communities rely heavily on groundwater supplies for everyday use (ie. tapwater). Volatilized BTEX can also enter into peoples homes via basements that are underneath contaminant plumes (Piver, 1984, USEPA, 2004). The United States Environmental Protection Agency (USEPA) has set a limit of 1mg/L of toluene in drinking water and 200 ppm of toluene in indoor air samples (USEPA, 2004).

The following is a list of common adverse health effects associated with exposure to BTEX: central nervous system toxicity, dizziness, vertigo, hematotoxicity (benzene), and respiratory tract irritation (USEPA, 2004).

Some microbes have the ability to move toward BTEX and other aromatic compounds. Bacterial chemotaxis is the movement either toward or away from a particular chemical due to the influence of a gradient (Pandey and Jain, 2002). Bacteria perform this task in order to find optimum conditions for their growth and survival (Pandey and Jain, 2002). This phenomenon is particularly relevant in regard to bioremediation as the degradation of contaminants requires the physical proximity of microorganism and substrate.

1.5 Microbial Chemotaxis to Aromatic Compounds

Parales described a series of chemoattraction experiments performed using toluene, benzene and trichloroethylene (Parales, 2000). Five motile bacterial strains were used in an agarose plug assay. All strains had the capability to degrade toluene and furthermore all strains employed a different oxidative enzyme in the initial step of toluene breakdown. Three of the five strains were attracted to toluene and the authors attribute this to the ability of these strains to grow using toluene as a substrate. The authors also noted that toluene metabolism was not necessary for chemoattraction; but rather the presence of toluene was sufficient for the three strains which showed chemotaxis to toluene (Parales 2000).

Harwood et al. (1984) reported the chemotaxis of *P. putida* PRS2000 to aromatic acid benzoate as well as *o-*, *m-* and *p-* toluate using a capillary assay. They also noted that while cells were attracted to *m*-toluate, it was not a growth substrate of the *P. putida* strain. Chemotaxis to benzoate by soil microorganisms *Azospirillum lipoferum* and *Azospirillum brasilense* has also been documented using a modified capillary assay (Lopez de Victoria and Lovell, 1993).

Law and Aitken (2003) described the chemotaxis of *Pseudomonas putida G7* in capillary experiments to naphthalene, a hydrophobic contaminant that was dissolved in the model NAPL 2, 2, 4, 4, 6, 8, 8-heptamethylnonane (HMN). While degradation and desorption of naphthalene was observed with a non-motile *P. putida G7*, chemotaxis toward the NAPL-water interface increased naphthalene desorption and degradation by an order of magnitude. The authors noted; however, that positive effects of chemotaxis were mitigated once the cell density increased from 9.2×10^6 CFU/ml to 6.7×10^7

CFU/ml.   The authors attribute this drop in desorption and degradation to nutrient limitations.

Grimm and Harwood reported the chemotaxis of *Pseudomonas putida* G7 and *Pseudomonas* sp. strain NCIB 9816-4 to naphthalene in a modified capillary assay (Grimm and Harwood, 1997). The ability to migrate toward the naphthalene source was dependant on the strains harbouring a plasmid with the degradative genes necessary for naphthalene metabolism.

1.6 Enzymatic Degradation of Toluene

Aerobic degradation of BTEX compounds can be accomplished by microorganisms which have the ability to express either monooxygenase or dioxygenase enzymes. TOL plasmids, including pWWO, carry two genetic cassettes which encode monooxygenase enzymes (Fig. 2). One cassette, consisting of enzymes *xly*CAMBN, encodes an "upper-pathway" that oxidizes methyl-benzenes to methyl-benzoates (Harayama et al., 1986). The second cassette, consisting of *xyl*E, *xyl*G, *xyl*F, *xyl*J, *xyl*Q, *xyl*K, *xyl*I, and *xyl*H, is referred to as the "lower-pathway" (Harayama et al., 1989). The upper-pathway enzymes have similar functions while the lower pathway enzymes each have more distinct, individual functions. The transcription of the lower pathway is controlled by the *xylS* gene product. The interaction of the *xylS* gene product with a primary substrate of the degradation pathway will in turn cause the binding of the upper cassette's promoter and initiate the transcription of the upper cassette's genes (Glick and Pasternak, 2003).   The products of these two genetic cassettes act in tandem to systematically degrade BTEX compounds into pyruvate, acetaldehyde or acetate, which

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are further metabolised and eventually mineralised by the microorganism (Jindrova et al, 2002).

1.7 The TOL plasmid

There are various Incompatibility group 9 (IncP-9) TOL plasmids which have been isolated; the most well known and widely used in laboratory settings is pWWO. Incompatibility groups identify plasmids which are not able to exist within the same cell (thus a cell containing pWWO cannot contain another IncP-9 plasmid). The IncP-9 TOL plasmid pWWO is 116,580 bp in size (Greated et al., 2002) and was initially isolated from *Pseudomonas putida*-mt2, as reported by Williams and Murray (1974)**.** The plasmid contains genes that encode enzymes for the degradation of toluene/xylene in addition to containing, and constitutively expressing, *traA, traB and traC,* which are necessary for its transfer from host to recipient (Fig. 2) (Worsey and Williams, 1975, Greated et al, 2002). There have been a number of hosts in addition to *Pseudomonas* strains that have been shown to successfully receive the TOL plasmid. *Pseudomonas* species as well as *Escherichia coli*, *Erwinia chrysanthemi*, *Hydrogenophaga palleronii*, *Serratia* and *Burkholderia* species have been shown to be recipients of the TOL plasmid (Benson and Shapiro, 1978**;** Nakazawa, 1978**;** Ramos-Gonzalez et al., 1991**;** Sarand et al., 2000; Nancharaiah et al., 2003)**.**
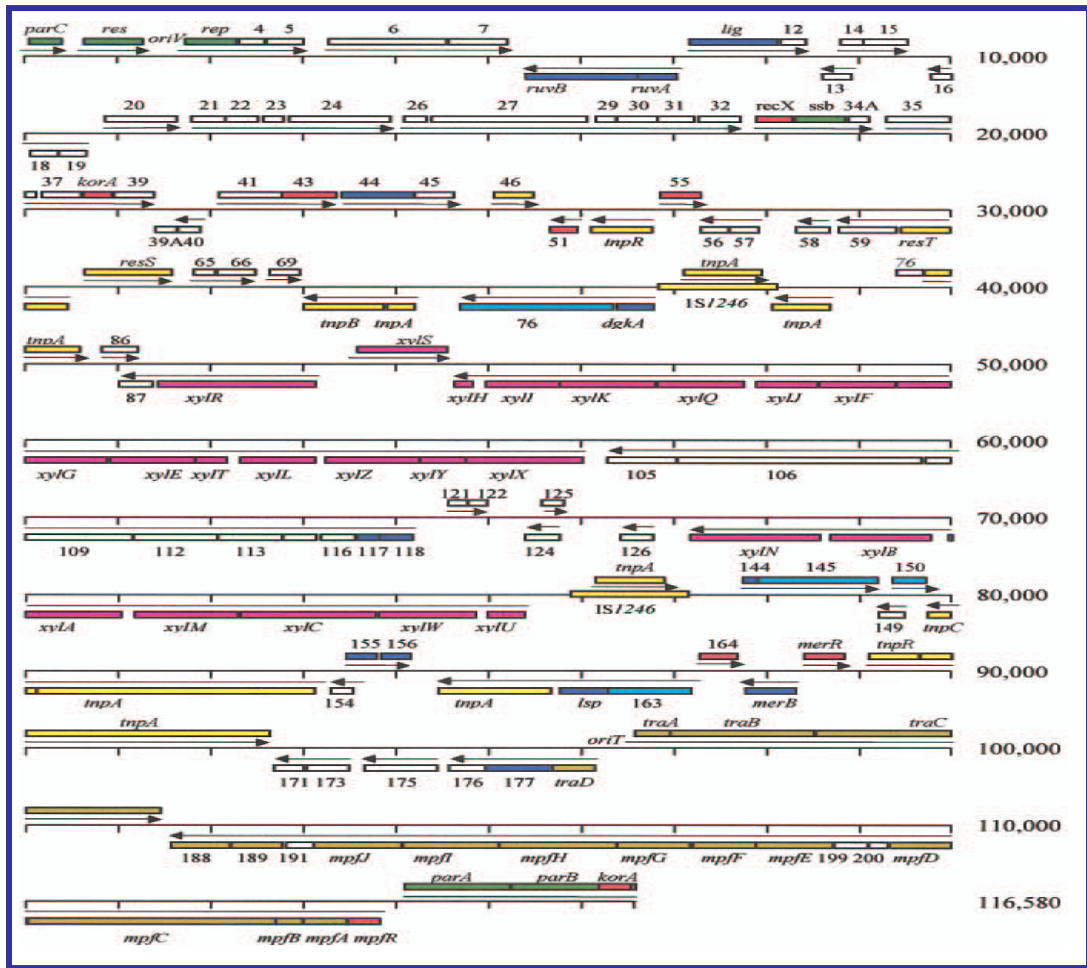
Figure 2. Schematic Representation of the TOL Plasmid, adapted from Greated et al. (2002). *xyl* genes responsible for the degradation of BTEX products are coloured magenta. *tra* genes involved in the transfer of the TOL plasmid are coloured dark yellow.

1.8 Groundwater Remediation

Groundwater which is contaminated with NAPLs must be cleaned to a certain standard before it is fit for consumption and use. The process of cleaning the groundwater of NAPLs is referred to as remediation. Remediation strategies fall into two broad categories: *Ex-Situ* and *In-Situ*.

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1.8.1 *Ex-Situ* Remediation

Ex-situ remediation refers to the removal of contaminants from groundwater after the water has been drawn from its source and transported to a treatment facility. While the relative monetary cost of ex-situ technologies is high, they provide a means for a controlled and predictable remediation of groundwater (Pandey et al., 2008). Environment Canada lists the following as ex-situ remediation technologies that are used to treat groundwater contaminated with fuel hydrocarbons (Environment Canada c, 2002):

1) Free product recovery – passive collection of un-dissolved liquids

2) Bioreactors – use of microorganisms in a bioreactor where physical conditions can be controlled

3) Air stripping – induction of contaminant volatilization by using an air current

4) Carbon Adsorption – groundwater contaminants adsorb to activated carbon as groundwater flows through a packed column

5) UV Oxidation – groundwater is treated with ultraviolent radiation and oxygen enhancing agents

1.8.2 *In-Situ* Remediation

In cases of non-aqueous phase liquid contamination there can be entrapment of contaminants in the subsurface (Abriola and Bradford, 1998). Thus, in-situ remediation techniques may be appropriate in such cases. Environment Canada lists the following as in-situ remediation techniques appropriate for groundwater contaminated with fuel hydrocarbons (Environment Canada, b, c, 2002):

1) Passive treatment walls – permeable barriers are placed in the path of a migrating contaminant plume. The treatment wall catalyzes the degradation of the contaminant.

2) Air sparging – air currents are used to induce volatilization of contaminants

3) Slurry walls – contaminant plumes can be contained or redirected using bentontite-water slurry walls that are introduced into desired locations

4) Permeability enhanced groundwater extraction – the extractability of groundwater supplies are increased by inducing subsurface fractures with pressurized water

5) Oxygen enhanced biodegradation – oxygen is introduced into contaminated areas in order to promote aerobic degradation of contaminants

6) Natural attenuation – natural biological, chemical and physical processes are deemed sufficient to resolve the contamination

Bacteria (and other microorganisms) have a vast metabolic diversity and thus, in some cases have the ability to naturally attenuate pollutants which enter the environment. The onset of contaminant degradation by bacteria can be accomplished by an increase in the population of specific degraders within a community, adaptation by way of mutation, or a spread of genetic information throughout a community by way of horizontal gene transfer (Top et al., 2002, Top et al., 2003). It is in this vein of thought that natural attenuation of environmental contaminants can be anthropogenically altered. Practically speaking, it would be a difficult task to perform site-directed mutagenesis to a population of microorganisms in a natural environment with a high-degree of precision, such that the

population would be able to degrade the contaminant in question. The addition of nutrients to an environment with the intention of stimulating the growth of specific degraders is perhaps a more practical avenue of procedure, and is commonly referred to as biostimulation (oxygen-enhanced biodegradation is an example of biostimulation). The approach of biostimulation is in essence dependant on the existence of a bacterial strain with specific metabolic capabilities in the vicinity of the contamination. The addition of new genetic elements can circumvent this caveat to biostimulation. This approach is referred to as bioaugmentation. A bacterial strain which has chromosomally encoded genes for the metabolism of a specified contaminant can be introduced into the contaminated area; or alternatively, a strain which harbours a plasmid that contains the relevant genetic components can be introduced into the contaminated area with the intention of plasmid transfer via a horizontal gene transfer mechanism.

There exist two significant pieces of literature which support the approach of horizontal gene transfer on a theoretical level. First, Ghigo reported that bacterial plasmids which constitutively express transfer factors responsible for the formation of specific pili, induce the formation of biofilms (Ghigo, 2001). Microorganisms which reside in biofilms are more closely situated to each other, and thus this arrangement provides a greater chance of horizontal transfer of plasmid DNA to recipient cells. The second, a report by Venugopolan et al. that competition between bacterial species leads to enhanced plasmid-mediated substrate utilization, specifically in *Pseudomonas putida* (Venugopolan et al., 2009). Using shake flasks containing minimal media and benzyl alcohol as a carbon source, they found that Pseudomonas KT2440 harbouring a modified version of pWWO degraded benzyl alcohol faster in co-culture as opposed to

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monoculture, regardless if the other bacterial species was a benzyl alcohol degrader or not. They also found that inert glass beads and cells with cell wall deficiencies were not able to induce an increase in benzyl alcohol breakdown by *P. putida* KT2440, thus leading them to conclude that cell-cell interaction is critical in eliciting a competitive response.

Combining these two reports, it is plausible to suggest that upon introduction of a donor strain carrying the TOL plasmid into an environment that the donor strain should promote biofilm formation (the TOL plasmid constitutively expresses all necessary transfer factors). Biofilm formation should in turn increase the chances of horizontal gene transfer, as cells in a bioflm are in close contact with each other. Biofilms in natural environments are often mixed cultures. Relatively high levels of expression of the *xyl* degradative genes should result as a competitive response to being in a mixed community biofilm.

1.9 Horizontal Gene Transfer

Horizontal gene transfer (HGT) is an important mechanism for the acquisition of new genetic information by bacteria in order to cope with changes in the surrounding environment (Grohmann, 2006). Often, the genetic material which is transferred resides in a plasmid. Self-transmissible plasmids, of which the TOL plasmid is an example, contain the genes which are necessary for the formation of a mating pilus. The family of genes which are responsible for the formation of the mating pilus is the transfer (*tra)* family (Holmes and Jobling, 1996, Gretaed et al, 2002). In addition to forming the mating pilus, these genes are also responsible for the replication and transfer of DNA

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(Holmes and Jobling, 1996). Briefly, horizontal gene transfer involves four steps: i) attachment of donor cell to recipient cell by a pilus ii) formation of a cytoplasmic bridge between donor and recipient, iii) replication and transfer of genetic elements, and iv) re-circularization of plasmids and dissociation of donor and recipient (Anthony et al, 1994; Holmes and Jobling, 1996; Thomas and Nielsen, 2005). It should be noted that this information pertains to gram-negative bacteria. Gram-positive bacteria are able to transfer plasmids; however, gram-positive bacteria have a different mechanism of attachment between donor and recipient (Grohmann, 2006**).**

1.10 Non-Aquatic Horizontal Transfer of the TOL plasmid and its Derivatives for Bioremediation

While this study explores the transfer of the TOL plasmid in a model groundwater flow system, work has been done in regard to the transfer of the TOL plasmid in non-aquatic model systems. These model systems are not similar to the one employed in this study, but the principles and findings of these studies can provide insight into the dynamics of the horizontal transfer of the TOL plasmid.

Normander et al. examined the horizontal transfer of a derivative of the TOL plasmid from donor strain *Pseudomonas putida* KT2442 to a *Pseudomonas putida* KT2442 culture (Normander et al., 1998). Donor cells and recipients were mixed together and then evenly coated on leaves of a bush bean plant (*Phaseolus vulgaris)*. Results suggested that the highest rate of conjugal gene transfer took place where metabolic activity of bacteria was most stimulated by root exudates, which supply

nutrients to adjacent bacteria. They estimated from their observations that under ideal conditions that 33% of potential recipients received the TOL plasmid.

In a similar study Molbek et al. showed conjugal transfer of two plasmids on alfalfa sprouts (Molbek et al., 2003). A modified *Pseudomonas putida* KT2442 was used as a donor strain and harboured either pWWO or pJKJ5. Again, the highest rates of transfer were observed in a region where metabolic activity of recipients was highest. Horizontal gene transfer of both plasmids took place despite the presence of selective pressure.

Taghavi et al. were able to confer greater phytoremediation capabilities upon Poplar plants inoculated with the endophytic bacterium *Burkholderia cepacia* VM1468 that harboured plasmid pTOM-Bu61, which encodes enzymes for toluene degradation (Thagavi et al 2005). Bu61 refers to its natural host *B. cepacia* Bu61. TOM refers to the operon which regulates the constitutive expression of toluene degradation enzymes. While plant growth increased and toluene evapotranspiration decreased with donor strain inoculation, there was no evidence that the donor strain became established in the community. This result highly suggested a horizontal gene transfer (HGT) mechanism for increased toluene breakdown; however, the authors did not show direct evidence that the plasmid was incorporated into the endophytic community.

Jussila et al. (2007) showed transfer of the TOL plasmid from *Pseudomonas putida* to *Rhizobium* in plate matings. Tryptone yeast extract agar was used for mating experiments. *Rhizobium* transconjugants which were initially isolated with selective media after mating experiments were either not able to maintain or express the TOL plasmid upon subculture in minimal media containing m-toluate. Although these

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transconjugants were not able to express the genes encoded in the TOL plasmid the authors suggested that these bacteria could act as a genetic reservoir.

1.11 Horizontal Transfer of Catabolic Plasmids in Model Aquatic Systems

There are several accounts of plasmid transfer in model aquatic systems. Below are the relevant findings and experimental parameters related to plasmid transfer in model aquatic systems.

In 2004 Bathe et al. (2004a) reported success in an attempt to bioaugment a sequencing batch biofilm reactor (SBBR). The SBBR was a bench-scale reactor filled with glass beads that acted as a biofilm carrier. The reactor was operated on an 8 hour SBR cycle. 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 in order to supply nutrients to biofilms within the system, as well as glass beads which served as an attachment surface for biofilm formation. Several carbon sources were supplied to the biofilms – sodium acetate, citric acid, sodium gluconate, D (+)-glucose and model contaminate 2, 4 dichlorophenoxyacetate (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 available. Transconjugants were detected by culture-dependant and culture-independent methods. Bioaugmented reactors showed more than 50% greater 2, 4-D removal (relative to initial concentrations) as compared to control reactors.

In 2005 Bathe (Bathe et al., 2005) slightly modified the experimental procedure outlined above. Most importantly: 3-chloroanaline (3-CA) was used as a model

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contaminant, the donor strain contained plasmid pNB2 which carries degradative genes for 3-CA and finally, the plasmid containing donor strain was unable to degrade 3-CA. Once again, transconjugants were detected and in this experiment it was possible to conclude that the observed increase in 3-CA degradation was totally attributable to an HGT mechanism.

Nancharaiah et al. investigated HGT in lab scale Sequencing Batch Reactors (SBRs) with artificial wastewater (Nancharaiah et al., 2007). Carbon was supplied to the microorganisms in the form of sodium acetate and benzyl alcohol. In contrast to the work of Bathe et al. (2004, a), no attachment surface was supplied for biofilm formation. Thus, the goal of the study was to assess HGT in microbial aggregates which were not anchored to a surface. *Pseudomonas putida* KT2442 which contained the TOL plasmid pWWO was used as a donor strain. HGT of pWWO as well as enhanced degradation of benzyl alcohol was observed.

Venkata Mohan et al. (2009) reported successful bioaugmentation of a lab scale SBBR using *Pseudomonas putida* KT2442 carrying the TOL plasmid (Mohan et al. 2009). The same success; however, was not replicated in a pilot scale SBBR. Plasmid recipients as well as increased benzyl alcohol degradation were observed in the lab scale SBBR. Addition of the donor strain to the pilot scale SBBR was not followed by the emergence of transconjugants or an enhanced reactor performance. The authors attributed this to an inadequate selection pressure in the pilot-scale reactor.

Bathe et al. showed successful bioaugmentation of a semi-continuous activated sludge reactor (Bathe et al. 2009). Reactors were bioaugmented with samples from a previously bioaugmented reactor (either biomass attached to a wood chip, or a sample of

free-floating biomass). The initial reactor contained donor strain *C. testosteroni* SB3 carrying the plasmid pNB2::*dsRed,* which is capable of degrading 3-chloroaniline. It was found that reactors supplied with biofilms attached to the wood chips outperformed those which received suspended biomass. This result may indicate that an attached mode of growth is more conducive to gene transfer and furthermore bioremediation.

Pei and Gunsch observed HGT of the TOL plasmid pWWO from donor strain *Pseudomonas putida* BBC443 to a donor consortia derived from activated sludge (Pei and Gunsch, 2008). These experiments were carried out in lab-scale batch reactors. While recipients of plasmid pWWO were detected, levels of toluene breakdown did not correlate with recipient numbers.

Limited information exists pertaining to plasmid transfer in groundwater systems; however, there has been some success. Jain et al. (1987) reported the dissemination and maintenance of the TOL plasmid in a model system of a porous aquifer regardless of selective pressure. *Pseudomonas putida* harbouring the TOL plasmid was used as a donor strain. Microcosms consisted of porous aquifer samples placed in a glass vial sealed with Teflon kept at 22°C. Toluene was added at a concentration of 400µg/L, which is below the saturation of toluene in water.

Enhanced horizontal gene transfer was demonstrated in subsurface soil microcosms under heavy metal stress (cadmium) by Smets et al. (Smets et al. 2003). Soil microcosms were supplied with artificial groundwater amended with Peptone (10g/L). The study employed *Escherichia coli* DH5α containing plasmid RP4 as a donor strain. Enhanced HGT and as well as enhanced cadmium retention was observed under the highest cadmium concentrations used (1,000µM).

In summary, HGT has been successfully demonstrated in model wastewater systems. HGT has been demonstrated less frequently in model groundwater systems.

1.12 Factors Affecting Horizontal Gene Transfer

While studying conjugal transfer rates in batch reactors between donor strain *Pseudomonas putida* PAW1 carrying the TOL plasmid and pure culture *P. aeruginosa* PAO 1162, Smets et al. (1993) reported that conjugal transfer rates were sufficient to support the propagation and maintenance of the plasmid in a dense microbial population. They also concluded that transfer rates depended on the specific growth rate of the donor strain. It should be noted that there was no attachment surface supplied for the microorganism, and that even though they carried out batch mating experiments, their conclusions were based on a mathematical model. Rittmann et al. also suggest that the specific growth rate of the donor strain is a factor in gene transfer frequency. To that, they add that the increasing the substrate concentration (in their case, glucose) positively influences horizontal gene transfer rates (Rittmann et al, 1995). Sudarshana and Knudson (1995) reported that the specific growth rate of donor cells is a factor in gene transfer rates. They also noted that the initial cell density of donor strain cells did not affect transfer rates, especially when nutrient availability was low.

Pinedo and Smets (2005) reported on the pre-mating exposure to toxicants as well as restriction proficiency (the ability of recipients to enzymatically degrade foreign DNA) with regard to transfer of the TOL plasmid. In their filter mating experiments between *Pseudomonas putida* KT2442-derived strain and two *Pseudomonas aeruginosa* strains

(one restriction proficient, one deficient) they found that pre-mating exposure to toluene or phenol had no appreciable effects on the ability of the restriction proficient strain to accept and retain the TOL plasmid. When comparing the transfer rates of the TOL plasmid to the restriction proficient and deficient strains, they noted that the transfer rates were 3 to 4 orders of magnitude lower for restriction-proficient strains.

Fox et al. described a set of filter mating experiments using donor strain *Escherichia coli* which harboured plasmid pB10, a multidrug resistance plasmid isolated from wastewater (Fox et al, 2008). They reported that the replenishment of nutrients provided a positive effect on the transfer of the plasmid. They also report that the infiltration of the plasmid into spatially static biofilms was much less than that in biofilms which were disturbed on a daily basis and were thus made to be spatially dynamic (two sets of filters were transferred into new medium daily; however one set was scraped, centrifuged, re-suspended and spread onto a new filter daily). These findings are consistent with those of Christensen, who performed flow-cell experiments which examined the integration of the TOL plasmid into biofilms. They show that TOL plasmid integration was restricted to the peripheral areas of the biofilm structure (Christensen, 1998).

Johnsen and Kroer showed again that metabolic activity of the donor strain is an important factor for horizontal gene transfer when investigating matings between *Escherichia coli* and *Pseudomonas putida* strains in microtitre wells (Johnsen and Kroer, 2007). They determined that the optimal temperature for transfer events was 29 ^{0}C. They also note that pre-mating exposure to selective pressure in the form of toxicants may have

a detrimental effect on the recipient community, thus decreasing the number of successful transfer events.

Examining horizontal gene transfer of the TOL plasmid to recipients in activated sludge, Pei and Gunsch reported that the highest transfer rates occurred when the recipient to donor ratio was high (20:1). The authors suggest that this result indicates that plasmid transfer rates are more closely associated with recipient cell characteristics. They describe an optimal situation for plasmid transfer as being one where potential recipients are present at the optimal cellular density. Batch reactors containing basal medium and toluene, activated sludge and donor strain *P. putida* BBC443 were used to carry out conjugation experiments (Pei and Gunsch, 2009).

Summary

Transfer of the TOL plasmid has been documented in both non-aquatic and aquatic model systems. The success of transfer of plasmids and the expression of genes contained on those plasmids is function of: donor-recipient compatibility, donor-recipient ratio, rate of the donor strain, nutrient availability, donor-recipient proximity and pre-mating toxicant exposure. There is not a significant amount of literature regarding use of the TOL plasmid in groundwater model systems, much less rock fracture apertures.

2. Materials and Methods

2.1 Strains, Culture Conditions and Media

Tryptic soy broth (TSB) (EMD, Brampton, Ontario. lot vm187759) was used for all flow cell experiments. Concentrations of TSB listed are based on a 100% concentration of 30g/L dissolved in distilled water. Luria Bertani (LB)-agar plates were prepared using 15g of LB powder per 1litre of distilled water (Bio Basic Inc., Markham, Ontario. lot 01148).

Donor strain *Pseudomonas putida* SM1443::gfp2x-pWW0::dsRed was used in all flow cell and mating experiments where indicated (referred to as donor 1) (Bathe 2004). Donor 1 constitutively expresses green fluorescent protein (GFP). Expression of red fluorescent protein that is encoded on the TOL plasmid is repressed via a lac-operon related mechanism in the donor cell, as described by Tolker-Nielson et al. (2000). Expression of RFP is not repressed in recipients of the TOL plasmid as recipients lack a chromosomally-encoded lac-repressor. It was maintained on LB- agar amended with kanamycin (50µg/ml) and gentamycin (25µg/ml).

Pseudomonas putida BBC443-pWW0::gfp (referred to as donor 2) was maintained on LB-agar amended with kanamycin (25µg/ml)(donor 2 was donated by Claudia Gunsch, Duke University, and was described in Pei and Gunsch, 2009). Donor 2 does not express any fluorescent proteins. The TOL plasmid in donor 2 encodes GFP. Expression of GFP in the donor is repressed by the same mechanism as that of donor 1. GFP on the TOL plasmid is expressed by recipients.

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Pseudomonas putida ATCC 15070 and *Pseudomonas aeruginosa* PAO1 were maintained on LB- agar plates. *Pseudomonas* sp. CT07 was maintained on 10%TSB (Bester et al, 2005; Wolfaardt 2008). Where indicated, 150µl of 50mM Isopropyl-ß-D-thiogalactopyranosid (IPTG) was applied topically onto agar plates. IPTG powder was dissolved in sterile distilled water.

2.2 Inoculum Preparation

The inoculum for flow cell experiments was prepared using a modified dual-dilution method adapted from Caldwell and Lawrence (1986). After autoclaving of the flow-through system, 1% TSB media was pumped through the system overnight to prepare the system for inoculation with groundwater. Glass beads of 2mm diameter contained in a 200ml beaker were used as an attachment surface for microorganisms. The beads were filled up to approximately the 50ml marker on the beaker. Groundwater, which was collected from an aquifer in Cambridge, Ontario, was used to inoculate the flow through system. Approximately 50 ml of groundwater was added to the beaker and the flow was stopped for 2 hours to allow for attachment of microorganisms to the glass beads. The flow was resumed after 2 hours at a rate of 3.2ml/hr with 0.1%TSB medium for two weeks. The influent tubing was not in contact with the glass beads or the medium/groundwater mixture. The effluent tubing was immersed in the medium/groundwater mixture. This allowed for a constant volume in the beaker, as well as the removal of unattached microbes from the media/groundwater mixture. After 2 weeks of flow, the beads were collected with a sterile scupula and deposited into a mixture of equal parts sterile 1%TSB and sterile 100% glycerol contained in a 50ml

plastic tube. The beads were then stored at -20°C. When inoculum was needed for a flow cell approximately 10 beads were removed from the frozen stock and placed into 5ml of 1%TSB in a test tube. The tube was then placed in a shaking incubator, set to 30°C and 250rpm, and incubated overnight.

2.3 Medium-Scale Flow Cell

Our collaborators at the Department of the Civil Engineering at the University of Toronto constructed and operated a medium-scale model rock-fracture aperture flow system. The construction and operation of this model system was described in Hill and Sleep (2002). Briefly, the glass plate fracture was inoculated with a groundwater-derived microbial consortium that was produced as outlined in section 2.2 (this was the same inoculum which was used in all experiments reported on here). Toluene was introduced into the plate fracture and resided within the fracture in a distinct, un-dissolved phase termed the toluene "globule." At the end of the experimental period (approximately 6 months) the glass plates were separated and biofilm samples were collected using sterile cell-scrapers. Samples were collected and labelled based on the location within the fracture (Fig. 3). DNA was extracted from the various samples and used for PCR/DGGE (Fig. 6).
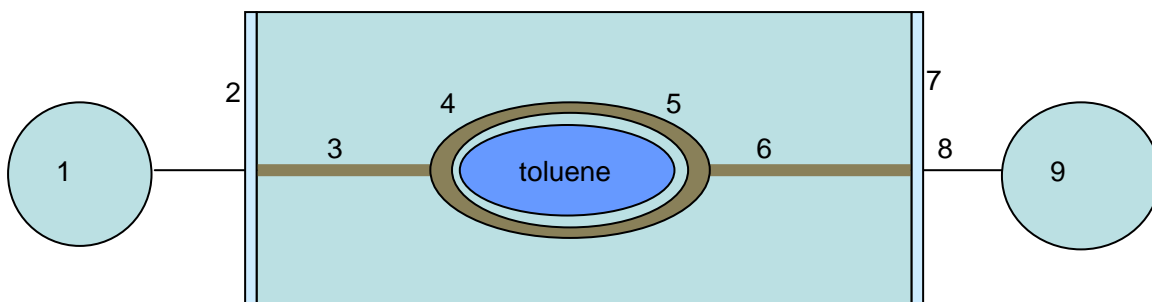
Figure 3. Simplified Schematic of the Medium-Scale Glass Plate Fracture Flow System. The system was constructed and operated by our collaborators at the University of Toronto. 1) Medium reservoir, 2) Inlet gate, 3) Biomass, "proximal trail," 4) Biomass, "proximal fence," 5) Biomass, "distal fence," 6) Biomass, "distal trail," 7) Outlet gate, 8) Outlet tubing, 9) Effluent reservoir

2.4 Small-Scale Flow Cell Construction and Other Apparatus

Flow cells were constructed with the goal of simulating a model rock-fracture aperture. Teflon blocks were used for flow cell construction (outer dimensions: 2.5cm height, 5cm width, 7.5cm length; inner dimensions: 2.5cm height, 3.25cm width, 5cm length). Gutters were milled at both ends of the Teflon block in the vertical plane using a 3mm endmill piece. Holes were drilled in both ends in the horizontal plane using a 1/8th inch drill bit and subsequently threaded. Swagelok brass straight male tube connectors (1/8NPT) were inserted into the threaded holes. Shale was used as a rock surface for the flow cells (2 cm height, 2.5cm width, and 5cm length). The wafer of shale was secured in the opening of the Teflon block using a two-part epoxy glue (Devcon plastic steel putty (a) 10110). Cover slips (60mm x 35mm) were secured over the rock wafer with liquid viton (Pelseal, Newtown, Pennsylvania. item #2077) in order to prevent volatilization of toluene and benzyl alcohol. Cover slips were used to allow for non-invasive microscopic examination of biofilms on the rock wafer surface. Microorganisms and toluene were introduced into the flow-through system through a minninert valve (1/8in) in a swagelok brass female branch tee (1/8in). Teflon tubing

segments were used to connect the branch tee and the straight tube connector as well as connecting the flow cell to the effluent receptacle. The remainder of the tubing used was silicon. Tubing which connected the bubble trap and flow cells was raised above the height of the flow cell in order to avoid the movement of flow cell microorganisms toward the medium reservoir. A bubble trap, which was fashioned from a 10ml syringe, was placed between the peristaltic pump and flow cell. A multi-channel peristaltic pump (Watson-Marlow model 205S) was used to create flow. The flow rate was set to 0.5 rpm, which corresponded to a volumetric flow rate of 3.2ml/hr (this was the slowest flow rate that could be achieved with this pump) was used to simulate groundwater flow. A schematic of the flow system can be seen in figure 4. A photograph of two flow cells can be seen in figure 5.

a

b
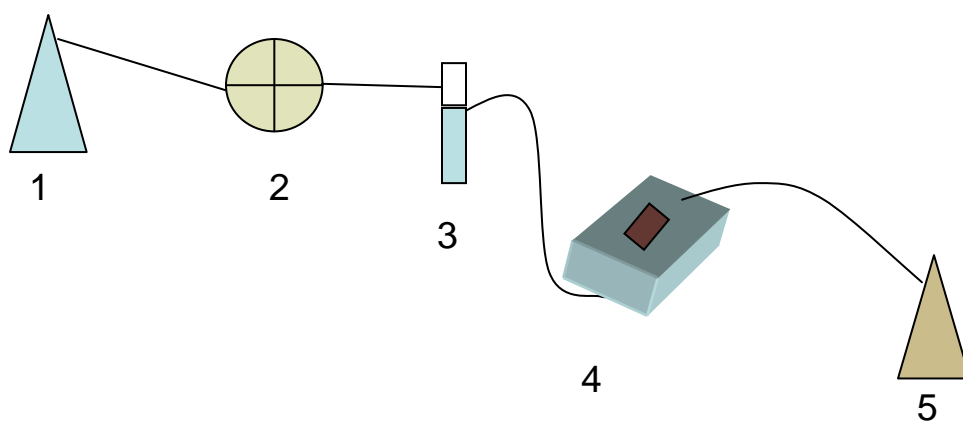
Figure 4. Experimental Set-up. a) Simplified schemtatic and b) photograph, of experiemtal set-up 1) medium reservoir, 2) peristaltic pump, 3) bubble trap, 4), Flow cell containing the rock wafer, 5) effluent. Silicon tubing was used to the right of the tee junction. Teflon tubing was used to the left of the tee junction – seen between 3 and 4 - in b.
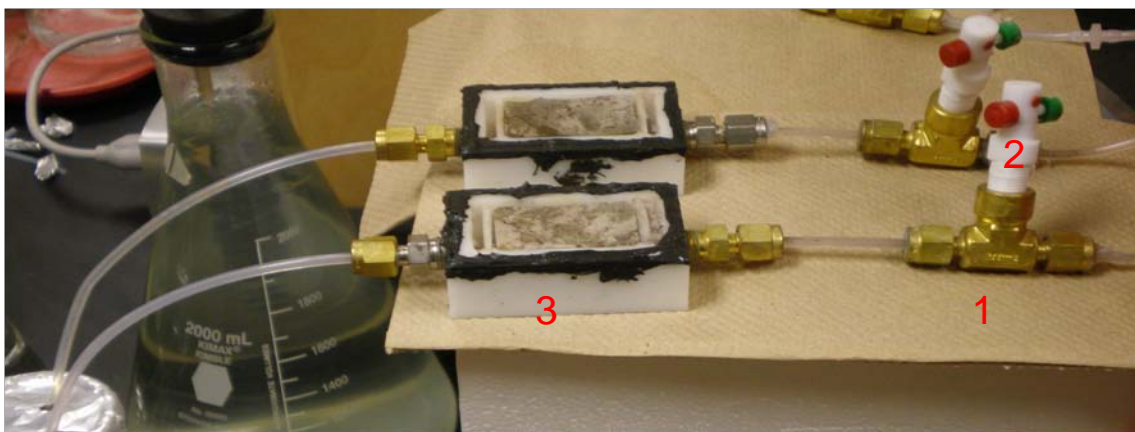
Figure 5. Image of Flow Cell Which Contain a Rock Wafer. 1) Tee junction, 2) Minninert valve, 3) Flow cell

2.5 Flow Cell Inoculation and Operation

All tubing, media and glassware were autoclaved before use at 121°C for 15min. Upon assembly, a 2% sodium hypochlorite solution (v/v) was pumped through the system for 3hrs. Autoclaved distilled water was then pumped through the system for 24hrs. 1.0% TSB was then pumped through the system for 3hrs. The flow cell was then inoculated with 1ml of overnight culture from the groundwater-derived bead stock. Flow was stopped for 2hrs following inoculation, and then resumed at 0.5rpm (3.2ml/hr) with 1.0% TSB. After 24hrs of flow, 0.1% TSB was pumped into the flow cell for the remainder of the experiment. After 3 more days of flow with 0.1% TSB, toluene and donor strains were inoculated into the flow cells, and this was taken as "day 0" of the experiment. A volume of 600µl of toluene was introduced into the flow cells before the flow cell was inoculated with the donor strain. An overnight culture of the donor strain was used for inoculation. The overnight culture was prepared with LB amended with gentamycin (25µg/ml) and kanamycin (50µg/ml); shaking overnight at 30°C and 250rpm.

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After inoculation of the donor strain flow was temporarily stopped for 30 minutes to allow for integration into biofilms.

2.6 Toluene and Benzyl Alcohol Measurements

Effluent samples were collected by removing the tubing from the effluent receptacle and placing it in a 1.5ml Eppendorf tube. Effluent samples were subsequently prepared for plating on TSA plates amended with gentamycin and LB-agar plates amended with kanamycin and gentamycin. Dilutions for plating were prepared with sterile saline solution. Effluent samples sent for toluene (tol) concentration analysis were collected in glass vials and sealed to avoid volatilization. Samples were sent to our collaborators at the department of Civil Engineering at the University of Toronto for gas chromatography analysis/ flame ionization detector (GC/FID). Samples were processed using a headspace analyser (Hewlett Packard, Ramsey, Minnesota. HP5890 II series).

After two weeks of flow and wash-out of toluene from the flow cell (confirmed visually) benzyl alcohol was added to the medium at a concentration of 500ppm. At this point effluent reservoirs were changed. A separate effluent reservoir was added at this point. It received media, directly from the medium reservoir via silicon tubing. Benzyl alcohol concentrations taken from this new effluent reservoir was taken as the "reservoir" concentration and given a relative value of 1. Benzyl alcohol concentrations were measured by UV-VIS (Beckman, Streetsville, Ontario), recording the absorption at a wavelength of 260nm. One millilitre of effluent was collected in a 1.5ml Eppendorf tube. Samples for spectrophotometry were prepared by mixing 100µl of effluent with 900µl of autoclaved distilled water. The Absorbance at 260nm was used as a measure for

benzyl alcohol concentration. Absorbance readings were recorded as the average of duplicate samples. Figure 6 depicts the sampling times and overall experimental procedure.

Day:	0 2	5	8	11	14 15	17	19 20

Medium: 0.1%TSB | 0.1%TSB + BA

Effluent Sample: TSA plating and [tol] measurement

Effluent Sample: [BA] measurement

CLSM investigation
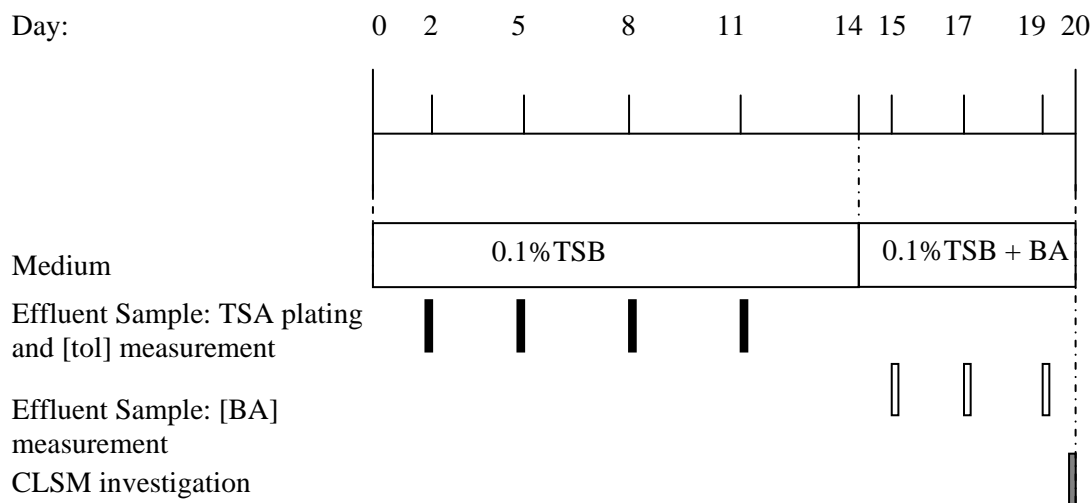
Figure 6. Gantt Chart of Experimental Procedure. Chart shows sampling times and experimental procedures taking place over the experimental period of 20 days

2.7 Biofilm Architecture Analysis

Two weeks after inoculation flow was terminated and biofilms were stained with 500µl of 50mM acridine orange dissolved in sterile water. After addition of acridine orange to the flow cells the flow was stopped for 15 minutes to allow for binding and the flow cell was covered with aluminum foil to avoid photo-bleaching of acridine orange signal. After the 15 minute period without flow, flow was resumed for 5 minutes to remove any unbound stain or stained planktonic cells. Flow cells were then examined with a confocal laser scanning microscope (Zeiss, LSM510, Jena, Germany). The surface of the flow cell was divided into six sections – front left, centre and right, and back left,

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front and right. Equal numbers of image stacks (approximately 5-10) were taken from each area. Images used for analysis were primarily generated from the front left, right and centre areas, as the highest quality images came from the three areas. Images were obtained using a 488nm laser and a 505-530nm band-pass emission filter. A 20x objective lens was used in addition to a 2x digital zoom, for a total magnification of 400x. The distance in the z-plane between individual images in a stack was 2µm. The full thicknesses of the biofilms were captured in the image stacks. 15 image stacks were selected from toluene and non-toluene treated flow cells for COMSTAT analysis. COMSTAT image analysis runs in MATLAB (the Math Works, Massachusetts, U.S.A,) The resulting image stacks were analysed using COMSTAT image analysis software for average biofilm biomass and biofilm thickness (Heydorn et al. 2000). Average biofilm biomass was measured as   the volumes of biomass per substratum area ($\mu m^3/\mu m^2$). Thickness was measured as the maximum thickness of the biofilm (µm). A single factor ANOVA analysis using a p value of (p=.05) was used for statistical analysis.

2.8 Plate Matings

Overnight cultures of the specified strains were prepared for plate mating experiments in a shaking incubator set to 30°C and 250rpm. Relevant antibiotics were used in overnight cultures (section 2.1). 500µl of donor strain cultures and 750µl of recipient cultures were used for preparation of mating mixtures. The donor strains and recipient strains (*P. putida* ATCC 15070 or *P. aeruginosa* PA01) or mixed cultures (inoculum) were combined in a 1.5ml Eppendorf tube and centrifuged at 7,000rpm for 10 minutes. After centrifugation the supernatant was discarded and the cell pellet was

suspended in 100µl of sterile saline solution and transferred to an LB plate in one droplet (ie. cells were not spread). Plate matings were left at room temperature for 4 days and then transferred to a refrigerator set to 4°C for 10 days. The two week incubation period was used in order to allow for folding of the red fluorescent protein (Bathe and Hausner, 2009). After 2 weeks of incubation mating patches were sampled and examined using a confocal laser scanning microscope (Zeiss, LSM510). Images were taken using a 100x oil immersion lens, for a total magnification of 1000x. GFP was visualized using a 488nm laser and a 505nm long-pass emission filter. RFP was visualized using a 543nm laser and a 560nm long-pass emission lens.

2.9 Batch Culture

Batch cultures were prepared in sterile, 50ml sterile screw-cap tubes. Cultures consisted of overnight cultures of the specified strains, prepared as described in section 2.1. One millilitre of each specified overnight culture was added to 62.5µl and 0.1%TSB filled to 25ml. Caps were loosely screwed onto the tubes in order to allow for oxygen entry into the tubes. Tubes were placed ain a shaking incubator set to 250rpm and 30°C. One millilitre of the batch culture was removed and samples were analysed for benzyl alcohol concentration as described in section 2.5. All biomass was visualized in transmission mode in the LSM510 channel D using a 543 nm laser.

2.10 PCR

Polymerase chain reaction was used to amplify portions of the 16s rRNA, *rfp,* and *gfp* genes. DNA was extracted for amplification of *gfp* and 16s rRNA using a genomic

DNA extraction kit (Sigma-Aldrich, sm2110-1kt). This kit was used for plasmid extraction as well. Overnight cell cultures were used for DNA extraction. Cell suspensions could also be used in which case an initial denaturation cycle of 15 minutes at 94°C was used. TRI reagent (Sigma, T2414) was used for extraction of DNA from biofilms in flow cells. All PCR protocols employed 30 cycles of amplification. Other cycle parameters and primer sequences can be found in Table 2. Reactions were prepared as follows: 25µl of 2x Lucigen TaqSelect DNA Polymerase (cat# 30041-1), 3µl of forward and reverse primers at a concentration of 5µM, 4µl of 5mM dNTP mixture, 2µl of 5mM BSA, 4µl of template DNA. Reactions were adjusted to 50µl with autoclaved, distilled water.

Gel electrophoresis was used to assess PCR results. 1.5g of agarose was added to 100ml of TAE buffer and heated in a microwave for 70s. Ethidium bromide was used at a concentration of 2µl/100ml to visualize DNA.

Table 2 – Polymerase Chain Reaction Primers and Thermal Cycler Temperatures

Primer Target	Sequence	Initial Denature	Denature	Primer Annealing	Extension	Final Extension	Reference
rfp	**Forward** - 5' -TTGAAGACGGTGGCGTCGTTAC- 3' **Reverse**- 5'-CCTTTCAACACGCCATCACGAG- 3'	94°C, 15min	94°C, 30s	62°C, 1min	72°C, 2min	72°C, 2min	Bathe (2004)
gfp	**Forward**- 5'-TTTCAAGAGTGCCATGCCCGAAGG- 3' **Reverse**- 5'-CTGGTAAAAGGACAGGGCCATCGC- 3'	94°C, 2min	94°C, 30s	60°C, 45s	72°C, 1min	72°C, 2min	Bathe (2004)
16s rRNA	**Forward**- 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3' **Reverse** - 5'ATTACCGCGGCTGCTGG-3'	94°C, 2min	94°C, 30s	65°C, 45s	72°C, 1min	72°C, 2min	Muyzer et al. (1993)

2.11 Denaturing Gradient Gel Electrophoresis (DGGE) and DNA Sequencing and Analysis

The protocol which was provided in the manufacturer's manual was followed for the preparation of all solutions used in DGGE experiments (D-Code, Biorad laboratories, Mississauga, Ontario. catalogue# 170-9080 through 170-9104). All DGGE experiments were prepared - with one exception - with a denaturing gradient of 55%-30% denaturant solution consisting of formamide and urea. The DGGE profile of biofilms sampled from the medium-scale model fracture used a denaturing gradient of 70%-30% denaturing solution. Gels were prepared with 8% acrylimide solutions (20ml acylamide/bis in 100ml of denaturing solution). Bands were visualized by the use of ethidium bromide. Ten microlitres of ethidium bromide (EMD, Brampton, Ontario. 2158B015) was added to 250ml of TAE (Tris, EDTA, Acetic Acid) buffer and the gel was immersed in the solution for 10 minutes. The gel was then de-stained for 15 minutes in 250ml of TAE buffer. Gels were photographed using a gel-doc system (UVP, Mississauga, Ontario BioDoc-it 210). DNA bands of interest were cut out using a sterile razor. Cut-out bands were then placed in 25µl of TE (Tris, EDTA) buffer in a 1.5ml eppendorf tube. Eppendorf tubes were incubated overnight at 30°C to allow for DNA elution into the TE buffer. Samples were sent to the DNA sequencing facility at the Hospital for Sick Children in Toronto, Ontario for sequencing. Resulting sequences were compared to those of the BLAST genome database.

2.11 Varied Nutrient Flow-Cells

Flow cells were prepared and inoculated as described in section 2.5; however, toluene was not introduced into the flow-cell. All flow cells were inoculated with donor 1 four days after inoculation with the groundwater-derived microbial consortium. One flow cell was supplied with 0.1%TSB, a second flow cell was supplied with 1.0% TSB and a third flow-cell was supplied with 10% TSB. Ten days after donor strain inoculation 50ml of effluent taken from the effluent reservoir was filtered and examined microscopically (as described in 2.8).

3. Results

3.1 Medium Scale Rock Fracture Biofilm Analysis

The composition of biofilm samples from the medium-size model of a rock fracture aperture were analyzed by PCR-DGGE (fig.7). Samples were taken from 8 different locations in the flow cell (section 2.3). Three of the 5 main bands which can be seen in the DGGE profile of the inoculum can be seen throughout the other profiles (indicated by arrows in figure 7). The DGGE profiles of the biofilm samples appear to contain bands which do not appear in the inoculum (lane a). While most bands appear consistently in lanes b-i, the intensity varies. Most notably, profiles from biofilms sampled from the distal fence and distal trail (lanes e and f, respectively) have a decreased intensity in most bands and an increased intensity in two other bands (within the boundaries of the superimposed rectangle in figure 7). These two regions were directly downstream of the toluene globule and thus likely had the highest toluene concentrations. Sequence analysis performed on the most intense band at the bottom of lanes e and f indicated that these bands represent *Rhodococcus erythropolis.*
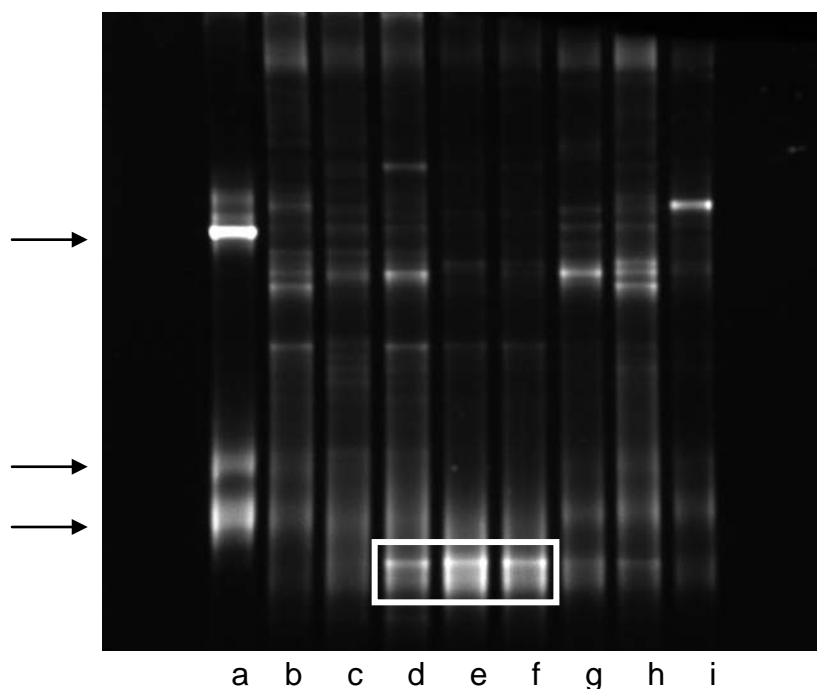
FIGURE 7. DGGE Image of Biofilms Sampled From a Medium-Scale Model Rock Fracture Aperture. Lanes correspond to biofilms sampled from different areas of the rock fracture; a) Inoculum, b) Inlet gate, c) Proximal trail, d) Proximal fence, e) Distal trail, f) Distal fence, g) Outlet gate, h) Outlet tubing, i) Effluent. Bands inside the white box were sequenced. Arrows indicate comn bands between inoculum and plate fracture samples.

3.2 DGGE Analysis of Inoculum

PCR-DGGE analysis of several inoculum stocks were performed to ensure the consistent composition of inoculum for flow cell experiments (fig 8, lanes a-c). While lane b has two extra bands, all lanes share 4 common bands (as indicated by the superimposed rectangle) (fig 8). These profiles represent microorganisms which attached to the glass beads. Lanes d represents the DGGE profiles of microorganism enriched with 1.0% TSB from groundwater in batch culture without an attachment surface. Lane e represents the DGGE profile of microorganisms from effluent collected during the enrichment of the inoculum bead stocks. While the intensity of bands in lane d is greater than that in lane e, there are at least 5 common bands in these two profiles.

40
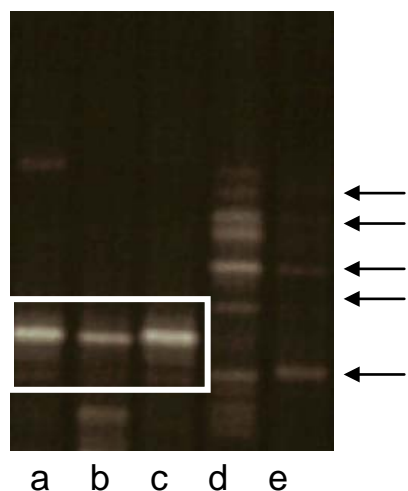
Figure 8. DGGE Profiles of Inoculum and Enriched Groundwater : a-c) profiles of inoculum used for flow cell experiments. Samples from each lane originated from unique bead collections which were produced on different dates. d) profile of groundwater community which was grown in batch culture with 1%TSB media. No attachment surface was provided for microorganisms in the batch culture. e) profile of effluent collected from a single dual-dilution inoculum generation trial. Arrows indicate common bands between lanes d and e.

3.3 Confirmation of Plasmid Presence in Donor 1

Repression of *rfp* expression in the donor cells is achieved through a lac-operon related mechanism (Tolker-Nielsen et al. 2000). The presence of IPTG interferes with this mechanism and allows for the expression of the *rfp* gene in donor cells. Donor strain *P. putida* is normally a yellow color when cultured on LB-agar plates (fig. 9, a). When plated on LB-agar with topically applied IPTG, donor cultures appear pink (fig. 9, b).

<center>a   b</center>
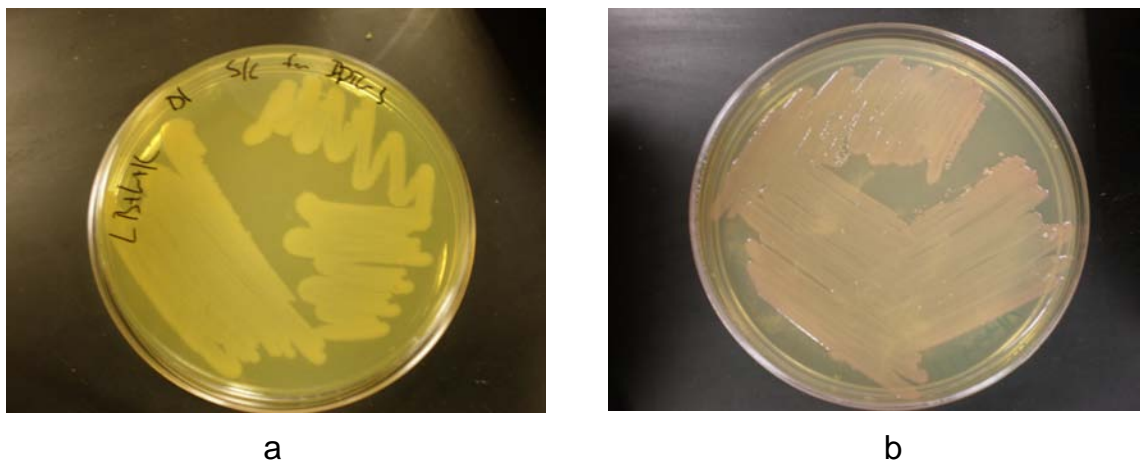
Figure 9. RFP Confirmation on pWWO Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed spread plated on a) LB with gentamycin (25mg/l) and kanamycin (50mg/l) and b) LB with gentamycin (25mg/l), kanamycin (50mg/l) and topically applied IPTG.

3.4 Confirmation of *gfp* in Donor 1

Our donor strain contains chromosomally encoded *gfp*. This was confirmed by PCR directed at the *gfp* gene (fig. 10), which yielded the expected product of 300 base pairs (lane b). Positive control, PCT07 which contains the *gfp* gene showed a similar result.
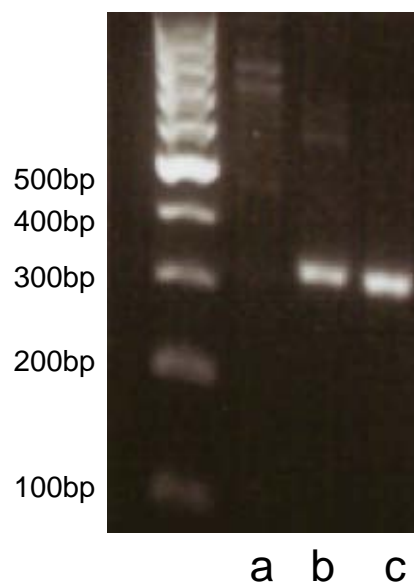
Figure 10. PCR Confirmation of Donor Strain *gfp*. Agarose gel electrophoresis of PCR products. Primers used were designed for the green fluorescent protein (gfp) gene. A DNA ladder can be seen on the left of the gel image. a) Inoculum, b) donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed, c) gfp-containing PCT07.

3.5 Biofilm Biomass Analysis

Average biofilm biomass in flow cells which were, and were not exposed to toluene (tol+ and tol-, respectively) was measured by analysis of confocal microscopy images with COMSTAT software (fig 11). The overall trend is that biofilms that are exposed to toluene have less biomass than biofilms which were not exposed to toluene; however, there is a high variation in biomass measurements in both types of flow cells. Using a single factor ANOVA test with a significance level of ($p=.05$) it was determined that there was a significant difference between the average biomass of biofilms in tol+ and tol- flow cells. Images were taken from 6 areas of the flow cell (as described in section 2.6). While figure 11 represents the average of values taken from all areas, a similar trend was seen when comparing measurements from each tol+ and tol- flow cells from each individual area.

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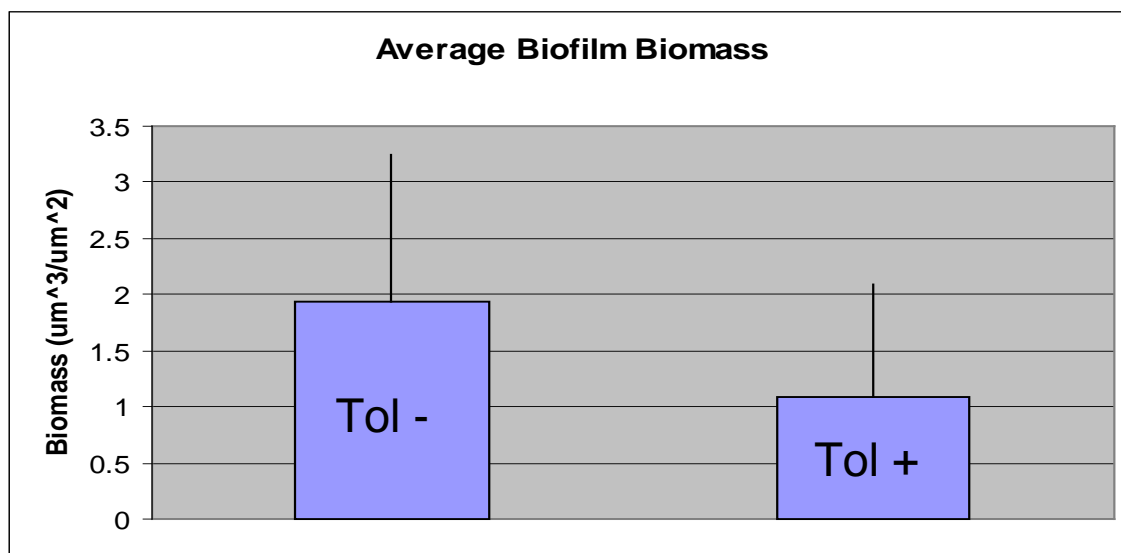
Figure 11. Biofilm Biomass. Graph showing the difference in biomass between biofilms in flow cells which were and were not exposed to toluene. Measurements were done by analysis of confocal microscopy images using COMSTAT software.

3.6 Plasmid Transfer Analysis in Flow Cells

Isolation of plasmid recipients was attempted by plating effluent samples onto tryptic soy agar (TSA) plates with gentamycin. While colonies did grow on these plates, after two weeks of incubation to allow for RFP production pink colonies which are typical of colonies expressing *rfp* were not observed. Furthermore, these colonies did not display green fluorescence typical of donor cells. We were not able to isolate DNA from colonies on TSA plates with gentamycin.

No observable trend in effluent toluene concentration was seen in flow cells regardless of donor strain inoculation (tables 3 and 4). Benzyl alcohol was added to the medium after 2 weeks in order to more easily assess (spectrophotmetrically) the

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breakdown of BTEX compounds in our flow system. Benzyl alcohol levels in effluent samples did not significantly vary between flow cells which were, and were not, inoculated with the donor strain (88.5% and 87.9%, respectively of reservoir levels) (fig. 12). There was however a decrease in benzyl alcohol concentrations in both flow cells compared to the medium reservoir.

Table 3 – Quantitative and Qualitative Data from a Control Flow Cell, (Toluene+, Donor-)

	Day 2	Day 5	Day 8	Day 11
CFU/ml in Effluent, TSA+Gentamycin	2.7x10^5	1.04 x 10^6	9.5 x 10^5	7.6 x 10^5
[Toluene] (ppm) in Effluent	47, 67, 178	21.8	78.9	17.83
GFP	negative	negative	negative	negative
DNA Extractable	negative	negative	negative	negative

Table 4 – Quantitative and Qualitative Data from a Flow Cell Inoculated with the Donor Strain, (Toluene+, Donor+)

	Day 2	Day 5	Day 8	Day 11
CFU/ml in Effluent, TSA+Gentamycin	4.8 x 10^5	1.23 x 10^6	3.4 x 10^6	3.3 x 10^5
[Toluene] (ppm) in Effluent	590, 124, 73	55.46	87	3.73
GFP	negative	negative	negative	negative
DNA Extractable	negative	negative	negative	negative
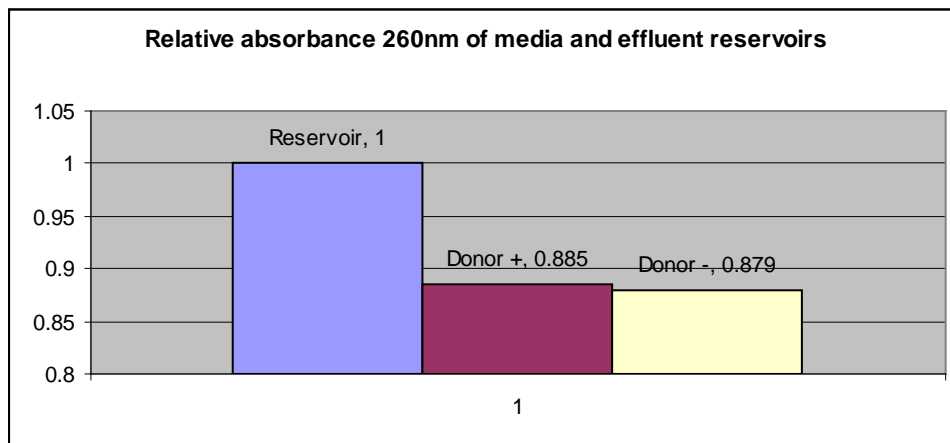
Figure 12. Cumulative Benzyl Alcohol Utilization in Flow Cells. Graph depicting relative benzyl alcohol levels in the effluent receptacles of: media that did not pass through a flow cell (Reservoir), effluent from a flow cell that was inoculated with the donor strain, and effluent from a flow cell that was not inoculated with the donor strain

3.7 Benzyl Alcohol Breakdown in Batch Cultures

Batch cultures supplied with 0.1%TSB containing 500ppm benzyl alcohol were prepared in order compare benzyl alcohol breakdown by our groundwater-derived inoculum and our donor strains in a different system (batch vs. continuous flow). While 4 of the 5 batch cultures showed a decrease in the relative concentration of benzyl alcohol, the un-inoculated control also decreased at a similar rate (fig. 13).
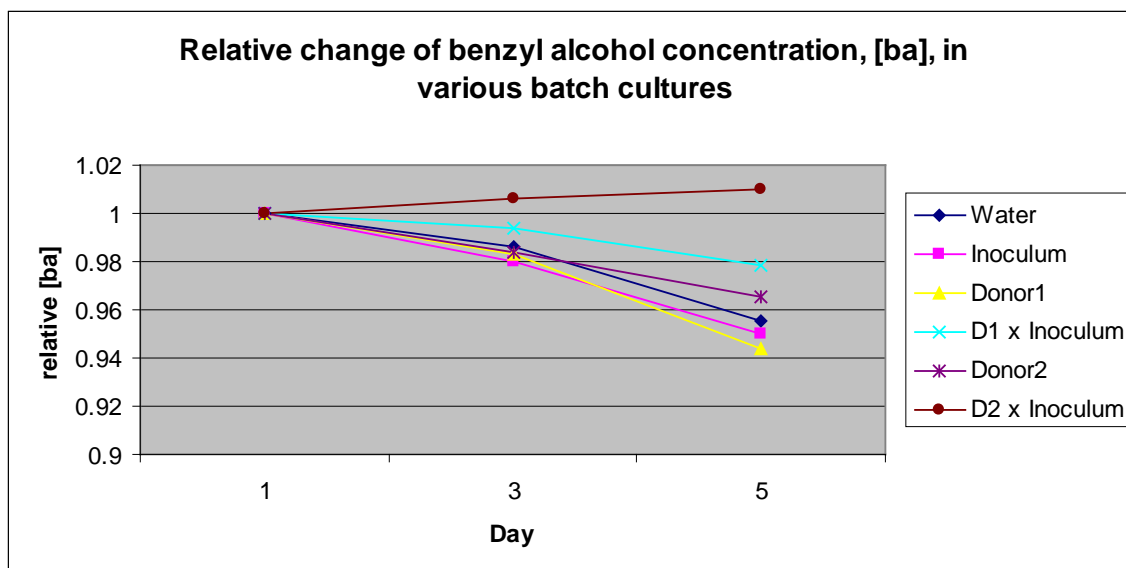
Figure 13. Benzyl Alcohol Degradation in Batch Cultures. Graph depicting the relative change in benzyl alcohol concentrations in batch cultures. Cultures consisted of 25ml of 0.1%TSB, 62.5µl benzyl alcohol and the indicated strain and consortia.

3.8 Effect of Toluene on Biofilm Composition

DGGE profiles of biofilms in flow cells with, and without toluene, were produced in order to determine the effects of toluene on the groundwater-derived microbial consortium (donor 1 and donor 2 were not involved in this experiment). The two profiles (fig. 14) appear to have 4 common bands (as indicated by arrows on the left), while each has unique bands (as indicated by arrows on the right). The intensity of bands in the DGGE profile of the flow cell in which toluene was not introduced (lane a) is greater than that of the flow cell in which toluene was introduced.
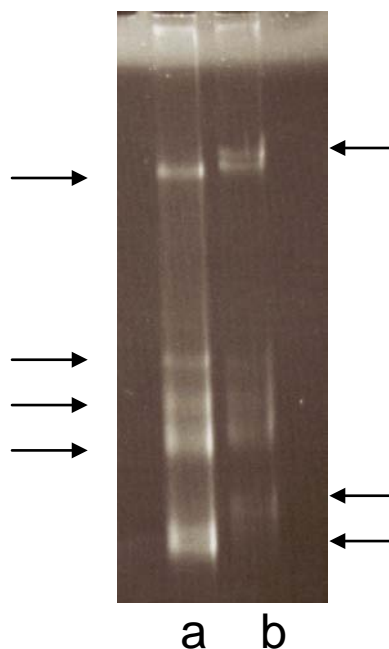
Figure 14. DGGE profiles of Flow Cell Biofilms. Image of DGGE profile of biofilms isolated from flow cells. a) DGGE profile of flow cell in which toluene was not introduced and b) DGGE profile of flow cell in which toluene was introduced. Arrows on the left of the image indicate common bands. Arrows on the right of the image indicate unique bands.

3.9 Plate Matings

Two-way plate matings between our groundwater-derived microbial consortium, *Pseudomonas putida ATCC15070* , *Pseudomonas aeruginosa* PAO1 as recipients and our 2 donor strains were performed in order to assess transfer of the TOL plasmid in a static, nutrient rich environment. Image a in figure 15 through 18 shows that the groundwater-derived microbial consortium does not exhibit any red or green fluorescence. Image b in figures 15 and 16 show that the *Pseudomonas putida* recipient strain does not exhibit red or green fluorescence. Image b in figures 17 and 18 shows that the *Pseudomonas aeruginosa* recipient strain does not exhibit red or green fluorescence. Image c in figures 15 through 18 show that the 2 donor strains exhibit the expected fluorescence (green

fluorescence by donor 1, no fluorescence by donor 2) As expected, both donors were able to transfer the TOL plasmid to *Pseudomonas putida* (Fig. 15 and 16, image e). Unexpectedly, plate matings between our 2 donor strains and *Pseudomonas aeruginosa* did not produce any transconjugants (Fig. 17 and 18 image e). Plate matings between the 2 donor strains and our groundwater-derived microbial consortium produced inconsistent results. Transconjugants have been detected at very low rates in plate matings between both donor strains and our groundwater-derived microbial consortium (Donor1 – image not shown, Donor 2 – Fig 18, d). These results were not consistently repeatable as seen in figures 15-17 (image d).

Figure 15. Donor 1 Mating with *P. putida* and Inoculum. 1,000x Confocal microscopy images of plate matings: a) groundwater-derived microbial consortia b) *P. putida* c) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed, d) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed allowed to mate with groundwater-derived microbial consortia and e) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed allowed to mate with *P. putida*. Clockwise from top left of individual images: 488nm wavelength excitation (GFP); 543nm wavelength excitation (RFP); transmission mode (all biomass); overlay.
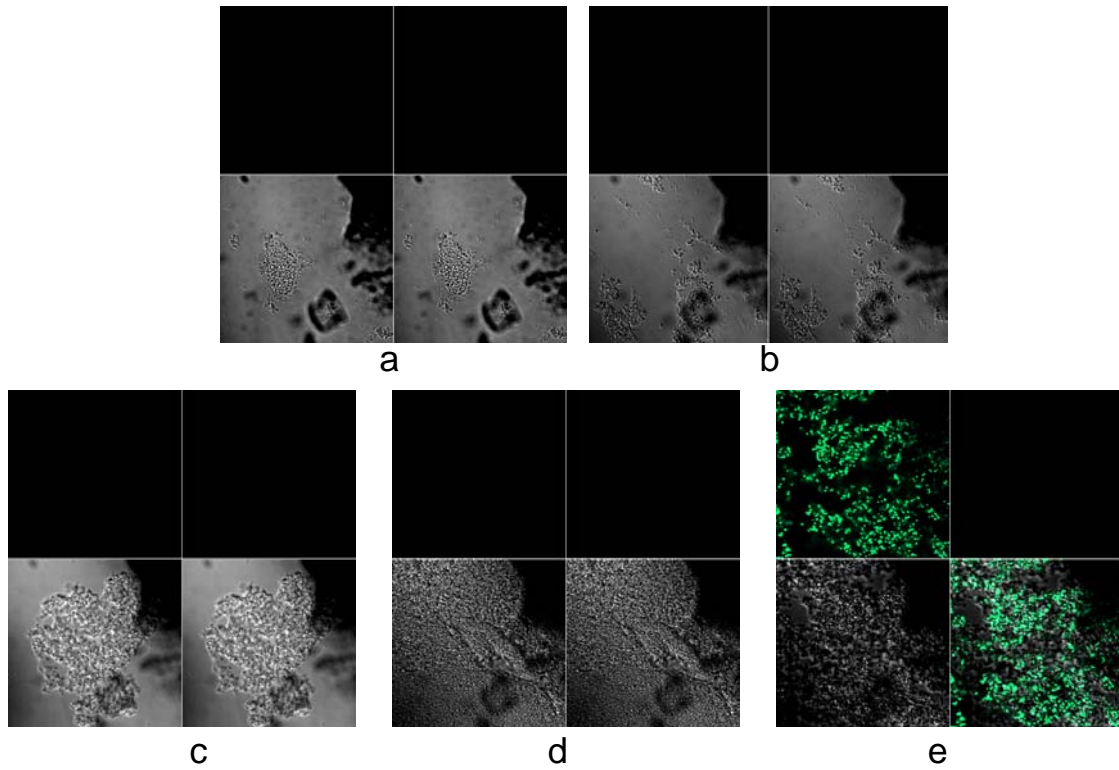
Figure 16. Donor 2 Mating with *P. putida* and Inoculum 1,000x Confocal microscopy images of plate matings: a) groundwater-derived microbial consortia b) *P. putida* c) Donor strain *P. putida* BBC443-pWW0::gfp, d) Donor strain *P. putida* BBC443-pWW0::gfp allowed to mate with groundwater-derived microbial consortia and e) Donor strain *P. putida* BBC443-pWW0::gfp allowed to mate with *P. putida*. Clockwise from top left of individual images: 488nm wavelength excitation (GFP); 543nm wavelength excitation (RFP); transmission mode (all biomass); overlay.
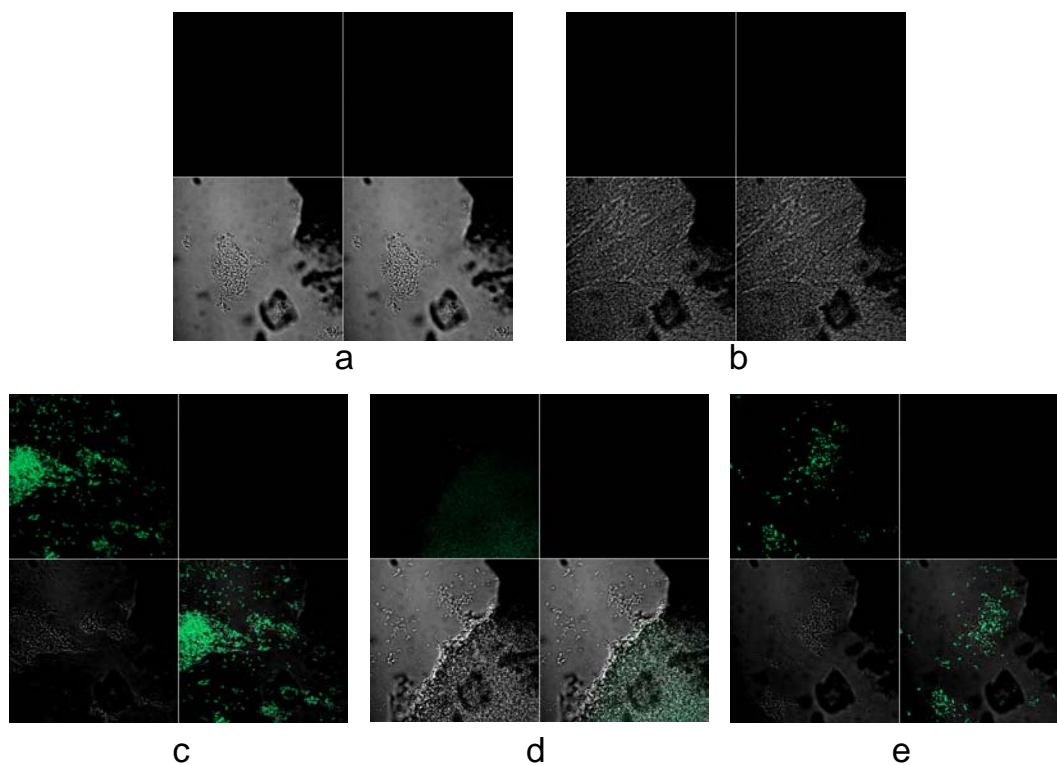
Figure 17. **Donor 1 Mating with *P. aeruginosa* and Inoculum.** 1,000x Confocal microscopy image of a) groundwater-derived microbial consortia b) *P. aeruginosa* c) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed, d) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed allowed to mate with groundwater-derived microbial consortia and e) Donor strain *P. putida* SM1443::gfp2x-pWW0::dsRed allowed to mate with *P. aeruginosa*. Clockwise from top left of individual images: 488nm wavelength excitation (GFP); 543nm wavelength excitation (RFP); transmission mode (all biomass); overlay.
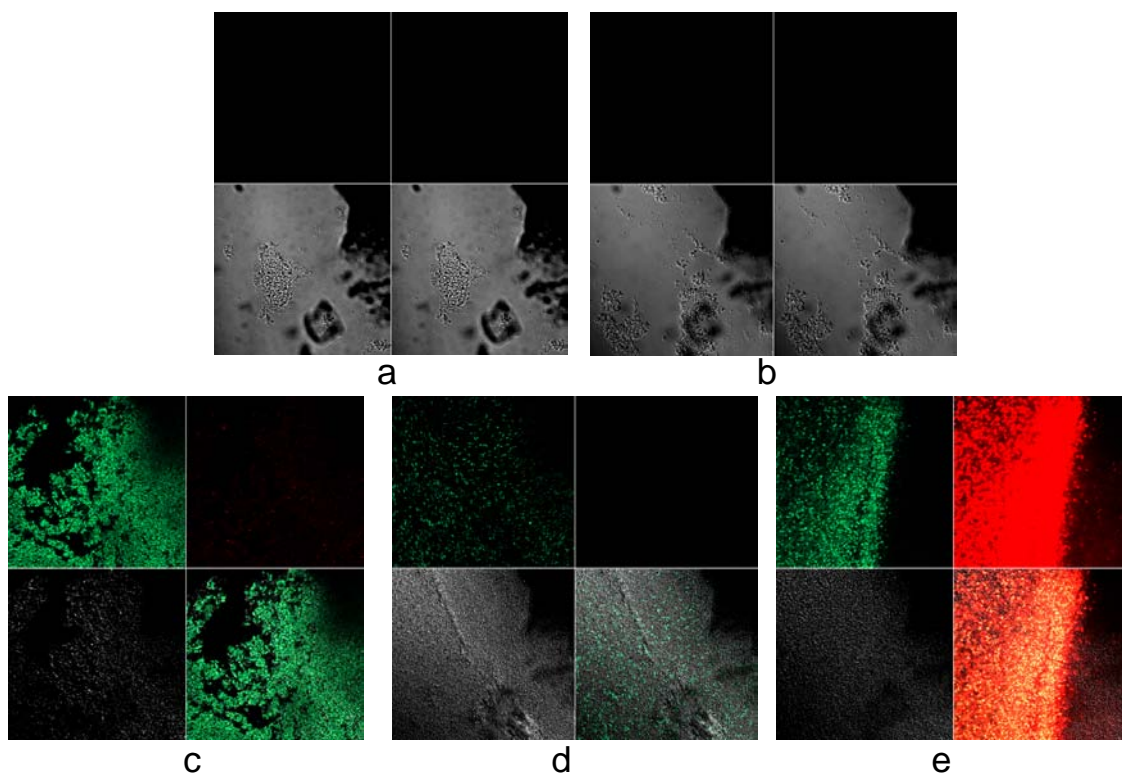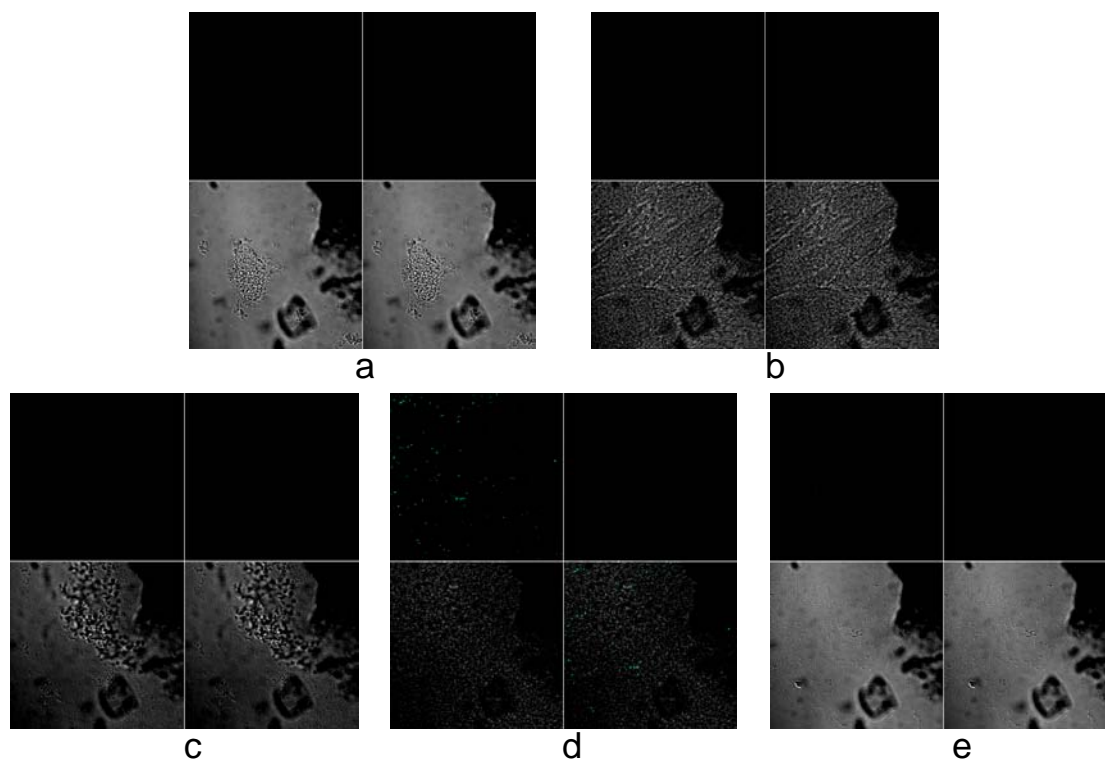
Figure 18. Donor 2 Mating with *P .aeruginosa* and Inoculum 1,000x Confocal microscopy images of plate mating of: a) groundwater-derived microbial consortia b) *P. aeruginosa* c) Donor strain *P. putida* BBC443-pWW0::gfp, d) Donor strain *P. putida* BBC443-pWW0::gfp allowed to mate with groundwater-derived microbial consortia and e) Donor strain *P. putida* BBC443-pWW0::gfp allowed to mate with *P. aeruginosa*. Clockwise from top left of individual images: 488nm wavelength excitation (GFP); 543nm wavelength excitation (RFP); transmission mode (all biomass); overlay.

3.10 Effects of Nutrient Concentration on Plasmid Transfer in Flow Cells

Flow cell experiments with 0.1%TSB, 1.0%TSB and 10%TSB medium were performed in order to assess the impact of nutrient concentration on plasmid transfer in our flow system. Effluent was filtered and microscopically examined for the presence of donor and transconjugant cells (Fig.19). Regardless of the concentration of nutrients, no donor or transconjugant cells were detected. A significant amount of biomass was observed on all filters (Fig 19, image a, b, c)

a   b   c
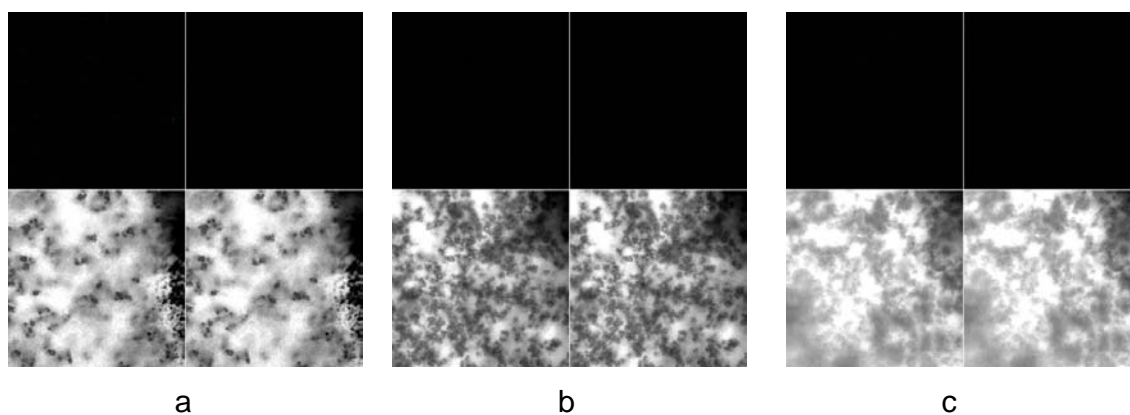
Figure 19 Images of Filtered Effluent from Flow Cells with Varying Nutrient Concentrations
1,000x confocal microscopy images of filtered effluent from: a) flow-cell which was supplied with .1%
TSB media, b) flow-cell which was supplied with 1% TSB media and c) flow-cell which was supplied
with 10% TSB media. All flow cells were inoculated with the groundwater-derived microbial
consortium and donor 1. All flow cells were also exposed to toluene. Individual images can be read
from the top left in a clockwise fashion as follows: 488nm wavelength excitation (GFP), 543nm
wavelength excitation (RFP), transmission mode (all biomass), overlay.

4.0 DISCUSSION

**Inoculum generation**

A modified dual-dilution procedure (Caldwell and Lawrence, 1986) was used to produce a source of inoculum. The rationale behind this approach was that it would be best to have a consistent, surface attached consortium of groundwater microorganisms that could be stored over a long period of time. A comparison of the DGGE profiles of attached and unattached microbes can be seen in figure 8. The profile of microbes which were isolated from the bead stock was almost completely different in comparison to effluent collected from the inoculum generation and the profile generated from groundwater enriched with TSB. The profiles of the enriched effluent and enriched groundwater were similar to each other; with 2 bands that were clearly common, as well as 3 other bands which are faintly visible that also appear to be in common (fig 8). This result indicated that there is a distinct difference in profiles between surface attached and planktonic cells in groundwater. All samples were prepared in the same manner (enriched with 1.0%TSB), thus the only difference between the samples was the presence or absence of an attachment surface. Our inoculum is best described as a groundwater-derived microbial consortium. Although we do not appear to have the full spectrum of microbial diversity found in the groundwater sample, theoretically this should not affect the hypothesis or expected outcome, as horizontal-gene transfer is hypothesized to occur predominantly in biofilms rather than planktonic cells.

Several trials of inoculum generation were performed and were stored for future use. Figure 8 shows the DGGE profiles of the microbial consortia generated from three separate trials of inoculum generation. Although one of the resulting DGGE profiles (fig

8, lane b) showed an extra dominant band, the three profiles shared 5 bands of similar intensity. This was a good indication that the groundwater-derived microbial consortium used as inoculum used for our flow cell experiments was consistent and reproducible over time.

Biofilm Architecture and Community Composition

Average biofilm biomass, as determined by COMSTAT image analysis software, was highly variable in biofilms regardless of whether or not toluene was introduced into the flow cell. Biofilms that were not exposed to toluene were significantly thicker and had more biomass than biofilms in flow cells that were exposed to toluene (fig. 11). The highly variable topography of the rock wafer is likely responsible for the highly variable thickness of biofilms.

COMSTAT software is a useful tool for determining trends in biofilm structure rather than determining absolute values. This evaluation stems from the subjective nature of the conversion between a grayscale and binary image (thresholding), which is necessary for processing of confocal microscopy images.

Plate mating experiments with both donor strains and the groundwater-derived microbial consortium showed a very low level of plasmid transfer (Fig 18, image d). Flow cell experiments in which the flow cells were inoculated with both the groundwater-derived microbial consortium and donor 1 did not yield any evidence of plasmid transfer (table 3, fig 19). Given these results in addition to the high architectural variability of biofilms in our system, it is impossible to determine from these results what effect, if any, biofilm architecture may play in the transfer of the TOL plasmid in our

model system. If the observation that biofilms have a heterogeneous architecture in our model system holds true in natural systems, biofilm architecture is likely not a significant factor in plasmid transfer in rock fracture apertures.

DGGE profiles of biofilm samples taken from flow cells that were, and were not, exposed to toluene were not significantly different (fig 14). There was however an extra band observed in the DGGE profile of microbes from the tol+ flow cell. There was; however, a lower level of staining intensity in the profile of biofilms collected from toluene inoculated flow cells. Interpreting this qualitative result with caution, it is possible that the presence of toluene in the flow cell inhibited the extent growth of microbes. A decreased staining intensity in the profile of microbes from the toluene treated flow cell (fig. 14, lane b) is consistent with observations that toluene treated flow cells have biofilms which are less thick and have less biomass than biofilms in flow cells which are not treated with toluene (fig. 11).

Medium-Scale Rock Fracture Biofilm

The results of the DGGE of biofilm samples from the model rock fracture of our collaborators gave insight into community dynamics of a NAPL-contaminated aquifer (Fig. 7). The microbial consortium used to inoculate the model system (Fig. 7, lane 1) was that which we produced in our lab using the modified dual-dilution method (section 2.2). There are several noteworthy observations that can be made from this result. First, the difference in microbial diversity between the inoculum and all other lanes appears to be significant. As described in section 3.1three of the 5 main bands which can be seen in the DGGE profile of the inoculum can be seen throughout the other profiles. There are at

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least 12 bands which appear in the DGGE profiles of the biofilms sampled from the plate fracture do not appear in the profile of the inoculum. The *Bacillus* genus that was determined by sequence analysis to be the dominant species in the inoculum may be selected for by the TSB over the short growth period, or in other words, the *Bacillus* genus from the beads may have grown most rapidly. The biofilms which were isolated from the model rock fracture were supplied with artificial groundwater at a constant flow and toluene over an approximate period of six months. Thus, our inability to detect what would appear to be a wide diversity of microorganisms in the inoculum is a likely result of some members of the inoculum consortium having an abundance which is below the detection limits of the PCR/DGGE protocol that was used (as seen in fig. 7).

Biofilm samples were collected in relation to the direction of flow as well as the proximity (or lack thereof) to the highest toluene concentrations. While these "boundaries" were not arbitrary, they did not correspond to any particular coordinate in the model system. The banding patterns of these regions appear to indicate that most regions contain a similar microbial profile, although different regions show different intensities for bands in the same position on the DGGE gel. The distal fence and distal trail which were immediately downstream of the toluene "globule" showed a relative increase in the intensity of two particular bands, while the intensity of all other bands from those two particular samples appeared to be significantly reduced, although still visible. The intensity of these bands in regions further downstream recovered their intensity (to approximately the level that was seen upstream of the toluene "globule"). The two bands which were more intense in the regions of high toluene concentration were still visible in other regions; the intensity was however, reduced. Both bands that

increased in intensity from samples taken in close proximity to the toluene globule were determined by sequence analysis to be *Rhodococcus erythropolis,* a documented degrader of toluene (Fahy, 2008). Overall this result indicates that overall diversity in different regions of the model rock fracture stayed relatively consistent; however, the relative amounts of each particular species can vary relative to its proximity to (in this case) a contaminant. As mentioned, the inoculum used in this experiment was that which we generated using a modified dual-dilution method, strongly indicating that we had toluene degraders present in the groundwater-derived microbial consortium.

Visually, after approximately 3 months of flow, it was possible to see a "fence" of microorganism forming around the toluene "globule" in the centre of the fracture flow system. It was observed that this microbial fence became denser over time. Presumably, the presence of toluene selected for certain members of the microbial consortium and over time these selected members multiplied significantly in the area surrounding the toluene globule. Chemotaxis of toluene-tolerant species toward the toluene/water interface may have played a role in the initial stages of the formation of this "fence;" although there was no experimental data that would directly support that claim. Time-lapse microscopic analysis of the flow system would be necessary in order to determine if chemotaxis took place and this was not possible due to the size of the model fracture (it cannot fit on a microscope stage).

In relation to plasmid transfer, two questions arise from these results. First, does the introduction of the TOL plasmid result in a different profile of microorganisms that inhabit the "fence?" A different profile upon introduction of a TOL-plasmid- bearing donor strain would indicate that the plasmid has been transferred and consequently

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conferred toluene resistance to a new group of microorganisms. Second, can the introduction of the TOL plasmid increase the rate at which the microbial "fence" can form? A faster rate of "fence" formation would indicate that TOL plasmid introduction positively influenced the rate at which microbes can move toward the toluene and begin degradation.

Mating Experiments

Mating experiments were performed to ensure that donor strain 1 had the ability to transfer the plasmid to our groundwater-derived microbial consortium. The mating experiments allowed us to put the donor strain and groundwater-derived microbes in close proximity in a static fashion with significantly more nutrients (as compared to the flow cell system, which is spatially dynamic and has limited nutrients). Matings were also performed with donor 2 (referred to as donor 2, as described in section 2.1) to control for any transfer deficiencies in our original donor strain.

Using confocal microscopic imaging to detect fluorescence, it was observed that both donor strains were able to transfer the TOL plasmid to *Pseudomonas putida* (figs 15, 16). This was the expected result, as *P. putida* has been documented in literature as a capable recipient of the TOL plasmid (Ramos-Gonzalez et al, 1994). The observation that both donor strains were able to horizontally transfer the TOL plasmid indicated that our original donor strain was not deficient in its plasmid-transfer abilities.

Mating experiments which used *Pseudomonas aeruginosa* PAO1 as a recipient strain did not give positive results for either donor strain (figs 17, 18). This result was not expected as *P. aeruginosa* has previously been shown to be a potential recipient of the TOL plasmid (Smets et al, 1993; Pinedo and Smets, 1995). Repetition of this mating

with other *P. aeurginosa* strains would be necessary to draw any conclusions regarding this result.

Mating experiments that were performed with the two donor strains and the groundwater-derived microbial consortium gave unclear results. In preliminary mating experiments (image not shown) our original donor strain appeared to transfer the TOL plasmid at very low, but detectable levels to our recipient community. Repetition of this mating experiment with the original donor strain did not show any red fluorescence indicative of plasmid transfer (Fig 16, image d). Matings that were performed with the second donor strain; however, showed the low, yet detectable level of gene transfer that was characteristic of the initial mating experiments (Fig 18, image d). As mentioned, both donor strains are capable of transferring the TOL plasmid to *P. putida,* indicating that the cellular machinery necessary for plasmid transfer is in tact in both donor strains. At first glance it may seem reasonable to conclude that the groundwater-derived recipient communities used in the mating experiments and the flow cell experiments did not have a consistent composition; however, the same overnight culture was used to prepare matings between both donor strains and the recipient community in these experiments. It is thus unlikely that the composition of the recipient communities on a plate-to-plate basis was significantly altered. Only a small portion of the mating patches were used to prepare slides for confocal microscopy analysis, so it is possible that transconjugants that were present in the mating between our original donor strain and the groundwater-derived recipient community were not sampled. Overall this result indicated that the groundwater-derived recipient community did not contain a significant amount of potential recipients for the TOL plasmid.

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Flow Cell Experiments

The genetic contents of the TOL plasmid and the donor strain used in our experiments 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 *xyl* genes encoded in the TOL plasmid which enables breakdown of BTEX compounds.

When LB-agar plates with kanamycin and gentamycin that had effluent samples spread on them were placed on a UV-transilluminator no green fluorescence was observed from any of the colonies that were isolated. This is in contrast to plates of the donor strain which do exhibit green fluorescence on a UV-transilluminator. Green fluorescence can be observed from donor strain 1 regardless of whether it is plated on LB-agar or TSA (image not shown). After ten days in the refrigerator to allow for expression and folding of RFP the colonies isolated from effluent samples exhibited a flat and colorless morphology, which was in contrast to the initial state of the colonies, and more importantly in contrast to the appearance of the donor strain on control plates. These two observations indicate that the colonies that were isolated from the effluent on LB-agar plates with kanamycin and gentamycin were likely not the donor strain.

There was no green fluorescence detected from colonies isolated from effluent samples. This result in theory should indicate that all colonies isolated on the TSA-gentamycin plates were transconjugants. The plates spent 10 days in the refrigerator to allow time for expression and folding of the RFP (Bathe and Hausner, 2009). At that time the colonies displayed morphology similar to that seen on the LB-agar plates with

kanamycin and gentamycin. Colonies which were initially round and white were flat and colorless. This is in contrast to colonies which were isolated from effluent samples using TSA plates without antibiotics. Subsequent attempts to isolate DNA from these colonies for the purpose of PCR were not successful. It can be speculated that cell death and subsequent DNA degradation was the cause for the inability do isolate DNA from these colonies. These results likely indicate that the colonies isolated from the effluent were neither donor cells nor transconjugants, as they did not display the characteristic antibiotic resistance or fluorescence of donor cells or transconjugants. At best, these colonies arose from cells which received the TOL plasmid but were not able to express the encoded gentamycin resistance gene or the *rfp* gene, although there is no additional evidence to support that possibility. Again, the cell density of the effluent samples may have allowed for initial growth of colonies, but over the two week incubation period, the eventual exposure to the antibiotics resulted in cell death. This was confirmed when no colony growth was observed after re-streaking colonies onto fresh TSA plates.

In addition to plating effluent samples, the toluene concentrations of effluent samples were also determined using gas chromatography. The change in effluent toluene concentration could not be directly correlated with toluene metabolism by microorganisms. The toluene was introduced directly into the flow cell in an amount such that it would not dissolve into the aqueous medium, thus it was not possible to determine an exact mass balance between the inflow and outflow. Also, attributing any differences in toluene effluent concentrations solely to degradation of toluene by TOL plasmid recipients was not possible as the donor strain also had the ability to degrade toluene. Furthermore, the total amount of effluent sampled over the period of the

experiment amounted to approximately 1.4 ml per flow cell, while the total amount of effluent produced was approximately 2L per flow cell. The toluene concentrations were measured to determine a general trend in toluene effluent concentrations that could be used in conjunction with the results of the effluent plating and microscopy in order to assess the success or lack thereof of the transfer of the TOL plasmid from our donor strain to the recipient community. Measurements of toluene concentration in effluent samples showed no discernable trend in degradation over the experimental period regardless of the inoculation of the donor strain (table 3 and 4).

Benzyl alcohol degradation genes are also encoded by the TOL plasmid and thus any cell that harbours the TOL plasmid should have the capability to degrade benzyl alcohol (Jindrova et al., 2002). This allowed us to effectively perform a balance between the concentration of benzyl alcohol in the medium and the concentration of benzyl alcohol in the effluent reservoir. The medium reservoir measurement was not taken directly from the reservoir itself; medium was pumped out of the reservoir into a collection flask. This allowed us to minimize any volatilization that may have occurred through the silicon tubing as well as control for any sorption to the silicon tubing. Results indicated that there was no difference in the breakdown of benzyl alcohol between the flow cells which had received the donor strain and those that had not. While there was no difference, it may be noteworthy that in both flow cells there was approximately a 12% decrease in the relative concentration of benzyl alcohol concentration as compared to the control measurement. This may indicate that BTEX degraders which were in the recipient community had been selected for by the toluene exposure and had similar absolute numbers in both of the flow cells. The result does not

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give any indication that gene transfer occurred in the flow cell which the donor strain was introduced.

After the three weeks of flow, the flow cells were examined with a confocal microscope using the 488nm and 543nm wavelength lasers (images not shown). Any donor cells or transconjugants should have in theory been fluorescent, and thus detectable by this method. While no green or red fluorescence was detected, it should be noted that there was a lack of a control flow cell which contained freshly inoculated donor cells or cells which express *rfp*. These images are also less meaningful in the sense that the microscope cannot be used in transmission mode due to the opaque nature of the rock wafer in the flow-cell and therefore biofilms could not be visualized to confirm that the microscope was properly focused. While there was no indication of TOL plasmid recipients in our experiments, controls such as those mentioned abovewould allow for a stronger conclusion

None of the aforementioned observations or measurements indicates that transfer of the TOL plasmid from our donor strain to members of the recipient community had taken place. These results give rise to the obvious question: why are we unable to observe any indications of horizontal gene-transfer of the TOL plasmid in our model system when numerous accounts of TOL plasmid transfer exist in the literature, particularly in wastewater systems (Normander et al., 1998; Molbek et al., 2003; Jussila et al., 2007; Nancharaiah et al., 2007; Mohan et al., 2009; Pei and Gunsch, 2008; Jain et al., 1987; Smets et al, 2003; Pinedo and Smets, 2004; Christensen, 1998)? Two possible factors came to mind and were explored in subsequent experiments: the ability of

members of the recipient community to take up and express genes on the TOL plasmid, and the low nutrient levels in the aqueous medium (0.1%TSB).

Donor Survival and Integration

The donor strain was not isolated from any of our flow cell experiments. This strongly indicates that the donor strain was not able to survive in the flow cell environment. In previous gene transfer work performed in model wastewater reactors the donor strain did not survive the duration of the experimental period. For example after 8 days of operation Bathe et al., (2004, a) were not able to detect their donor strain. In that model system plasmid transfer was observed, so the long-term survival of the donor strain is not necessary for successful transfer of the plasmid to the recipient consortium. However, we were not able to detectthe donor strain after periods as short as 24 hours. Given that we were not able to detect the donor after a relatively short period, it is unlikely that there was any significant degree of integration of the donor strain into the biofilms in the flow cell.

Wild type *Pseudomonas putida* KT2440 has been shown to disperse from biofilms in a flow through systems when dissolved oxygen levels were gradually decreased from 250μM to <3μM (Hanson et al. 2007). In a similar fashion it has been observed that *Pseudomonas putida* Ous82 biofilms disperse under conditions of low carbon (Gjermansen et al, 2005). Although we did not measure dissolved oxygen or carbon levels in our flow system, upon disassembly of the flow through system a significant build-up of biomass could be seen dwelling in the tee junction (Fig. 5). Microbes present in this build-up likely consumed a large portion of the available

dissolved oxygen as well as available nutrients. Oxygen-impermeable Teflon tubing was used after the tee junction; therefore atmospheric oxygen could not reach the medium past this point.

Low levels of dissolved oxygen do not necessarily provide an explanation for the inability to isolate the *Pseudomonas putida* donor strain in the short-term. Low dissolved oxygen levels may provide an explanation as to why we did not see evidence of donor integration into flow cell biofilms, and by extension, plasmid transfer. In addition, plasmid transfer is an active process, thus microbes which require oxygen for aerobic respiration may be hindered in their ability to transfer a plasmid (Palmen et al, 1994). It does not; however, explain the lack of donor cells in effluent samples.

Dissolved oxygen concentrations in groundwater tend to be heterogeneous, although they are generally low. For example, two particular studies found that maximum dissolved oxygen at Sturgeon Falls, Ontario and Long Point, Ontario measured 5mg/L and 3mg/L, respectively (3µM and 1.8µM, respectively)(Robertson et al, 1992; Robertson and Schiff, 1994; Malard and Hervant, 1999). Given that groundwater has a characteristically low level of dissolved oxygen, further studies in groundwater plasmid transfer may call for a donor strain which does not disperse from biofilms under conditions of low dissolved oxygen and nutrients.

Varied Nutrient flow cells

To address the question of nutrient sufficiency for plasmid transfer flow cell experiments were repeated using 0.1%TSB, 1.0%TSB and 10%TSB in three different flow cells. In this experiment cumulative effluent samples were filtered as opposed to taking intermittent effluent samples in order to examine a larger number of cells in the

hopes of finding transconjugants. Observation of filtered effluent from these three flow cells did not yield any transconjugant or donor cells, although biomass was observed (Fig 19). In our experience cells in liquid medium need a less concentrated dilution of a given medium as compared to cells which are grown on plates. This, in our experience, was applicable to batch cultures as well, for example, recipient liquid cultures were turbid after overnight shaking in 1.0% TSB, while 1.0%TSA plates were not sufficient to support growth. We were able to culture the donor strain, and observe its green fluorescence with a UV-transilluminator, using 10% strength TSA plates. Thus it would then stand to reason that 10%TSB in solution should provide sufficient nutrients for both donor cells and recipient cells in the flow cells. In spite of this, we were still not able to detect a donor or recipient cells in the filtered effluent from the flow cell with 10%TSB, much less the flow cells which were supplied with 1.0%TSB and 0.1%TSB. This result indicates that the concentration of nutrients is not the limiting factor to plasmid transfer in our flow system.

5. Conclusions

A model of groundwater flow through a rock-fracture aquifer was assembled and optimized. The flow cell that was assembled was amenable to non-invasive microscopic investigation. It was found that biofilms in our model rock fracture aquifer had a highly variable architecture in terms of average biomas and thickness. Biofilms which were exposed to toluene had significantly less average biomass and were significantly less thick. Subsequent flow-cell experiments did not support the initial hypothesis. Evidence to suggest that transfer of the TOL plasmid took place in our model system between our *Pseudomonas putida* donor strain and a groundwater-derived microbial consortium was not collected. Plate matings between the two donor strains and *P. putida* indicated that the donor strains were able to transfer the TOL plasmid to *P. putida*. However, low and inconsistent levels of transfer of the TOL plasmid in plate mating experiments were observed between the donor strains and the groundwater-derived microbial consortium, indicating that an additional parameter other than donor-recipient compatibility played a role in our inability to observe plasmid transfer in our model system. The availability of nutrients did not appear to be the limiting factor in plasmid transfer, as 10- and 100-fold increases in nutrient concentration had no effect on the ability to detect recipients of the TOL plasmid.

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6. Recommendations

1)   In early experiments and trials, TSB was the medium of choice as it was used by colleagues with success, specifically with the growth of groundwater-derived microbes (Dr. Mahendran Busavaraj, personal communication). While the donor strain is capable of growing on TSA plates, we observed "healthier" growth of the donor strain on LB-agar plates (colonies appear larger and grow faster on LB, individual cells are larger in confocal microscope images). After failing to isolate donor cells from flow cells which were supplied with TSB at concentrations as high as 10% it may be worthwhile repeating the flow cell experiments with low percentage LB medium. In addition to this, it is recommended that LB is also used in the generation of the groundwater-derived inoculum. In this situation all strains used throughout the experiment would be enriched and maintained on the same medium. If this approach is successful, flow cell experiments could be repeated using a defined mineral medium or artificial groundwater (as opposed to LB or TSB) to supply microbes with nutrients in order to closer simulate a groundwater environment. The timeframe of experiments may need to be considered if minimal medium or artificial groundwater is used. Past attempts by colleagues to enrich groundwater microorganisms with artificial groundwater were not particularly successful in that there was not any significant growth after several weeks of enrichment in a flow cell (Mahendran Busavaraj, personal communication). The three week experimental period may not be suitable when using minimal medium or artificial groundwater as a nutrient source.   Our collaborators at the University of Toronto used artificial groundwater in their medium-scale flow cell with success, but the experiment took place over approximately half of a year, rather than a period of several weeks.

2)   In our flow cell experiments with groundwater-derived microbial consortium as potential recipients we did not detect any transconjugants. In our plate mating experiments we were able to detect transconjugants; however, in low numbers and inconsistently. In our plate matings using *P. putida* as a recipient we observed evidence of plasmid transfer. If *P. putida* were added to the groundwater-derived inoculum and transconjugants were detected in flow cell experiments, this would strongly indicate that the lack of success in our model system is a function of the recipient community used. Conversely, if the addition of *P. putida* to the recipient consortium makes no difference in the results obtained, this strongly indicates that the parameters of the flow system negatively influence the horizontal transfer of the TOL plasmid. To carry out the suggested experiment it would first be necessary to determine if *P. putida* has the ability to survive under experimental conditions.

3)   Given that we were not able to detect plasmid transfer with a donor strain that was previously used with success in model wastewater systems by other investigators, it would be worthwhile to attempt to repeat their experiments in our own lab (Nancharaiah et al., 2007; Pei and Gunsch., 2009; Venkata Mohan et al., 2009). If positive results were obtained in a model wastewater system with the donor strain that was used in this, that would provide a strong control for the ability of the donor strain used in this study to transfer the TOL plasmid. Although we did observe plasmid transfer on the static LB-agar plates, the model wastewater system would provide a dynamic, nutrient-rich and recipient-rich system where our donor strain could be tested. In addition, it would be

worthwhile to examine the ability of the donor strain to establish a biofilm and fluorescence in a flow cell in order to establish its viability in the model system.

4)   Rock fracture apertures, and groundwater in general, are regarded as harsh environments for microbes (Coombs, 2009). While *Pseudomonas putida* has been used successfully as a donor strain in model wastewater systems, it may not be suited for groundwater applications. An ideal donor strain for groundwater studies would be a strain that is able to survive and exist in a biofilm in low nutrient and low dissolved oxygen conditions.

5)   Plasmid transfer could be tested in a medium-scale model fracture, such as that of our collaborators at the University of Toronto (as described in section 2.3). Relevant questions in regard to the applicability of plasmid transfer as a technique for bioremediation enhancement could be answered. By addition of a donor strain is the profile of microorganisms which inhabit the regions of high toluene concentration altered? Does the formation of the microorganism "fence" occur at a different rate? Does the toluene "globule" disappear faster when a donor strain is added?   And ultimately, is there direct evidence of the presence of the plasmid and/or donor strain at the end of the experimental period? Addressing these questions by performing plasmid-transfer experiments in a medium-scale flow cell could aid in determining the potential effect that the introduction of a degradative plasmid could have on microbial segregation and degradation of a NAPL.

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