Stability and expression of a plasmid-borne TCE degradative pathway in suspended and biofilm cultures
by Robert Raymond Sharp III

A thesis submitted in partial fulfillment of the requirments for the degree of Doctor of Philosophy in Civil Engineering
Montana State University

Abstract:
Trichloroethylene (TCE) is a United States Environmental Protection Agency Priority Pollutant. TCE is mutagenic and a suspected carcinogen. TCE is recalcitrant in the environment and has been found to be ubiquitous in soils and ground waters, globally. Use of TCE degradative pathways moderated by enzymes, the expression of which redides on recombinant plasmids, to biologically degrade TCE is a promising new technology to eliminate TCE.

One such TCE degradative pathway is the toluene ortho-monooxygenase (TOM) pathway which is borne on the recombinant plasmid pTOM. Research presnted here examines the ability of pTOM pathway to degrade TCE in two different host cells, the original plasmid host Pseudomonas cepacia PR1 and a transconjugant Pseudomonas cepacia 17616. The goal of this research was to determine those mechanisms that cause the loss of the TCE degrading phenotype, associated with pTOM, in both suspended and biofilm cultures.

These mechanisms include plasmid instability within the host cell, cell toxicity caused by TCE exposure, and cell injury caused by TCE exposure. In addition this research developed a protocol to determine the severity of these phenotypic losses in any pTOM host organism. With the use of several novel reactor systems, analytical methods, and protocols were developed to determine the suitability of P. cepacia 17616 as a host for plasmid pTOM.

Batch studies indicate that P. cepacia 17616 is able to incorporate pTOM and degrade TCE at rates equal to those of other pTOM hosts. Plasmid stability experimental results showed significant segregational plasmid loss in P. cepacia 17616-pTOM in non-selective suspended and biofilm cultures, resulting in almost complete loss of plasmid-bearing cells within 30 days of operation. Continuous culture plasmid loss studies showed that the probability for plasmid loss per cell generation was a function of growth rate and ranged from 0.025/generation at a growth rate of 0.065 hr-1 to 0.035 at a growth rate of 0.17 hr-1. Results indicate there was no significant difference in the probability of plasmid loss between suspended and biofilm culture, suggesting that biofilm growth does not affect plasmid stability the P. cepacia 17616-pTOM system.

Use of a novel TCE vapor exposure reactor system showed the TCE exposure can cause serious injury and toxicity to P. cepacia 17616 and can result in catastrophic loss of the TCE degrading phenotype in P. cepacia 17616-pTOM cultures. In addition, the severity of both cell injury and toxicity was found to be a function of TCE exposure time and TCE concentration.

Research here indicates that plasmid loss, cell injury, and cell toxicity are significant mechanisms that can result in the detrimental loss of pTOM pathway expression and these mechanisms should be considered in any plasmid mediated catabolism of a toxic waste.

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APPROVAL

of a thesis submitted by

Robert Raymond Sharp III

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the college of Graduate Studies.

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ABSTRACT

Trichloroethylene (TCE) is a United States Environmental Protection Agency Priority Pollutant. TCE is mutagenic and a suspected carcinogen. TCE is recalcitrant in the environment and has been found to be ubiquitous in soils and ground waters, globally. Use of TCE degradative pathways moderated by enzymes, the expression of which redides on recombinant plasmids, to biologically degrade TCE is a promising new technology to eliminate TCE.

One such TCE degradative pathway is the toluene ortho-monooxygenase (TOM) pathway which is borne on the recombinant plasmid pTOM. Research presnted here examines the ability of pTOM pathway to degrade TCE in two different host cells, the original plasmid host *Pseudomonas cepacia* PR1 and a transconjugant *Pseudomonas cepacia* 17616. The goal of this research was to determine those mechanisms that cause the loss of the TCE degrading phenotype, associated with pTOM, in both suspended and biofilm cultures. These mechanisms include plasmid instability within the host cell, cell toxicity caused by TCE exposure, and cell injury caused by TCE exposure. In addition this research developed a protocol to determine the severity of these phenotypic losses in any pTOM host organism. With the use of several novel reactor systems, analytical methods, and protocols were developed to determine the suitability of *P. cepacia* 17616 as a host for plasmid pTOM.

Batch studies indicate that *P. cepacia* 17616 is able to incorporate pTOM and degrade TCE at rates equal to those of other pTOM hosts. Plasmid stability experimental results showed significant segregational plasmid loss in *P. cepacia* 17616-pTOM in non-selective suspended and biofilm cultures, resulting in almost complete loss of plasmid-bearing cells within 30 days of operation. Continuous culture plasmid loss studies showed that the probability for plasmid loss per cell generation was a function of growth rate and ranged from 0.025/generation at a growth rate of 0.065 hr^{-1} to 0.035 at a growth rate of 0.17 hr^{-1}. Results indicate there was no significant difference in the probability of plasmid loss between suspended and biofilm culture, suggesting that biofilm growth does not affect plasmid stability the *P. cepacia* 17616-pTOM system.

Use of a novel TCE vapor exposure reactor system showed the TCE exposure can cause serious injury and toxicity to *P. cepacia* 17616 and can result in catastrophic loss of the TCE degrading phenotype in *P. cepacia* 17616-pTOM cultures. In addition, the severity of both cell injury and toxicity was found to be a function of TCE exposure time and TCE concentration.

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Chapter 1

Goals And Objectives

1.1 Goal

The goal of this work is to determine the fate and activity of $pTOM_{31c}$ in the transconjugant host *Pseudomonas cepacia* 17616 under non-selective and "$pTOM_{31c}$-selective" growth conditions in both suspended and biofilm cultures in order to ascertain the host's applicability to a TCE biofilm reactor.

1.2 Thesis Statements

Hypothetically, the stability and activity of plasmid pTOM31c in the transconjugant host, *Pseudomonas cepacia* 17616, may be different in suspended versus biofilm culture. This research project will determine the stability and activity of plasmid $pTOM_{31c}$ in the transconjugant host *Pseudomonas cepacia* 17616, in both suspended and biofilm cultures.

Further, this project hypothesizes that TCE toxicity, plasmid stability, and injury caused by TCE exposure will result in the significant loss of the TCE degrading phenotype of *B. cepacia* 17616-$pTOM_{31c}$.

1.3 Motivations

This research was initiated to determine the applicability of $pTOM_{31c}$ to a TCE biofilm reactor, and determine the causes for the apparent loss of the TCE degrading phenotype in both suspended and biofilm cultures. Further, this research was concieved in order to develop an understanding of the extent and severity of phenotypic losses in caused by plasmid/host interactions and the exposure to TCE. Finally, there exists a need to will establish a protocol for determining toxicity, plasmid loss, and injury in $pTOM_{31c}$ bearing microorganisms.

1.4 Objectives

■ Determine growth characteristics for plasmid bearing and plasmid free *P. cepacia* 17616 cultures under non-selective (acetate) and selective (phenol) growth conditions.

■ Determine the plasmid loss coefficient for $pTOM_{31c}$ in *P. cepacia* 17616 under non-selective (acetate growth) conditions in suspended and biofilm cultures.

■ Determine the activity of plasmid $pTOM_{31c}$ in suspended and biofilm *P. cepacia* 17616 cultures when grown under selective and non-selective conditions.

■ Determine the cometabolic TCE degradation kinetics of *P. cepacia* 17616-pTOM$_{31c}$ cultures and the effect of TCE exposure on the metabolic activity and health of 17616-pTOM$_{31c}$.

Chapter 2

Introduction

Contamination of soil and groundwater by organic pollutants has been the focus of much research in recent years. Some of the most notable contaminants in America's aquifers are volatile organics, a large number of which are chlorinated aliphatic compounds. Trichloroethylene (TCE - $ClCH{=}CCl_2$) is a chlorinated ethene and a member of the chlorinated aliphatic family. Often used as a degreaser, TCE has remained a popular solvent and is used by a great many industries (Westerick et al 1990). TCE is a U.S. Environmental Protection Agency priority pollutant and is one of America's most ubiquitous and recalcitrant groundwater contaminants (Love and Eilers 1982).

2.1 General TCE Biodegradation

Many methods have been developed for remediating TCE laden soil and groundwater. The most common technologies are (1) volatilization into the atmosphere, (2) pump and treat methods using physicochemical processes, and (3) incineration. Many of these technologies involve transfer of the pollutant from one phase or state to another and do not involve actual destruction of the contaminant. This fact, along with the legalities, inefficiency, and cost of these non-biological TCE treatment technologies, have prompted intense research into the biodegradation of TCE (Travis and Doty, 90).

2.2 Anaerobic TCE Biodegradation

Although TCE is quite recalcitrant in nature, a number of anaerobic bacteria have been found capable of its degradation. These anaerobes include: the methanotroph *Methylosinus trichosporium* OB3b (Oldenhuis et al 89), a number of propane-utilizing *Mycobacterium* (Wackett et al 89), two isoprene-utilizing *Alcaligenes denitrificans* (Ewers et al 90), the autolithotroph *Nitrosomonas europaea* (Arciero et al 89) and two consortia of methanogenic bacteria (Fogel et al 86, Vogel and McCarty 85). Each of these anaerobic TCE degraders utilizes different oxygenases to carry out reductive dechlorination of TCE, which often results in the production of the highly recalcitrant and mutagenic vinyl chloride (Vogel and McCarty 85). Also, anaerobic biodegradation of TCE can be as much as ten fold slower and less efficient than aerobic biodegradation processes (Bouwer and McCarty 83, Bouwer et al 81, Freedman and Gossett 89, Kleopfer et al 85). For these reasons, researchers have been searching for aerobic cultures capable of degrading TCE.

2.3 Aerobic TCE Biodegradation

In recent years, a number of bacterial consortia and isolates capable of aerobic degradation of TCE have been discovered. Some of the more widely known aerobic TCE degraders include: a heterotrophic consortia (Fliermans et al 88), *Pseudomonas putida* F1 (Nelson et al 87) (Wackett and Gibson 88), *Pseudomonas mendencina* (Winter et al 89), *Pseudomonas pickettii* PK01 (Kaphammer et al 90), *Pseudomonas florescens* (Vandenbergh and Kunka 88), *Pseudomonas putida B5* (Nelson et al 88), and *Burkholderia cepacia* G4 (Nelson et al 86). The majority of these aerobic TCE degraders utilize a toluene oxidizing pathway to degrade TCE. Most notable of these toluene oxidizing TCE degraders are *P. putida* F1 and *B. cepacia* G4. The biochemistry and TCE degradative pathway of *B. putida* F1 have been well characterized (Finette et al 84; Gibson et al 82; Subramanian et al 85; Wackett and Householder 89; Wackett and Gibson 88). In addition, the TCE degradative capabilities of G4, and the novel pathway by which it is performed, have received significant research attention (Nelson et al 86,87,88; Folsom and Chapman 91; Folsom et al 90; Shields and Reagin 92; Shields et al 95).

Rates of aerobic TCE biodegradation obtained from batch reactor studies have been reported for a number of microorganisms. Monod TCE degradation kinetics for the inducible *Burkholderia cepacia* G4 include a maximum specific activity, Vmax, of 8 nMol TCE/min-mg protein and a half saturation constant, Km, of 4 uM TCE (Folsom et al 90). Initial TCE degradation rates for *M.*

trichosporium OB3b, *B. putida* F1, and *B. cepacia* G4 PR1-pTOM$_{23c}$ were found to be 35 nMol TCE/min-mg protein at 80 uMTCE, 1.8 nMol TCE/min-mg protein at 80 uM TCE, and 1 nMol TCE/min-mg protein at 20 uM TCE, respectively (Tsien et al 89, Wackett and Gibson et al 88, Shields and Reagin 92). These published TCE degradation rates differ greatly because many of the microorganisms utilize different toluene oxygenase systems. In addition, TCE degradation rates can vary greatly depending on the growth rate of the cells and the carbon/energy source used. It is difficult to determine which microorganisms are most efficient in degrading TCE because all of the physiological and environmental variables present during the respective studies.

2.4 *Burkholderia cepacia G4*

Resent research has been performed on the environmental isolate *Burkholderia cepacia* G4 to determine the mechanism by which it mineralizes TCE. Results show that *B. cepacia* G4 degrades TCE via a plasmid-borne cometabolic pathway that must be induced by one of the following: phenol, toluene, m-cresol, o-cresol, or catechol (Folsom et al 90). In addition, a novel pathway involving the toluene degradation pathway has been discovered (Nelson et al 87, Shields et al 95). This pathway involves the sequential hydroxylation of toluene at the ortho- and meta- positions to form 3-methylcatachol (Shields et al 89). Further, research has shown the involvement of a plasmid borne sequence that encodes for toluene ortho-monooxygenase (*tom*) in the TCE mineralization process (Shields et al 91).

Like most aerobic TCE degraders, *B. cepacia* G4 utilizes a cometabolic pathway to degrade TCE. "Cometabolism" is defined as the metabolic transformation of a substance (TCE) while a second substance serves as the primary energy and/or carbon source (Brock and Madigan 88). This definition of cometabolism is reserved for aerobic microorganisms only, and must involve oxygenase enzymes and the depletion of oxygen during the cometabolic process (Dalton and Stirling 82). Along with a co-metabolite, *B. cepacia* G4 and most other aerobic TCE degraders also require an inducer to activate the genes responsible for cometabolic TCE degradation. Induction of these cometabolic systems is typically by the primary carbon source/co-substrate, which is usually an aromatic compound that can induce the toluene oxidizing pathway required for TCE degradation.

A number of published papers have demonstrated the efficiency of *B. cepacia* G4 in degrading TCE under various conditions using the appropriate inducers (Folsom and Chapman 91, Folsom et al 90, Ensley and Kurisko 94, Landa et al 94). Results from these studies show that both toluene and phenol can be used effectively as inducers/co-substrates for TCE degradation. However, both toluene and phenol demonstrate competitive inhibition with TCE which can adversely affect the TCE degradation process (Folsom et al 90). The competitive inhibition demonstrated with these inducers/co-substrates, along with the need for induction of the cometabolic TCE pathway by an aromatic compound, has led researchers to derive a TCE constitutive strain of *B. cepacia* G4.

2.4.1 Plasmids and TCE degradation

Plasmids are circular, double stranded, extrachromosomal DNA sequences that are not essential for cell growth and have no extracellular form. Plasmids can be transmitted from one cell to another via replication, transconjugation, transduction, or transformation. Not only are plasmids self-replicating within the cell, but many cells carry multiple copies of their plasmids.

Most aerobic TCE degrading microorganisms carry the genes responsible for TCE degradation on a plasmid. In addition, these plasmids usually code for expression of the cometabolic toluene oxidizing pathway proteins that must be induced by an aromatic compound, the primary metabolite. One such plasmid is the large plasmid found in previously mentioned environmental isolate *B. cepacia* G4.

2.4.2 Plasmid pTOM$_{31c}$

The plasmid pTOM$_{31c}$ (Figures 1 and 2) is a transmissible plasmid that includes gene sequences which encode for all of the proteins needed for constitutive mineralization of TCE via a cometabolic pathway; the newly defined TOM (toluene ortho-monooxygenase) pathway. These proteins include toluene ortho-monoxygenase (TomA) and catechol 2,3 dioxygenase (C23O or TomB) (Shields et al 95). Plasmid $pTOM_{31c}$ also includes a Tn5 transposon insertion which is a transposable DNA sequence containing a kanamycin resistance marker (Figure 3). Two plasmid maps showing the locations of tomA, tomB (C230), and tomR (tom regulatory region), along with the areas of Tn5 homology, are shown in Figures 1 and 2.

The TOM pathway's involvement in the aerobic mineralization of TCE has been definitively characterized in the literature (Shields and Reagin 92, Shields et al 95). Plasmid $pTOM_{31c}$ could have a number of important applications in the field of bioremediation. For example, this plasmid could be transferred into indigenous bacterial populations to increase the TCE degradative activity of *in-situ* bioremediation, or it could be applied to highly effective reactor-based remediation technologies like TCE degrading biofilm reactors.

Figure 1 - Restriction Map of Plasmid $pTOM_{31c}$.

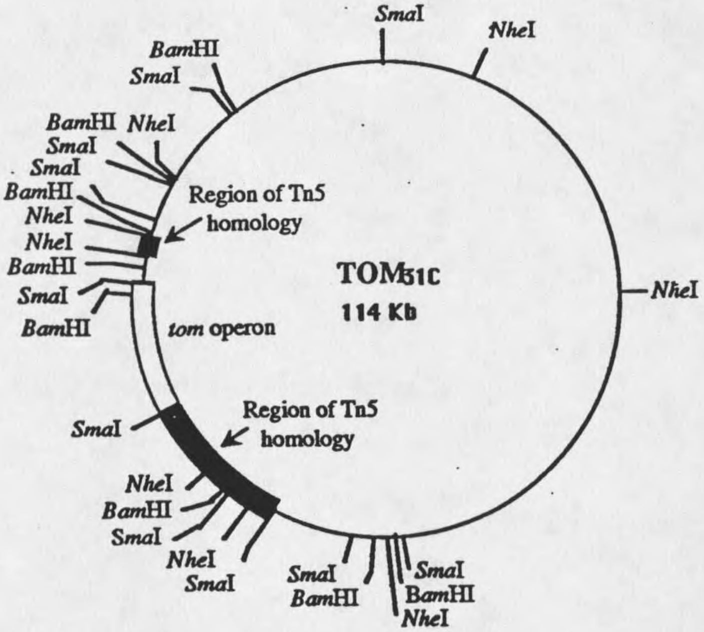


Figure 2 - Restriction Map of Plasmid pMS64, Derived from $pTOM_{31c}$. Showing position of TomA, C230, and areas of Tn5 homology (An EcoR1 digest of PR1-$pTOM_{31c}$ plasmid prep cloned into pGEM4Z, Reagin 92).

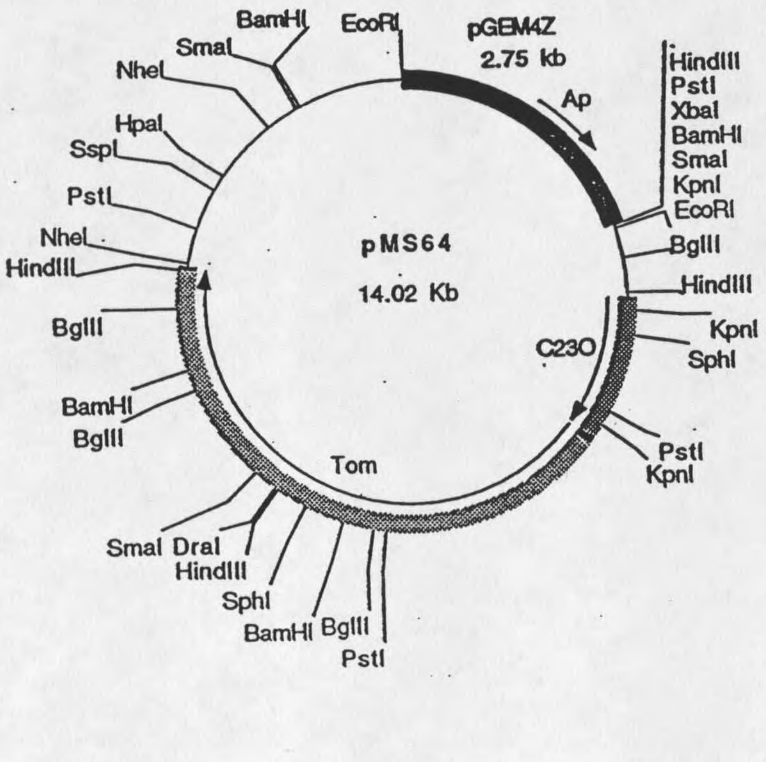
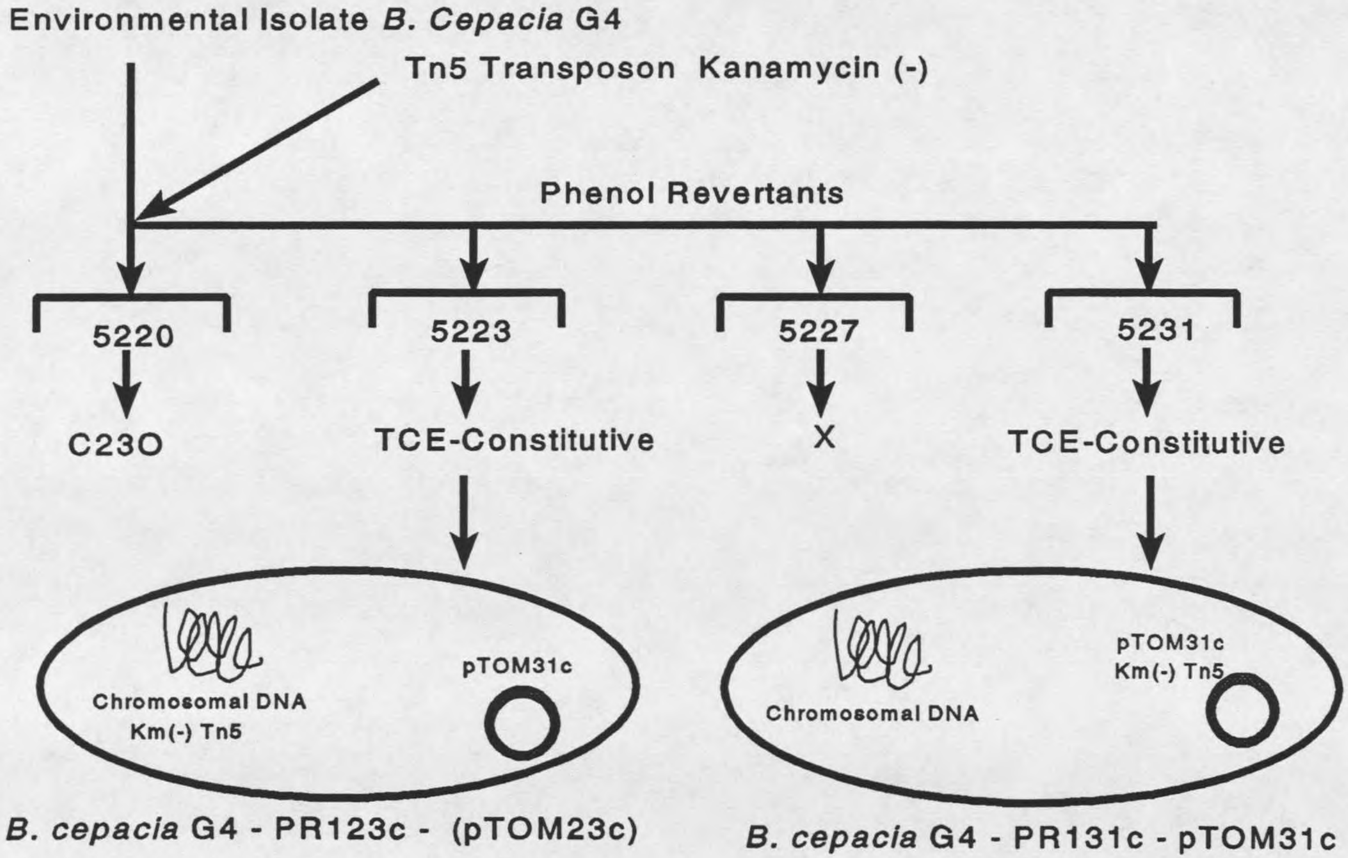


Figure 3 - Genesis of the Constitutive TCE Degrader *Burkholderia cepacia* PR1-pTOM31c Via Tn5 Transposon Mutagenesis



2.4.3 *B. cepacia* PR1-pTOM$_{31c}$

B. cepacia PR1-pTOM$_{31c}$ is a transposon mutant of the environmental isolate *B. cepacia* G4. Like *B. cepacia* G4, *B. cepacia* PR1-pTOM$_{31c}$ is capable of aerobic TCE degradation via the cometabolic TOM pathway. *B. cepacia* PR1-pTOM$_{31c}$ was obtained by the insertion of a Tn5 transposon into the large plasmid of *B. cepacia* G4, then re-inserting plasmid pTOM$_{31c}$ into *B. cepacia* G4. The resulting phenol revertants are capable of aerobic TCE degradation via a constitutive cometabolic pathway (refer to Figure 3) (Shields et al 91) without an aromatic inducer and without the possible detrimental effects of competitive inhibition. In addition, the simple Tn5 insertion resulted in a G4 mutant carrying the plasmid pTOM$_{31c}$, which includes all of the constitutive TCE degradative pathway and the kanamycin resistance associated with the Tn5 transposon (Shields et al 95).

2.5 Biofilms and Biofilm Reactors:

Most bacteria found in nature are associated with a surface (Marshall 76) Surface-associated bacteria are physiologically different than suspended bacteria and can be found embedded in an extracellular polysaccaridic (EPS) matrix. Interactions between attached cells and their environment are strongly affected by mass transfer effects, hydrodynamics, and other transport phenomena (Characklis and Marshall 90). Surface-associated bacteria have been designated as biofilms (Costerton et al 87). Biofilms include all surface associated microorganisms in any environment, and are considered a unique and relatively new area of microbiological/bioengineering research.

Biofilms can be both detrimental and beneficial. Some examples of detrimental biofilms include biofilms that mediate corrosion of pipes in water and oil distribution systems, biofilms that cause fouling of cooling towers and heat exchangers resulting in inefficiency and increased pressure drop, and biofilms that infect the human body and biomedical prosthetics which are difficult and/or impossible to kill. However, biofilms can also be beneficial. Biofilms that remove pollutants from water and wastewater have been used for hundreds of years. More recently, biofilms have been used in bioremediation technologies to degrade and attenuate xenobiotics found in industrial effluents, groundwaters, and soils. A list of various bioremediation applications of biofilms are shown in Table 1.

2.5.1 Biofilm reactors for biodegradation of organics including TCE

A number of papers have reported the use of anaerobic and aerobic biofilms to degrade organic compounds. The literature is full of instances, both bench scale and field scale, where biofilm reactors of one type or another have been used to treat simple aromatic pollutants such as phenol, cresol, and the less volatile gasoline components benzene, toluene, ethyl-benzene, and xylene (BTEX). There are only a few examples of biofilm reactors being used successfully to treat TCE laden effluents. Speital and Leonard (92) used a

Table 1 - Applications of Biofilms In Reactor Based and *In-situ* Bioremediation Processes

Bioremediation Need	Biofilm Process Application
1 - *In-situ* removal of xenobiotics from soil and groundwater (*in-situ*)	Bacteria associated with soil surfaces are considered a biofilm and may be capable of degrading various xenobiotics (BTEX, TCE, PAHs, etc.). Various techniques can be employed to enhance the degradative capabilities of the indigenous bacteria (addition of oxygen, nutrients, co-substrate).
2 - Reactor based technology for removal of xenobiotics in groundwater.	Pump and treat systems where the removed ground water is treated using engineered biofilm reactors (packed columns, fluidized bed reactors, and rotating disk biofilm reactors).
3 - Removal of metals from industrial waste streams and production effluents.	Reactor based biofilm processes with metal binding bacteria colonized on packing surface.
4 - Removal of organics, nitrate, sulfate, and ammonia from waste waters.	Trickling filters, activated sludge reactors, and fluidized bed reactors used in community and industrial waste water treatment facilities to maintain effluent water quality.
5 - Treatment of volatile organics in vapor waste streams, soil vapor extraction processes, concentrator effluents, and soil venting technologies.	Vapor phase biofilm reactors using engineered biofilms grown on inert packing. Biofilters using surface associated indigenous bacteria present on peat, compost, and other naturally occurring media.
6 - Biobarriers	Introduction of large quantities of bacteria down an injection well to develop a cylindrical biofilm "plug" radiating from the well screen. Used to attenuate polluted groundwater flow.
7 - Bioaugmentation	Introducing selectively enriched microorganisms into the subsurface to enhance biodegradation capabilities of attached microorganisms.

anaerobic biofilm reactor to treat TCE. Fennell et al (93) used a methonotrophic attached film expanded bed reactor to degrade TCE at a maximum rate of 0.8 mg TCE/g VSS-day. There are a few other accounts of anaerobic biofilm reactors used to treat TCE (Strandberg et al 1989). However, most of these processes involve slow growing and low yield anaerobic cultures that do not readily produce a biofilm. In addition, many of the anaerobic TCE biodegradation rates are extremely slow and would be impractical for long term use in reactor based bioremediation applications.

Researchers have been trying to apply aerobic TCE degraders to biofilm reactors with limited success and little literature on aerobic TCE degrading biofilm reactors can be found. A few attempts have been made to utilize biofilm reactors to treat either liquid and vapor TCE wastes using pure cultures of TCE degrading microorganisms. Some of these attempts have had limited success during short term operation. However, when these systems were used for long term treatment of TCE waste, many lost their ability to degrade TCE at a significant rate. This apparent loss of TCE degrading ability was noted by decreasing efficiency, lack of biomass accumulation, and failure to degrade TCE. There are a number of explanations for such failures. Figure 4 enumerates phenomena to which the apparent loss of a plasmid borne TCE degrading phenotype of a particular microbial population can be attributed. These phenomena include toxicity, plasmid instability, and cell injury. In addition, mono- and engineered mixed culture systems operated under field or

Figure 4 - Apparent Losses of Plasmid Borne TCE Degrading Phenotype

PHENOTYPE LOSS MECHANISMS

Toxicity

- Cell Lysis
- TCE Toxicity
- Intermediate Toxicity

Plasmid Instability

- Segregational
- Recombination
- Injury Induced

Injury

- Deactivation
- Inhibition
- Catabolic Repression

non-sterile conditions can suffer from competition and predation by invading microorganisms. Other TCE degrading microorganisms may be washed out of the biofilm system due to both their inability to attach to the reactor packing and their inability to produce a significant biofilm.

The key to developing an efficient, reliable TCE degrading biofilm reactor is finding a suitable host to harbor and express the desired TCE degrading plasmid phenotype. Suitable host characteristics include: ability to produce copious amounts of biofilm, resistance to TCE related injury and toxicity, and ability to retain and express plasmid during long term operation and other favorable plasmid-host interactions.

2.6 Loss of TCE Degrading Phenotype - Toxicity, Plasmid Stability, and Cell Injury.

Loss of a TCE degrading phenotype in TCE degrading biofilm processes has been noted by many researchers. Many times, the performance of TCE degrading biofilms in long term application is not as effective as observed in short term experiments. The lack of efficiency can be attributed to scale-up factors and poor reactor design. However, many times it is a decline in the health and activity of the TCE degrading biofilm cells that is affecting reactor performance. When TCE degrading cells are grown in a biofilm and exposed to TCE for a prolonged period of time, a number of physiological changes can occur that result in the loss of the desired TCE degrading phenotype. Such physiological changes include toxicity, plasmid instability, and injury.

2.6.1 Toxicity

Death or permanent inactivation of a given microbial population can occur when the population is exposed to a toxic substance either in a sufficiently high concentration or for a long enough period of time to fatally affect the function of the cells. Toxicity can involve severe damage to the cell wall and cell membrane which in turn can cause cell lysis and loss of the cell's structure and function. Cell lysis is usually caused by anti-microbial agents, strong solvents, endogenous proteolytic enzymes, strong acids, or highly reactive substrates dissolving the cell wall. Toxicity can also involve permanent damage to a desired phenotype or set of genes within the cell's chromosomal or accessory DNA. This form of toxicity may be caused by the toxin itself or the production of harmful intermediates or by-products. Toxicity of this nature is very common in TCE degrading microorganisms.

Intermediate and product toxicity among TCE degrading methane oxidizing cultures has been noted by Alvarez-Cohen and McCarty(91a and 91b) and Janssen et al (87). This type of toxicity results in decreased TCE degradation along with decreased methane conversion, thus leading one to believe that the whole pathway for methane oxidation (and TCE degradation) has been damaged (Ely et al 95). Other accounts of intermediate toxicity among phenol and methane oxidizing TCE and DCE degraders (Bielefeld et àl 95) and toluene oxidizing biofilm cultures (Arcageli et al 95) have been presented in the literature.

Toxicity is an obvious concern when designing TCE biofilm reactors. Unlike cells in continuous flow stir tank reactors or batch stir tank reactors, biofilm cells are continuously exposed to a relatively steady, low level concentration of TCE. Some environmental and nutrient conditions may decrease intermediate and product toxicity effects; but ultimately, the degree of TCE related toxicity will be a function of the TCE concentration, toxigenicity to the cell, and the pathway utilized to degrade TCE.

2.6.2 Plasmid Instability

Plasmids may place a metabolic burden upon their host cell because of the energy needed for their maintenance and replication. This burden may or may not be significant depending on the plasmid/host relationship and the total amount of accessory DNA the cell is maintaining. Depending on the growth conditions, plasmids may increase the fitness of the cell, even if those growth conditions do not select for the plasmid (Bouma and Lenski 88, Zund and Lebek 80).

The interactions between a given plasmid and its host can not be generalized. Each plasmid-host relationship has unique characteristics and differs from one plasmid type to another in the same cell. Plasmid-host relationships can be strongly influenced by growth and environment conditions.

Microorganisms carrying plasmids are susceptible to plasmid instability which can lead to the loss of a desired plasmid-borne phenotype, or complete loss or inactivation of a desired plasmid-borne genotype. Plasmid instability can occur in two ways: (1) segregational instability and (2) structural instability. Segregational instability is the consequence of random and irregular separation of a plasmid between daughter cells during cell division, and leads to new generations of daughter cells that do not contain the plasmid. The plasmid free daughter cells produced by segregational instability will have lost all of the genotypes/phenotypes carried on that plasmid, including all selection markers, anti-microbial resistant sequences, and any amended DNA sequences such as transposons and other insertion sequences. Structural instability of a plasmid involves the actual change or recombination (deletion, insertion, and rearrangement) of a single gene or several genes in the plasmid. Structural instability can result in a portion of the plasmid DNA being incorporated into the chromosomal DNA. In addition, structural instability may involve the loss of a certain plasmid borne phenotype, but not the loss of other phenotypes carried by the plasmid. For instance, structural instability of plasmid $pTOM_{31c}$ may result in the loss of the TCE degrading phenotype, but not the loss of the plasmid borne kanamycin resistance. Since most TCE degrading microorganisms carry their

TCE degradative ability on plasmids, plasmid stability will be a major factor affecting the performance of TCE bioreactors.

Plasmid stability in suspended culture

Due to increased environmental concerns and the promise of genetic manipulation in biotechnology related industries, much research on the stability of plasmids in suspended cell cultures has been performed (Sherrat 82, Ensley 86, Ollis 82, Noack et al 82). Such research has focused on determining the factors that govern plasmid stability and expression in well controlled suspended pure culture systems (Grandi et al 81, Kumar et al 91, and Seo and Bailey 85). In addition, a number of kinetic models have been developed to quantify and predict plasmid loss in suspended culture (Summers 91).

Researchers have found that plasmid maintenance can reduce the overall growth rate of cells in continuous culture (Uhlin and Nordstrom1978, Peretti and Bailey 87). While Chao et al (83), and Bouma and Lenski (88) note instances where plasmid maintenance did not result in a growth rate disadvantage relative to the plasmid bearing cells. Results indicate that plasmid maintenance can actually enhanced cell health even under non-selective conditions.

Researchers have measured significant segregational plasmid loss under both non-selective (Grandi et al 81, Kadam et al 87) and highly selective (Peretti et al 89, Wood and Peretti 91, Roth et al 80) continuous growth conditions. Noack et al (82) have shown structural instability of plasmid pBR325 in *E. coli* cultures where certain antibiotic resistances are lost at a slower rate than other plasmid borne antibiotic resistances. Other researchers have found that plasmid loss can be decreased or eliminated by using either a selective growth substrates or antibiotics that are selective for the plasmid (Tiedji et al 89).

Dyhuizen and Hartl (83) review the effects of continuous culture growth on plasmid stability and expression and suggest that continuous culture may either enhance or deter plasmid stability depending on the plasmid/host system and the growth factors involved. Dwilvedi et al (82) found plasmid stability was increased in continuous *E. coli* cultures, while stability significantly decreased when the cultures were grown in batch. Additionally, certain methods for increasing plasmid stability require continuous culture dynamics (Primrose et al 84, Roth and Noack 82).

It is obvious that the plasmid-host relationship is unique for any given plasmid-host system; however, a number of environmental and physiological factors have been found to influence plasmid stability that may have some general implications. A list of these factors are presented in Table 2. Kumar et al (91) present a review of strategies for improving plasmid stability. Many of these strategies include cellular/molecular techniques used either to control plasmid partitioning during cell division (Austin 81, Summers and Sherrat 84) or to kill plasmid free cells after segregation (Lauffenberger 87, Gerdes 88, and Rosteck and Hershberger 83). Other plasmid stabilizing strategies include bioprocess control strategies to separate plasmid free cells from the culture or to

Table 2 - Factors Affecting Plasmid Stability and Retention.

Environmental and Physiological Factors	References
Growth Rate - Increases plasmid loss with increased growth rate.	Stewart and Carlson 86 Taxis Du Poet 87 Seo and Bailey 86
Plasmid copy number - decreased plasmid loss rate with increased plasmid copy number. Plasmid copy numbers can range from 1 to 700.	Jones et al 80 Sayadi et al 89 Uhlin and Nordstrom 79
Carbon to nitrogen ratios - Increased nitrogen growth conditions can increase plasmid stability.	Huang et al 94 Sayaldi et al 89
Selection - Selection of plasmid using a selective carbon source or antibiotic resistance markers can increase plasmid retention in a given population.	Lauffenberger 87 Tiedje et al 89 Wood et al 90
Nutrient Limitations - Nitrogen, phosphorous, potassium, magnesium, and carbon limitations may either increase or decrease plasmid stability.	Godwin and Slater 79 Jones and Melling 84 Jones et al 80 Noack et al 82
Immobilization and Attachment - plasmid bearing populations that are immobilized or in a biofilm culture may display either increased or decreased plasmid stability.	Huang et al 93 Kumar and Schugerl 90 Inloes et al 83 Dykhuizen and Hartl 83
Exposure to injurious or toxic substances - injury and toxicity may lead to increased plasmid loss.	Ridgway 94

inhibit the growth of plasmid free cells (Stephens and Lyberatos 92, Siegel and Ryu 85, and Henry et al 90).

Plasmid stability in biofilms

When compared to suspended cultures, biofilm and immobilized cells may experience increased plasmid stability for two reasons: 1) the proximity of immobilized and biofilm cells to one another may improve intra-cellular transfer mechanisms and 2) the mass transfer limitations in biofilm and immobilized cell systems. Because immobilized and biofilm cells exist in close proximity to one another, the likelihood of plasmid transfer by conjugation could be increased. Thus the net rate of plasmid loss may be decreased when the number of plasmid transfers from plasmid bearing cells to plasmid free cells is included. Due to mass transfer limitations, there is the tendency for growth rate gradients to develop within immobilized and biofilm cultures systems. Since plasmid loss has been shown to be a function of growth rate, the cells experiencing a slower growth rate due to nutrient and carbon source limitations may also experience decreased plasmid loss.

Inloes et al (82) have reported increased plasmid stability in *E. coli* cultures immobilized in a non-selective hollow fiber system. Taxis du Poet et al (86) saw increased stability of plasmid pTG201 in k-carrageenan encapsulated *E. coli* cultures. These results are attributed to mass transfer effects resulting in slower growth rates, the possibility of increased plasmid transfer, and the reduction of structural instabilities. Similar results using different plasmid/host systems and different immobilization techniques have been reported by Nasri et al (87), Sayadi et al (89), and Kumar and Schrugel (90).

In biofilm cultures, a number of researchers have found significant plasmid transfer that may result in a net decrease in plasmid loss (Levin et al 79, Stewart and Carson 86, Saye et al 87). In contrast to these reports, Huang et al (93) found decreased stability of plasmid pMJR1750 in *E. coli* DH5α when grown in a biofilm. This decrease was attributed to copy number differences between suspended and biofilm cultures and the energy needed for production of the biofilm extracellular matrix polymers that might compete for plasmid maintenance/replication energy. As with suspended plasmid bearing cultures, biofilm plasmid bearing cultures have their own unique plasmid/host characteristics. In order to utilize certain recombinant plasmids in industrial microbial systems, specific plasmid/host interactions must be well understood irrespective of whether the system involves suspended or attached cells.

2.6.3 Cell injury caused by exposure to toxic or injurious substances

The word <u>injury</u> is generally used to explain the loss of a given phenotype under certain environmental conditions. Injury differs from toxicity in that injury implies that the lost phenotype can be regained under non-selective general growth conditions. The concept of injury in microorganisms arose in the water treatment industry where coliforms and other indicator microorganisms were found to be "inactivated" but not killed by traditional chlorine disinfection (Camper and McFeters 79, LeChevaliier and McFeters 85, Waters et al 89). The key element to injury that makes it such an intriguing phenomena is that the cells can recover to their full pre-injury capacity. A number of challenges including: chlorine and other antimicrobial agents, specific metabolites, oxidative conditions, DNA damaging agents, and hydrocarbon exposure have been shown to cause microbial injury (Ridgeway et al 94, Arcangeli et al 95).

Injury can be determined in a number of ways, the simplest of which are growth-related methods. Injured cells are non-culturable on selective media, yet they are recoverable on general growth media. By comparing the differential microbial counts on selective and non-selective plates, one can get an idea of the extent of injury experienced in a given microbial population (McFeters et al 86).

Injury, however, is a poorly understood phenomena and to date there are no mathematical models explaining its affect on engineered microbial systems. Nevertheless, along with toxicity and plasmid instability, injury may be an important element governing the performance of a TCE biofilm reactor.

2.7 Plasmid Transfer

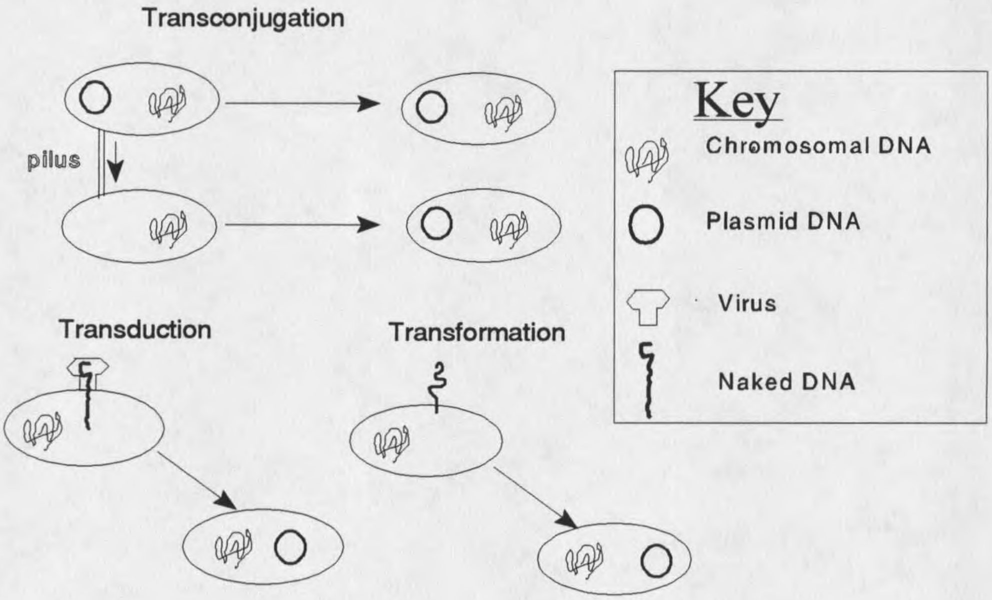
The growth of biotechnology and environmental-related industries has prompted the need to exploit certain recombinant organisms possessing desired phenotypes/genotypes in a variety of microbial systems. Since the persistence and expression of a desired recombinant plasmid is uniquely dependent upon the health, maintenance, and interactions of the plasmid-host system, techniques have been developed to increase the retention and expression of a plasmid in a given microbial system. One method consists of transferring the plasmid to a more suitable host. Plasmid transfer can be done three ways: transconjugation, transduction, and transformation. Figure 5 illustrates the three plasmid transfer methods.

The method most commonly used to transfer a plasmid from one microbial strain to another is transconjugation, often called " mating". In this method, a plasmid-bearing cell conjugates with a plasmid-free cell via a pilus, resulting in a new plasmid bearing cell. The new plasmid-bearing cell is called a transconjugant. Not all bacteria are capable of conjugation and not all plasmids can be transferred by this method. In order for a given plasmid to be transferred by conjugation, the donor cell must contain the proper transfer gene (*tran*) and the plasmid must be mobile (*mob* genes). In addition, the recipient cell must be able to incorporate, maintain, and express the plasmid in order for the transconjugation to be successful.

2.7.1 *Pseudomonas cepacia* 17616-pTOM$_{31c}$ - a transconjugant of *Burkholderia cepacia* PR1-pTOM$_{31c}$.

Over two dozen transconjugants of *Burkholderia cepacia* PR1-pTOM$_{31c}$ have been obtained. One of these transconjugants is *Pseudomonas cepacia* 17616-pTOM$_{31c}$, which is an environmental isolate capable of producing copious amounts of biofilm (Murgel et al 91). The DNA content of *Pseudomonas cepacia* 17616 has been well characterized by Cheng and Lessie (94), and it was chosen as a host for plasmid pTOM$_{31c}$ for both its ability to produce a biofilm and its apparent health and competitiveness in open systems.

Figure 5 - Methods of Plasmid Transfer



Chapter 3

Mathematical Models

3.1 TCE Degradation and Bacterial Growth Kinetic Models

3.1.1 Single substrate saturation kinetics

Michaelis-Menten kinetics are used to describe the saturation kinetics of enzyme-catalyzed reactions. Enzyme saturation kinetics can be used to model cometabolic TCE degradation because TCE degradation is driven by the enzymes produced by the TOM pathway (Folsom et al 90, Landa et al 94, Barrio-Lage et al 87, Shamat and Maier 80). Equation 3.1 is the Michaelis-Menten enzyme saturation kinetic expression, where V is the specific activty of the enzyme system ([TCE]/min. - protein), Vmax is the maximum specific activity of the enzyme system, and Km is the Michaelis constant ([TCE]). The amount of enzyme produced in a cell is a function of growth rate, thus, since Michaelis-Menten kinetics are a function of the total amount of enzyme present, they are only valid in describing whole cell TCE degradation kinetics at a single growth rate of the cells. The Michaelis-Menten kinetic expression can be used to describe the TCE degradation kinetics of cells at a single growth rate.

$$V = \frac{V_{max}\ TCE}{(K_m + TCE)} \qquad (3.1)$$

Monod kinetics are the whole cell growth rate analog of Michaelis-Menten specifc activity kinetics. Monod saturation kinetics have been historically used to model microbial growth that follows the same form as Michaelis-Menten enzyme saturation kinetics (Monod 49). Data for the Monod kinetic model can be obtained from either a series of batch growth studies, each initiated at a different substrate concentration or a series of continuous culture experiments, each run at a different dilution rate. The parameters used in Monod kinetics include the maximum growth or utilization rate (μ_{max}) and the half-saturation constant (Ks). Using methods presented in Appendix A, the specific growth rate for a given system can be determined and plotted against concentration to yield a typical Monod kinetics curve. The curve can be fit using the single substrate Monod kinetics expression presented in Equation 3.2.

$$\mu = \frac{\mu_{max}\ S}{(K_s + S)} \qquad (3.2)$$

3.1.2 Andrews substrate inhibition growth kinetics

The Andrews substrate inhibition growth kinetics model is a modified version of the Monod kinetic model (Andrews 68). Andrews kinetics account for inhibitory effects that may occur at high substrate concentrations. Using the same methodology presented in Appendix A, a series of initial growth rates can be plotted against initial substrate concentration to give a standard growth rate (μ) versus substrate concentration curve. This curve can be fit using Equation 3.3 to determine the following Andrews kinetic parameters: maximum growth rate (μ_{max}), half-saturation constant(K_s), and the Andrews substrate inhibition constant (K_i). From these parameters, the growth rate limit for a given set of Andrews kinetic parameters occurs at a substrate concentration equal to the square root of the product of half-saturation constant and the Andrews inhibition constant $(K_i{*}K_s)^{1/2}$.

$$\mu = \frac{\mu_{max}\, S}{(K_s + S + \frac{S^2}{K_i})} \qquad (3.3)$$

3.2 Continuous, Suspended Cell Culture Plasmid Loss Model

A model for plasmid loss in continuous growth cultures has been developed by Ollis (82). The mass balance on a plasmid-bearing population in a chemostat includes cell growth (μ), probability of segregational plasmid loss (p), and dilution rate (D). Equations 3.4 and 3.5 are sterile feed, continuous culture mass balances for the plasmid- bearing(+) and plasmid-free(-) chemostat populations, respectively.

Plasmid-bearing:

$$\frac{dX^+}{dt} = (1 - p)\mu X^+ - DX^+ \tag{3.4}$$

Plasmid-free:

$$\frac{dX^-}{dt} = \mu X^- + p\mu X^+ - DX^- \tag{3.5}$$

Equations 3.4 and 3.5 can be applied to a system where plasmid maintenance does not result in decreased cell growth rate ($\mu^+ = \mu^- = \mu$). Letting the total biomass be the sum of the plasmid-bearing and plasmid-free populations in the system ($X = X^- + X^+$) and adding Equations 3.4 and 3.5, the total biomass (X) will have a non-zero steady-state value (when dilution rate is equal to the growth rate, $D = \mu$). In a steady-state chemostat system, the value for plasmid-bearing cells (X^+) as a function of time, obtained from the integration of Equation 3.4, results in the following exponential decay expression:

$$X^+(t) = X^+(t)_* \exp(-p_* \mu_* t) \tag{3.6}$$

Equation 3.6 can be linearized to give Equation 3.7.

$$\ln X^{+}(t) = \ln X^{+}(0) - p_{*}\mu_{*}t \qquad (3.7)$$

Using least squares linear regression, Equation 3.7 can be applied to the linear portion of a ln X^+ versus time data set. The slope of the fitted line will give the plasmid loss factor (p) for a continuous culture when the growth rate μ is known (slope = $p_*\mu$ and the growth rate, μ, is equal to dilution rate, D). This model was used to determine the probability of segregational plasmid loss in suspended continuous cultures of 17616-pTOM$_{31c}$.

3.3 Biofilm Culture Plasmid Loss Model

A number of models have been developed to describe plasmid loss in biofilm cultures. One such model, proposed by Huang et al (92), is based on three biofilm processes that dictate a mass balance on a developing culture of biofilm cells in a given system: 1) Adsorption or deposition of cells on the systems surface; 2) attached cell growth; and 3) detachment of biofilm cells.

The Huang model requires a number of assumptions and operating conditions that are needed to simplify the system, including:

1) No cells are introduced in to the system after initial inoculation.

2) The system is operated at a high dilution rate, thus any detached cells will have a short residence time ($\mu_{max} \leq 0.2$ D). This operating condition, combined with condition one, enables the model to disregard the growth and deposition of suspended (detached and influent) cells within the System.

3) Plasmid presence or loss in a population does not affect attachment or detachment rates of these cells.

4) The relative amount of plasmid-bearing cells and plasmid-free cells, detached from the biofilm is identical to the relative amounts of each population in the entire biofilm.

With the above process controls and assumptions, the accumulation of plasmid-bearing cells and plasmid-free cells in a biofilm culture become functions of attached cell growth, plasmid loss, and biofilm detachment rate.

Plasmid-bearing:

$$dB^+/dt = \mu^+B^+ - p_*\mu^+B^+ - Kdet^+B^+ \tag{3.8}$$

Plasmid-free

$$dB^-/dt = \mu^-B^- + p_*\mu^+B^+ - Kdet^-B^- \tag{3.9}$$

Huang et al simplify this model for a system where the plasmid-bearing cells have a different growth rate than the plasmid-free cells. Using a number of substitutions and dimensionless population fractions, Huang et al derive a linear expression for the plasmid loss factor, p. The expression is strictly a function of plasmid-bearing population densities, plasmid-free population densities, and their respective Monod growth kinetic parameters.

For a system where there is no measurable growth rate differential between the plasmid-bearing and plasmid-free populations, the above simplifications cannot be made; and Equations 3.8 and 3.9 must be solved directly using finite difference methods. In order to directly solve either Equation 3.8 or Equation 3.9, expressions for growth rate and detachment rate must be determined.

The growth kinetics of a certain population on a specific substrate can be determined using the appropriate kinetic model. Examples of such models are presented in Equation 3.2, the Monod kinetic model, and in Equation 3.3, the Andrews substrate inhibition model.

A kinetic expression for the detachment rate (K_{det}) of biofilm cells, developed by Jones et al (94), was used to model the detachment of coliform biofilm cells in a rotating annular reactor. This model determines a biofilm detachment rate when the influent and effluent cell concentrations are known and when the dilution rate and the specific area for biofilm formation are constant.

$$K_{det} = 2(1/a \frac{(X_{bi} - X_{bi-1})}{t_i - t_{i-1}} - D/2((X_{bOi} + X_{bOi-1}) - X_{bi} - X_{bi-1}) \qquad (3.10)$$

The plasmid loss factor for biofilm cultures can be determined using the plasmid-free cell mass balance (Equation. 3.8), where μ can be expressed by the Andrews substrate inhibition model (Equation 3.3) and K_{det} can be expressed by the biofilm detachment model (Equation 3.10):

$$p = \frac{\frac{dB^-}{dt} - \mu + K_{det}B^-}{\mu B^+} \tag{3.11}$$

3.4 Nomenclature

V - specific activity (substrate conc./(time * biomass conc.)).

V_{max} - Maximum specific activity (substrate conc./(time * biomass conc.)).

K_M - Michaelis constant (substrate conc.)

μ - specific growth rate ($time^{-1}$).

μ_{max} - Maximum specific growth rate ($time^{-1}$).

K_s - Monod half-saturation constant (mMolar or mg/l).

K_i - Andrews substrate inhibition half-saturation constant (mMolar or mg/l).

[TCE] - Concentration of TCE (mMolar or mg/l).

S - Concentration of growth substrate (mMolar or mg/l).

X^+ - Plasmid-bearing cell concentration(cells/ml).

X^- - Plasmid-free cell concentration(cells/ml).

t - time(hr or days).

p - plasmid loss factor.

D - Dilution rate (/time).

B^+ - Plasmid-bearing biofilm cell density (cells/cm^2).

B^- - Plasmid-free biofilm cell density (cells/cm^2).

K_{det} - is the detachment rate (/time).

X_{bi} - Effluent bulk fluid cell concentration at time point i (cells/ml).

X_{bOi} - Influent bulk fluid cell concentration at time point i (cells/ml).

a - specific area (surface area/volume of reactor).

dB^-/dt - change in plasmid free cell over time (cells/cm^2-day).

Chapter 4

Materials and Methods

4.1 Bacterial Strains

Burkholderia cepacia PR1-pTOM$_{23c}$ were used for all initial lab and field scale studies to determine the feasibility of applying a constitutive, aerobic, TCE degrading plasmid pTOM to a TCE vapor phase bioreactor. *B. cepacia* PR1-pTOM$_{23c}$ and PR1-pTOM$_{31c}$ are the original Tn5 transposon mutants of the environmental isolate *B. cepacia* G4 (see Figure 3, Shields and Reagin 92). PR1-pTOM strains are capable of aerobic, constitutive TCE mineralization via the TOM pathway. Both *B. cepacia* PR1-pTOM$_{23c}$ and *B. cepacia* PR1-pTOM$_{31c}$ have the same metabolic capabilities; however, pTOM$_{31c}$ bearing strains carry a kanamycin resistance marker on their plasmid, while the PR1-pTOM$_{23c}$ cells carry a kanamycin resistance on their chromosomal DNA only.

The transconjugant *Pseudomonas cepacia* 17616-pTOM$_{31c}$ was used in all of the toxicity, plasmid loss, and injury studies. The 17616-pTOM$_{31c}$ transconjugant was obtained by solid surface transconjugation between PR1-pTOM$_{31c}$ and 17616. *Pseudomonas cepacia* 17616 was chosen to be the transconjugant host of plasmid $pTOM_{31c}$ for both its ability to produce a biofilm and its apparent activity and competitiveness in open systems (Murgel et al 91). The genotype of *P. cepacia* 17616 has been well documented by Cheng and Lessie (94). *Burkholderia cepacia* PR1-$pTOM_{31c}$ was also used for comparing pTOM activity and expression results with *P. cepacia* 17616-$pTOM_{31c}$. Plasmid-free strains of 17616 were used for growth kinetics and protein content comparisons. Glycerol/peptone frozen cultures (-70 °C) of plasmid free and plasmid-bearing PR1, and 17616 cell cultures were maintained and used for inoculating starter cultures in all of the experiments. Selective phenol-kanamycin agar plates of all plasmid bearing strains were maintained and re-streaked every week and phenol-kanamycin selective slants were re-streaked every month.

4.2 Plasmids

All of the research presented here was aimed at determining the factors affecting the application and exploitation of the recombinant plasmid pTOM. The plasmid $pTOM_{23c}$ was used in a PR1 strain to determine the persistence of *P. cepacia* PR1-$pTOM_{23c}$ in lab and field scale TCE biofilm reactors.

Plasmid $pTOM_{31c}$ is a 114 Kb plasmid containing the TOM pathway. Plasmid $pTOM_{31c}$ constitutively encodes for toluene ortho-monoxygenase (tom A) and catechol 2,3 dioxygenase (C230) genes, as well as for all of the other genes needed for the aerobic, cometabolic mineralization of TCE (Shields and Reagin 92). In addition, $pTOM_{31c}$ contains a Tn5 transposon carrying the kanamycin resistance marker. A detailed map of plasmid $pTOM_{31c}$ is shown in Figure 6. This plasmid (refer to chapter 1) was used in both a PR1 strain and a 17616 strain to determine its activity and expression in suspended and biofilm cultures. In addition, the stability of plasmid $pTOM_{31c}$ in transconjugant host *P. cepacia* 17616-$pTOM_{31c}$ in suspended and biofilm cultures was determined.

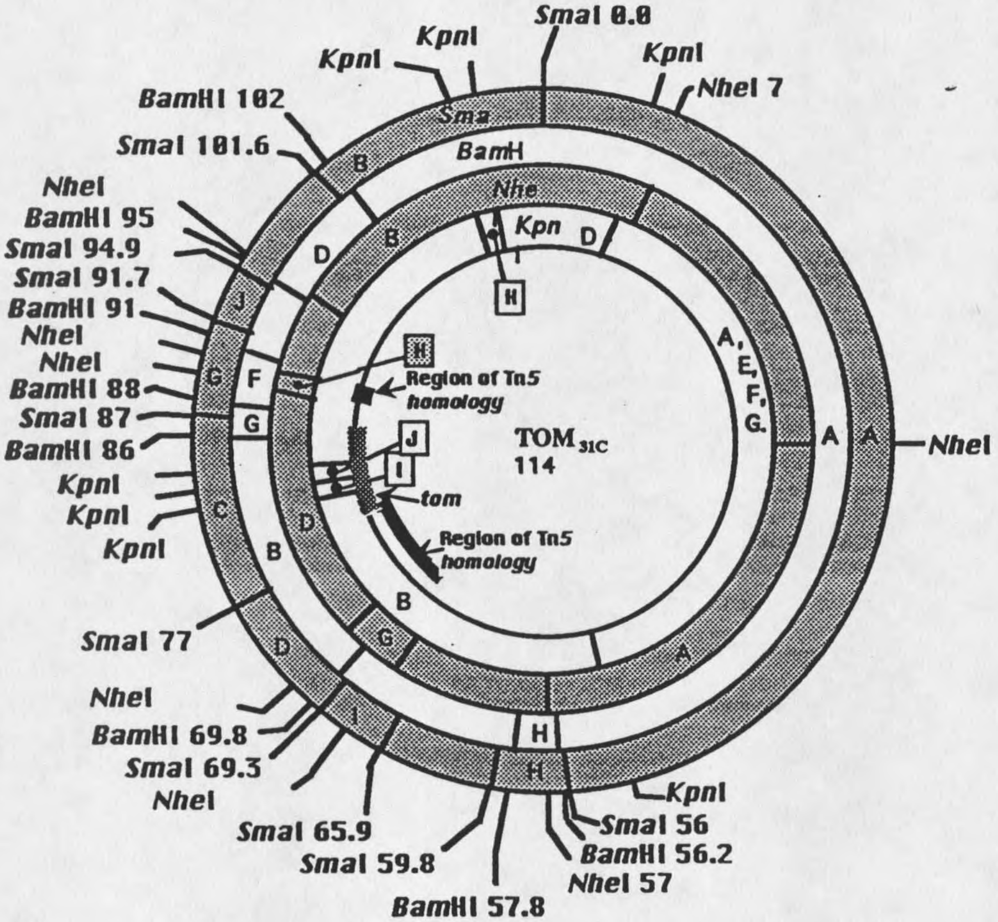
4.3 Media

Four standard media were used in this research: 1) basal salts media (BSM), amended with either non-selective phthalate or selective phenol; 2) rich general growth media - Luria Broth Glucose (LBG); 3) non-selective media, hydrocarbon minimal media (HCMM2) amended with a non-selective, non-inhibitory carbon source (acetate or phthalate); and 4) selective media, HCMM2 amended with kanamycin and phenol or toluene as a selective carbon sources.

4.3.1 Basal salts medium

Basal salts mineral medium (BSM) was used as a rich nutrient medium for all of the initial lab and field scale studies using *B. cepacia* PR1-$pTOM_{23c}$. BSM was amended with either non-selective phthalate or pTOM-selective phenol as carbon sources. The composition of BSM is given in Table 3. Agar plates of BSM phenol, BSM phenol-kanamycin, and BSM phthalate were used to maintain cultures and for determining both selective and non-selective cell counts during the initial lab and field scale studies.

Figure 6 - A Detailed Restriction Map of Plasmid $pTOM_{31c}$



4.3.2 Rich general growth medium - LBG

Luria broth glucose (LBG) medium was composed of 10.0 g/L tryptone, 5.0 g/L yeast extract, 5.0 g/L sodium chloride, and 1.0 g/L glucose in 1 liter of distilled water. LBG was used in agar (15 g/L Bacto agar, Difco, Detroit, MI) form to grow and maintain plasmid-free cultures of *P. cepacia* 17616 and PR1. In addition, LBG medium was used as a general growth source to recover injured 17616-pTOM$_{31c}$ and PR1-pTOM$_{31c}$ cells. Whenever a rich general growth source was needed, LBG was used. An all purpose growth agar medium was made with 15 mg/l Bacto Agar (Difco, Detroit, Michigan).

4.3.3 Non-selective growth medium - HCMM2 -sodium acetate

The non-selective growth media HCMM2, amended with sodium acetate as a carbon source, was used for all non-selective suspended and biofilm culture studies using 17616-pTOM$_{31c}$. The composition of HCMM2 is listed in Table 4. HCMM2 was typically amended with 0.5 to 50 mMoles sodium acetate. HCMM2-acetate medium does not exert a selection pressure for pTOM$_{31c}$. In addition, HCMM2- acetate medium does not inhibit pTOM activity, and both PR1 and 17616 grow and express the TOM pathway well with acetate as their sole carbon source. All non-selective plasmid loss and activity experiments and all non-selective growth experiments were performed using HCMM2-acetate medium. Phthalate was occasionally substituted for acetate as an alternative non-selective carbon source.

Table 3 - BSM Medium Formulation

Ingredients For Basal Media	Concentration
$K_2HPO_4 \cdot 3H_2O$ (anhydrous)	74.2 g/L
$NaH_2PO_4H_2O$	22.85 g/L
NH_4Cl	45.7 g/L
Note: pH to 7.2	
Ingredients for Trace Metals Media	
NTA diNa (nitrilotriacetic acid)	2.45 g/l
$MgS0_4 \cdot 7H_2O$	3.88 g/l
$FeSO_4 \cdot 7H_2O$	232.9 mg/L
$ZnSO_4 \cdot 7H_2O$	57 mg/L
$MnSO_4 \cdot H_2O$	57 mg/L
Dilute both media to 1X and combine 1:1 for final media.	

Table 4 - HCMM2 Medium Formulation

Ingredient	Concentration
Na_2SO_4	2.84 g/L
NH_4CL	1.37 g/L
KH_2PO_4	1.515 g/L
Na_2HPO_4	1.58 g/L
NaOH	to a pH of 7.2
$CaCl_2$	11.25 mg/L
$MgCl_2$	45 mg/L
Add $CaCl_2$ and $MgCl_2$ after pH=7.2 is reached.	

4.3.4 pTOM selective medium

Phenol-kanamycin-HCMM2 selective medium was developed to select for pTOM bearing cells. Neither PR1 nor 17616 plasmid-free cells can grow on phenol. In addition, neither plasmid-free strain can remain viable in the presence of kanamycin ≥ 10 gamma (mg/L). Phenol-kanamycin medium selects for both the TOM pathway and the kanamycin resistant marker associated with the Tn5 transposon. All plasmid-bearing starter cultures and plates were maintained on selective medium. Selective agar medium was used in all injury and plasmid loss studies. These plates were made with 15 g/L Noble Agar (Difco) to insure that phenol was the only significant carbon source.

4.3.5 Growth substrates

Toluene and phenol were used as selective growth substrates, because both select for the TOM pathway. However, both toluene and phenol competitively inhibit TCE degradation which is encoded for by the TOM pathway (Folsom and Chapman 90).

Phthalate and acetate were used as non-TCE-inhibiting, non-selective growth substrates. Concentrations of phthalate and acetate varied from 0.5 to 50 mM. LBG media was used as a rich general growth source. Kanamycin may be added to HCMM2 acetate/phthalate and LBG media resulting in a selective, non-inhibitory media. However, extensive use of this type of media was impractical due to the cost of kanamycin.

Table 5 shows a summary of the uses for each medium used in this research.

Table 5 - Substrates and Their Uses in the Studies Presented.

Function of Media	Specific Type of Medium	Bacterial Strain The Medium is Used For
1) Non-selective for pTOM31c and non-competitive with TOM pathway.	1) LBG Medium 2) Acetate/HCMM2 3) Phthalate/BSM	Used to grow all strains of 17616 and PR1.
2) Plasmid selective, non-competitive with TOM Pathway.	Any non-selective media amended with Kanamycin	Used to select for only the cells carrying $pTOM_{31c}$
3) Plasmid selective and competitively inhibits TOM pathway.	1) Phenol 2)Toluene	Used to grow and select PR1-pTOM and 17616-pTOM strains

4.4 Growth and Activity Studies

4.4.1 Growth of PR1-pTOM$_{23c}$ on non-selective, non-competitive phthalate - BSM medium.

Monod growth kinetics for PR1-pTOM$_{23c}$ on phthalate-BSM media were determined using a continuous culture reactor. Phthalate was chosen as the non-selective, non-competitive medium for PR1, because PR1 grows and expresses the TOM pathway well when grown on phthalate as its sole carbon source. Phthalate concentrations were determined by HPLC. Biomass concentrations were determined by protein assay and dilution plating on general media. A non-linear spreadsheet based curve fitting routine, using least squares error determination, was used to fit Monod kinetics to the phthalate growth data (refer to Chapter 3).

4.4.2 Growth of 17616, 17616-pTOM$_{31c}$, and PR1-pTOM$_{31c}$ on non-selective, non-competitive acetate medium.

Growth kinetics for *P. cepacia* 17616 pTOM$_{31c}$ on acetate-HCMM2 media were determined using batch reactor studies. Initial acetate concentrations in the batch studies varied between .5 mM and 50 mM of acetate. All batch inoculation cultures were taken from selective media starter cultures. Acetate concentrations were determined using ion chromatography. Biomass concentrations were determined by one or more of the following methods: colorimetric protein assay, direct cell counts, or dilution plating on general media (total cfu). Growth data was modeled using Andrews substrate inhibition kinetics. The observed Andrews kinetics for the *P. cepacia* 17616 strains were used for experimental design and implementation into the plasmid loss models presented in Chapter 3.

General growth studies for PR1-pTOM on acetate-HCMM2 medium were performed in batch culture to compare the growth and activity characteristics of PR1-pTOM$_{31c}$ to those of 17616-pTOM$_{31c}$.

4.4.3 Growth characteristics 17616-pTOM$_{31c}$ on selective medium

General growth characteristics of 17616-pTOM$_{31c}$ on phenol -HCMM2 media were determined using batch reactors. Phenol concentrations were determined using a phenol colorimetric assay and biomass concentrations were determined using the protein assay. Initial phenol concentrations ranged from 0.2 mM to 10mM.

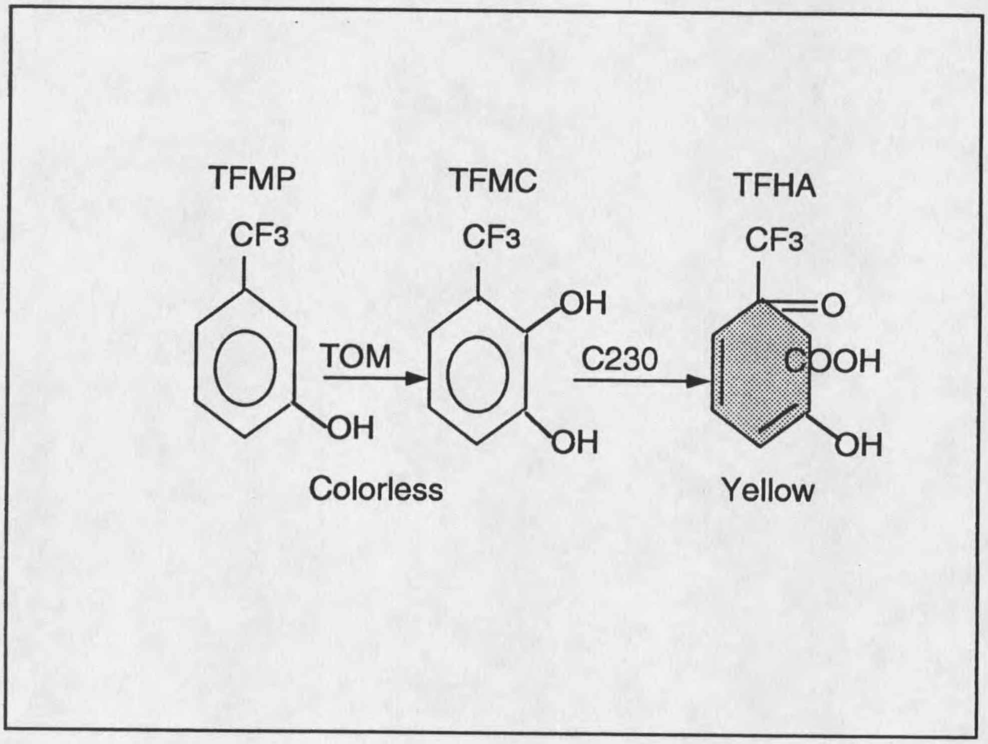
4.5 Activity and Expression of pTOM

The activity and expression of the TOM pathway (pTOM specific activities) were determined using two methods: the TFMP colorimetric assay and the TCE batch disappearance assay.

4.5.1 TFMP assays

The m-trifluoromethylphenol (TFMP) assay is a colorimetric assay used to indicate the expression and activity of the TOM pathway (toluene ortho-mono-oxygenase and catechol 2,3, dioxygenase). A diagram illustrating how the TFMP assay works is shown in Figure 7. The TFMP assay can be performed in three ways: 1) as a suspended culture assay to give the specific activity of the TOM pathway for a suspended cell culture , 2) as a biofilm culture assay to determine the activity of the TOM pathway for a biofilm culture, or 3) as a colony assay to make a positive/negative determination of TOM expression and activity. Details and equations for these assays, along with calibration data, are provided in Appendix A. The pTOM specific activities can be expressed as the pTOM activity of the total culture or the true pTOM specific activity. The true pTOM specific activity is the total pTOM specific activity corrected for the fraction of p(-), dead, and/or injured cells measured in a given culture. The correction is made by subtracting the fractions of biomass protein that are attributed to plasmid loss, cell injury, and toxicity from the total biomass, and then using that corrected protein value to determine the true pTOM specific activity.

Figure 7 - TFMP Colorimetric Assay Used to Determine the Expression and Specific Activity of the TOM Pathway.



4.5.2 TCE disappearance assays.

Batch reactor TCE disappearance assays were performed using 120 ml serum bottles equipped with gas tight mininert valves (Supelco, Inc., Bellefonte, PA). Assays were conducted with 25 mls of either a PR1-pTOM$_{31c}$ culture or a 17616-pTOM$_{31c}$ culture. PR1-pTOM$_{31c}$ cell samples were harvested from a 20 mM phthalate-BSM-40 gamma kanamycin continuous culture. The 17616-pTOM$_{31c}$ samples were harvested from a 2 mM phenol-HCMM2 continuous culture. All continuous culture samples were concentrated to an A_{600} of 1. Serum bottles were sealed with a mininert valve and Teflon tape. TCE was introduced into each assay to give an initial liquid TCE concentration between 0.5 and 70 uM. Bottles were shaken for 2 minutes to equilibrate, and a vapor sample from each assay was analyzed every 5-8 minutes until either the TCE detection limit was reached or a distinct initial TCE degradation rate was noted.

TCE analysis was performed by gas chromatography. A dimensionless Henry's law constant (H_{TCE} = vapor TCE concentration/Liquid TCE concentration) of 0.4 was used for the PR1-pTOM batch studies to determine liquid TCE concentrations from the vapor TCE concentrations (Folsom and Chapman 91). A dimensionless Henry's law constant of 0.38 (± 0.03) was determined and used for the 17616-pTOM$_{31c}$ batch cultures (Appendix B). Cometabolic TCE degradation kinetics for relevant TCE concentrations were determined for PR1-pTOM$_{23c}$ and 17616-pTOM$_{31c}$ strains using linear and Michaelis-Menten kinetic models, respectively (Appendix C). Proper cell free controls and TCE calibration curves were used for each set of experiments.

In addition, a positive/negative TCE disappearance assay was performed to conclude the active presence of the TOM pathway. This simple disappearance assay involved placing ~ 0.5 ppm TCE and a 5 ml cell sample into a 10 ml crimp top bottle and sealed with a Teflon lined septum. The sample was shaken and incubated at 30 °C overnight and then analyzed for residual TCE. Cell free controls were also performed.

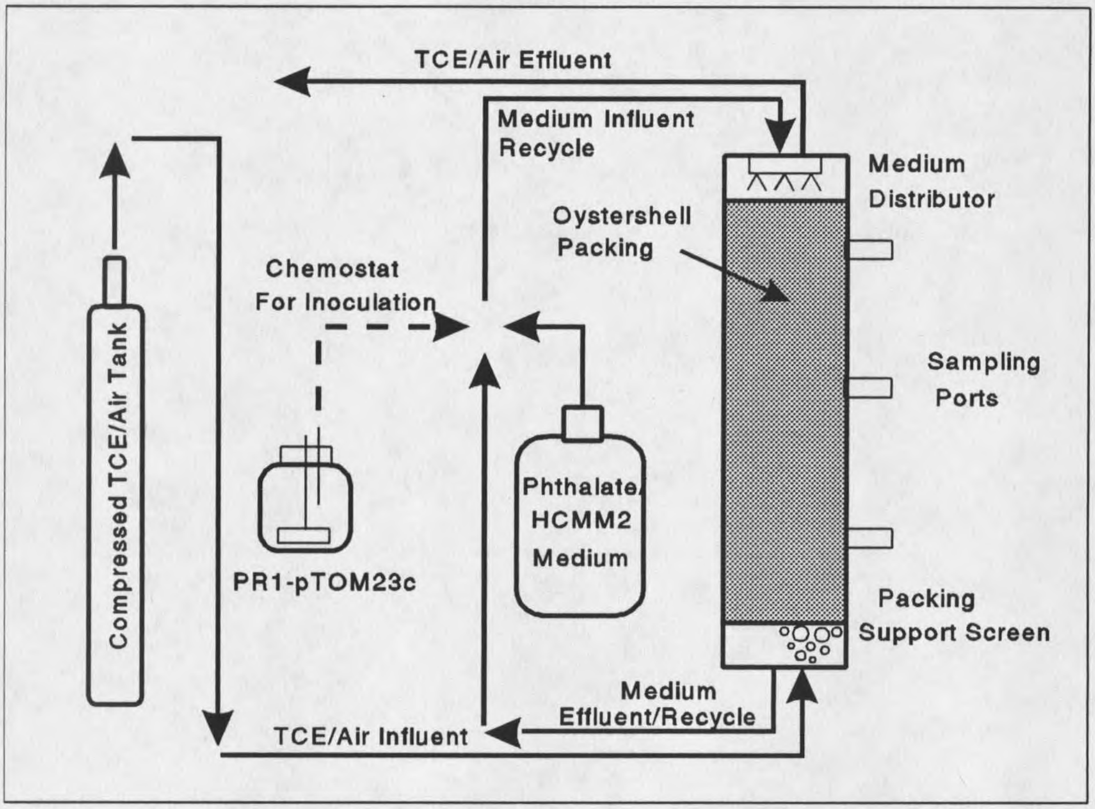
4.6 Methods for Initial Lab and Field Scale Column Studies Using PR1-pTOM$_{23c}$

Lab and field scale column studies were performed to determine the applicability of PR1 as host for the pTOM plasmid in a TCE degrading biofilm reactor.

4.6.1 Lab column studies

Column studies utilizing a glass column system (shown in Figure 8) packed with crushed oyster shell packing were performed using PR1-pTOM$_{23c}$ as the inoculum and phthalate as the primary growth source. The column system was used to determine if PR1-pTOM23c could produce a competitive biofilm population on oyster shell packing with phthalate as its only carbon source. The column system was run as a full-recycle, fed-batch, trickling packed bed reactor.

Figure 8 - Diagram of Lab Scale Bioreactor Columns



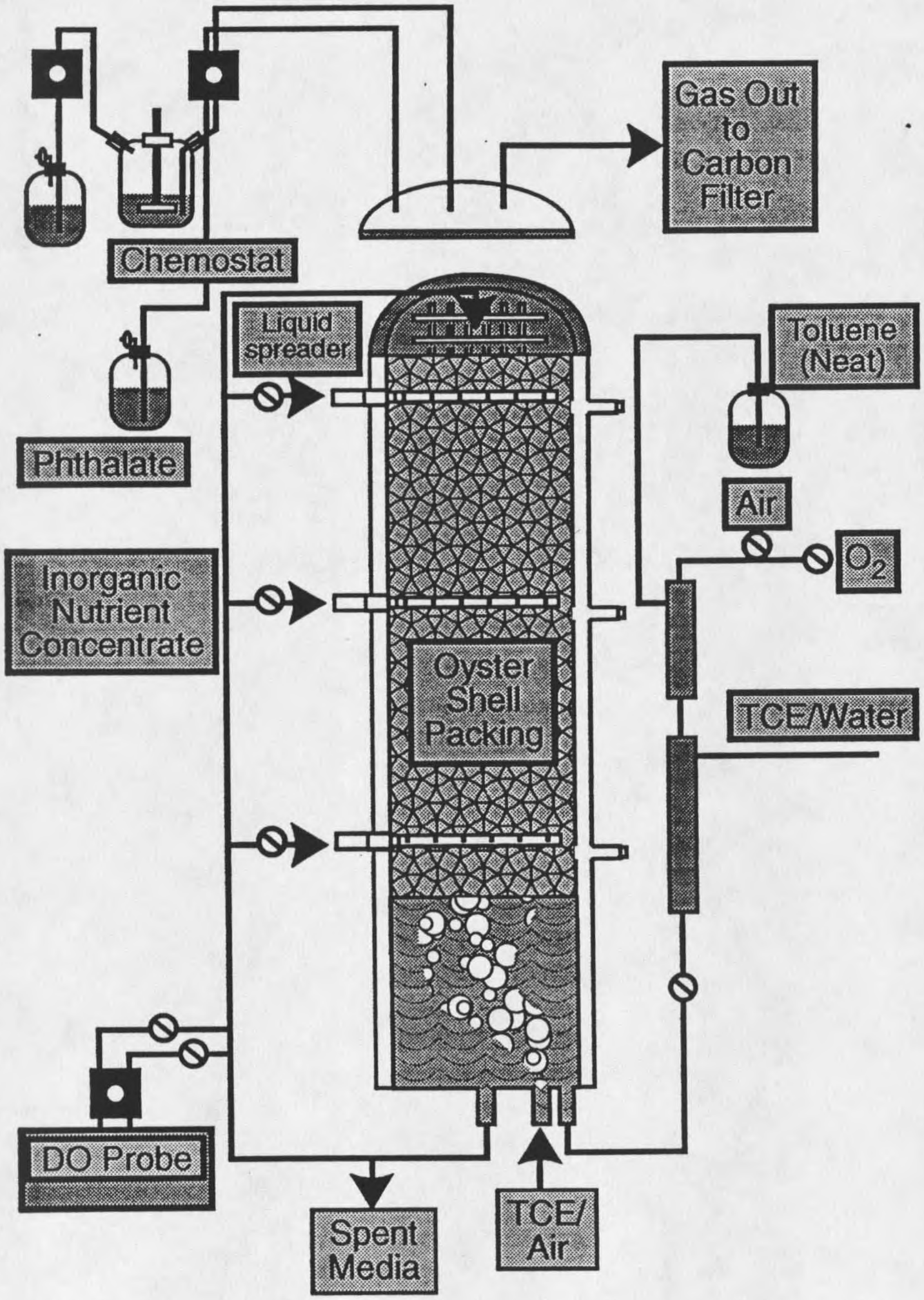
The reactor was operated in a batch fed mode with 2 liters of phthalate-HCMM2 medium which was drained and replaced every 2 days. During the two day period the medium was recycled through the column with a residence time of approximately 1 hour. The system was inoculated with 1 liter of PR1-pTOM$_{23c}$ cells at an A_{600} of 1 under heavy kanamycin selection. The system was not sterile because the oyster shell could not be sufficiently sterilized. Periodically the batch feed solution was amended with 40 gamma kanamycin to select for PR1-pTOM$_{23}$ cells.

Daily analysis of the system included: measurement of phthalate concentration, total heterotrophic cell counts in bulk fluid, selective cell counts in bulk fluid, biofilm protein measurements, and a TCE disappearance assay on biofilm/oyster shell samples.

4.6.2 Field scale column studies

A field study was conducted to determine the ability of PR1-pTOM$_{23c}$ to be utilized in a large scale TCE vapor phase bioreactor (VPBR) packed with crushed oyster shell using both selective/inhibitory and non-selective/non-inhibitory carbon sources for start-up and operation. The reactor system was set up at a TCE concentrator plant at the Hanscom Air force Base outside Boston, Massachusetts. A diagram of the 110 liter, counter current VPBRs used in the field is shown in Figure 9. Two VPBRs were used in this study, one cell-free control reactor (uninoculated) and one test reactor. TCE vapor to be treated

Figure 9 - Diagram of the Field Scale Vapor Phase Bioreactor System



was the effluent from a TCE concentrator. TCE influent was fed to the VPBRs from the bottom of the each reactor along with toluene vapor that was used as a selective growth substrate. Nutrient media and liquid growth media were introduced at the top of the columns at a rate of approximately 0.5 liters/hr.

Start-up of the test VPBR involved a large volume inoculation of PR1-pTOM$_{23c}$ cells harvested from a continuous culture. Over 5 liters of high cell density culture were added to the system at the top of the column for inoculation. The system was also fed toluene vapor as a selective growth source to enhance start-up and PR1-pTOM$_{23c}$ biofilm formation. A number of different feed scenarios involving toluene vapor, phenol-BSM medium, and phthalate-BSM took place during the operation of the system. TCE vapor was introduced to the columns after 27 days of reactor start-up and inoculation. When TCE was introduced, 20 mM phthalate was fed to the reactor systems in place of the inhibitory toluene vapor.

Oyster shell packing samples from both the test and control reactors were taken every day at each of the three sample ports. The oyster shell samples were analyzed for the following: total heterotrophic cell numbers using LBG, protein content using the protein assay, pTOM activity using the TFMP biofilm assay, and PR1-pTOM$_{23c}$ cell number using phenol-Km-HCMM2 selective plates. Samples of the effluent liquid were also analyzed for total cell counts, protein content; PR1-pTOM$_{23c}$ cell counts, and carbon source concentration. TCE vapor was analyzed at the inlet, outlet, and all three of the sampling ports in each column.

4.7 Methods for Plasmid Stability and Activity Studies Using *P. cepacia* 17616-pTOM$_{31c}$

Stability and activity of pTOM$_{31c}$ in the transconjugant host *P. cepacia* 17616 was determined in both suspended and biofilm cultures under non-selective growth conditions.

4.7.1 Suspended culture plasmid stability and activity studies

Batch experiments

Experiments using PR1-pTOM$_{31c}$ and the transconjugant 17616-pTOM$_{31c}$ growing on non-selective acetate-HCMM2 media were carried out to compare pTOM activities, acetate growth characteristics, and pTOM$_{31c}$ plasmid stability between the two strains during batch growth. A series of growth studies at acetate concentrations ranging from 2-20 mM were performed. Acetate growth characteristics were determined by monitoring acetate and biomass concentrations over the duration of each complete batch growth curve (Appendix D). Plasmid pTOM$_{31c}$ activities were determined periodically during each batch experiment using the TFMP suspended culture assay and the TCE disappearance assay.

Total, plasmid-free, and plasmid-bearing cell concentrations were determined periodically throughout each batch growth study to monitor the loss of plasmid $pTOM_{31c}$ in the batch 17616-$pTOM_{31c}$ cultures. Total cell numbers were determined by dilution plating on LBG agar plates. Plasmid-free cell count to total cell count ratios and plasmid-bearing cell count to total cell counts ratios were determined using the $pTOM_{31c}$ selective direct-colony transfer (PSDCT) method.

Continuous culture experiments

Acetate fed chemostat studies were carried out to determine the stability and activity of $pTOM_{31c}$ in host 17616 during continuous culture. The chemostat system used is shown in Figure 10. Chemostats were run at a number of different dilution rates ranging from 0.06/hr to 0.19/hr. Steady-state values for pTOM specific activity were determined every day for each dilution rate, using the suspended culture TFMP assay to determine if pTOM activity was a function of growth rate. Steady-state plasmid-bearing, plasmid-free, and total cell counts were determined daily for each dilution rate, using the PSDCT method. Cell counts were used to monitor plasmid loss during each continuous growth experiment to determine if plasmid loss was a function of growth rate. Continuous culture plasmid loss was modeled using the Ollis model presented in Chapter 3.

A set of pTOM selective, phenol fed chemostat studies were performed to determine if phenol could be used to either stabilize the $pTOM_{31c}$ plasmid in 17616 or select for $pTOM_{31c}$ bearing cells in continuous culture. Phenol concentrations were measured using a colorimetric phenol assay. Biomass measurements were made using the protein assay. Plasmid loss was determined using the PSDCT method.

All continuous culture experiments were inoculated with a pure culture of 17616-$pTOM_{31c}$ cells harvested from a highly selective starter culture.

4.7.2 Biofilm culture plasmid stability and activity studies

Pseudomonas cepacia 17616-$pTOM_{31c}$ biofilm cultures were grown on non-selective acetate-HCMM2 media to determine the stability and activity of $pTOM_{31c}$ in biofilm cultures. Biofilm cultures were grown using a rotating annular reactor shown in Figure 11. Annular reactors were initially colonized under batch operation with high kanamycin selection (80 gamma or ug/ml) to insure the initial biofilm was made up of all plasmid-bearing cells. No cells were added to the system after initial inoculation. Biofilm reactors were operated at two different influent acetate concentrations, 4 mM and 10 mM acetate-HCMM2. The annular reactors were run at a dilution rate of at least 1.0/h (at least 5 times greater than the maximum growth rate of 17616) to insure no replication of detached biofilm cells occurred in the bulk fluid phase.

Figure 10 - Diagram of Chemostat System Used for Plasmid $pTOM_{31c}$ Stability and Activity Studies.

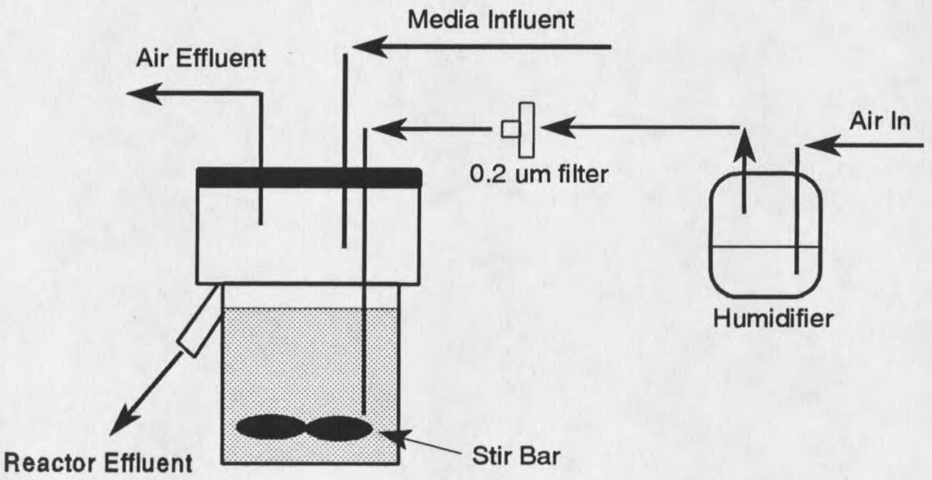
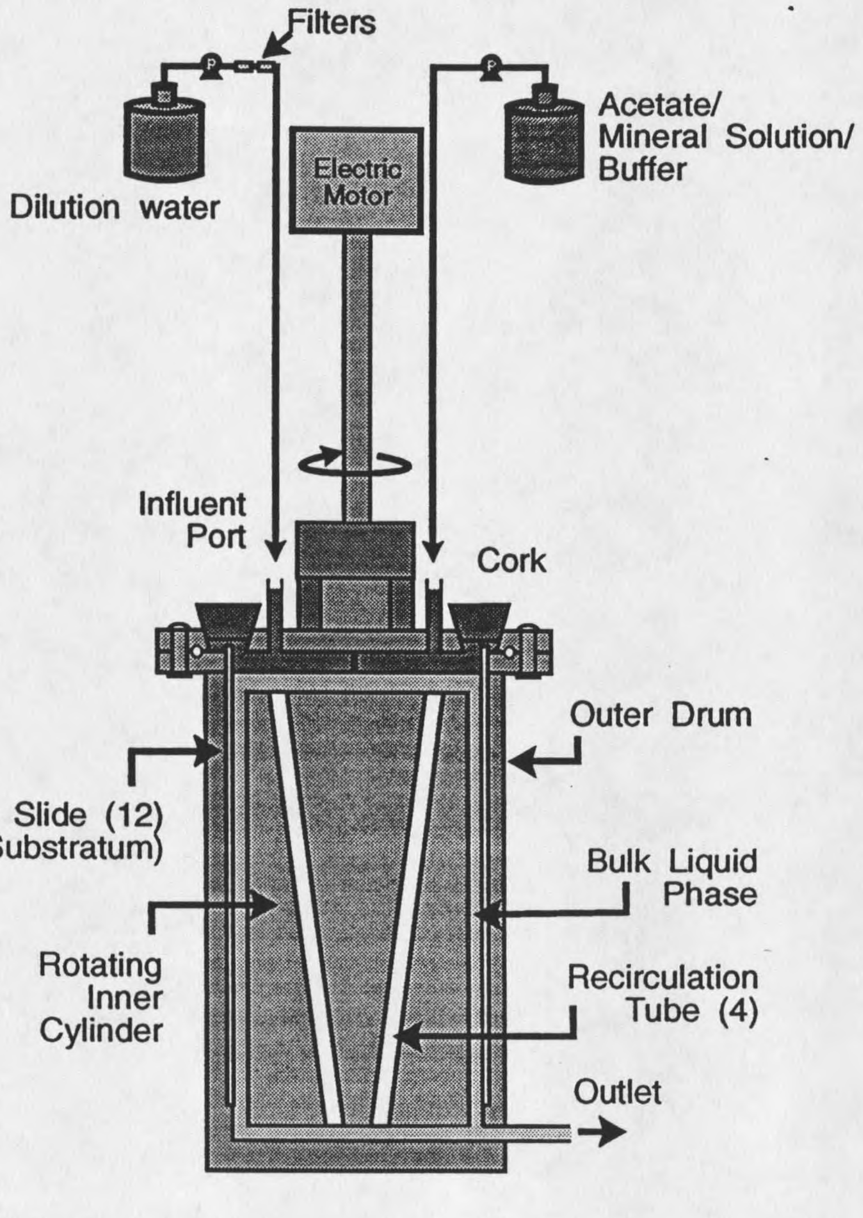


Figure 11 - Diagram of the Annular Reactor System Used for Biofilm Culture Plasmid $pTOM_{31c}$ Activity and Loss Studies.

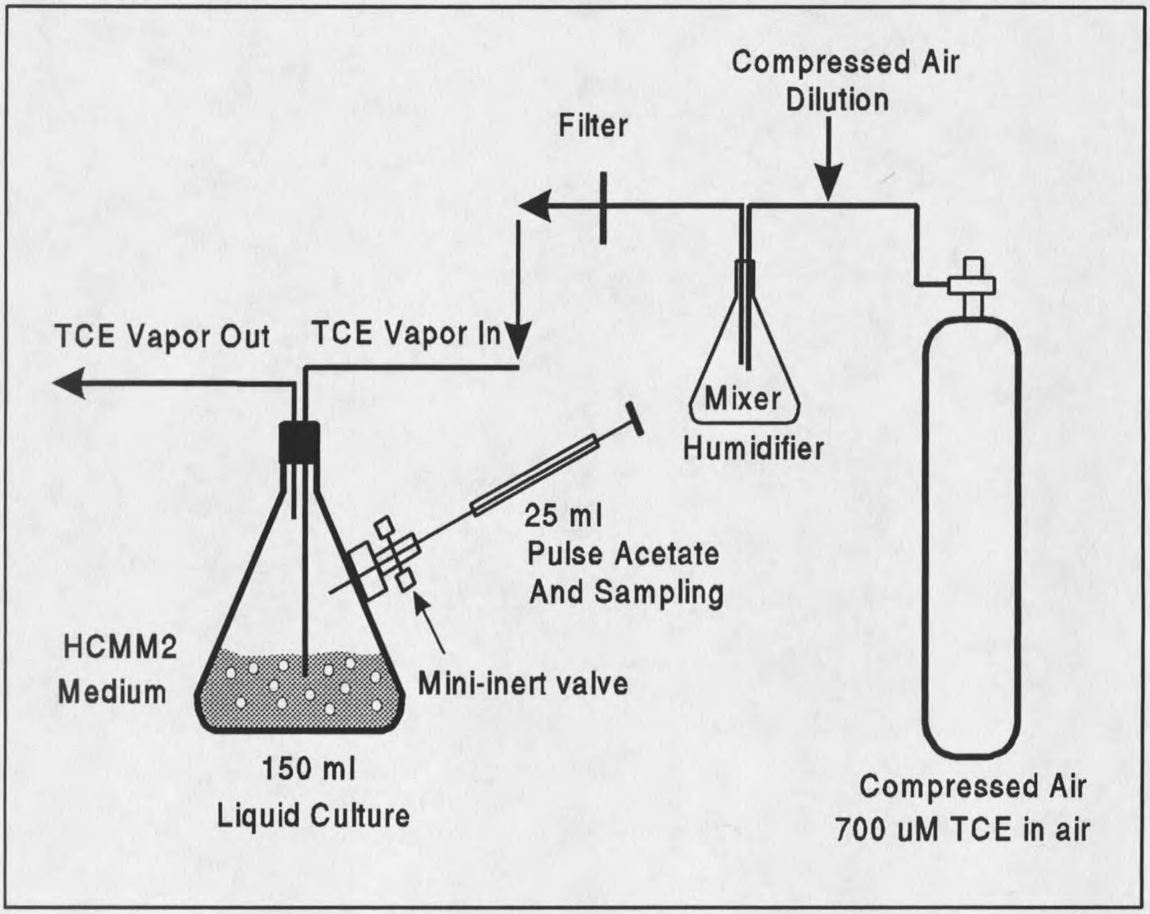


Protein content and acetate concentrations of effluent and biofilm samples were determined periodically throughout the biofilm experiments. Plasmid-bearing, plasmid-free, and total cell counts in both effluent and biofilm samples from each annular reactor were determined every two days using the PSDCT method. Samples for the PSDCT method were harvested from the annular reactor slides and effluent, and prepared using the biofilm culture suspension method (BCS). Cell counts were used to determine the growth and plasmid loss characteristics of 17616-pTOM$_{31c}$ in biofilm culture. Plasmid pTOM$_{31c}$ specific activities of the biofilm cultures were determined using the suspended culture TFMP assay on a 5ml aliquot of the BCS sample. Results were modeled using the biofilm plasmid loss model presented in Chapter 3.

4.8 Methods for TCE Exposure Studies Using an Acetate Fed-Batch, TCE Vapor Continuous Flow Reactor to Determine Cell Injury, Toxicity and Plasmid Loss During TCE Exposure.

An acetate fed batch reactor with continuous TCE vapor flow was designed to examine the effect of TCE exposure on the toxicity, plasmid-loss, and injury of 17616-pTOM$_{31c}$ cell cultures. The system used is shown in Figure 12. A fed batch system was used to mimic a biofilm system, in that the cells were continuously exposed to a pseudo steady-state TCE concentration (unlike a batch reactor), while they did not have a finite residence time (like in a continuous culture). Cells in the fed batch reactor system had a residence time

Figure 12 - Diagram of Fed-Batch, TCE Vapor Continuous Flow Reactor Used to Examine the Affects of TCE Exposure.



of 6 days. Two different experiments, with the appropriate controls, were run using the fed batch system.

4.8.1 Selective TCE exposure studies

The selective fed batch reactor system was operated by taking a 25 ml sample from the 150 ml liquid volume every 24 hours. The 25 ml sample was then replaced by 25 mls of 24 mM acetate-HCMM2 medium amended with 120 gamma kanamycin, resulting in a 6 hour residence time for the system. This volume extraction resulted in a concentration of 4 mM acetate/20 gamma kanamycin at the end of each batch feed. The kanamycin was used to select for 17616-pTOM$_{31c}$ by killing plasmid-free cells generated by segregational plasmid-loss. It was assumed that the 4 mM acetate was completely utilized within the 24 hour time period between batch feeds. In addition, the kanamycin was assumed to be utilized and degenerated to a substantially lower level between batch feeds. It was also presumed, with the combination of a 6 day residence time (dilution) and the utilization and degeneration of the kanamycin, that the concentration of kanamycin would not exceed the lethal limit for 17616-pTOM$_{31c}$, which was found to be approximately 280 gamma. If the maximum kanamycin concentration were ever reached, it would be noted by a severe decline in both total and selective cell counts.

A test and a control reactor were run. Each reactor was inoculated with the same concentration of 17616-pTOM$_{31c}$ cells harvested from a pure culture (high selection) 17616-pTOM$_{31c}$ batch culture. After 8 hours of unincumbered growth, 70 uM TCE (in air) vapor was introduced to the test reactor at a flow rate of 50 mls/min. After 15 days of 70 uM TCE exposure , the TCE concentration was raised to 700 uM for three days to determine the effect of concentration. No TCE was introduced to the control reactor.

Daily analysis of the reactors included: protein contents, acetate concentrations, pTOM specific activities, total cell counts, Tn5 selective plate counts, and pTOM selective plate counts. Total cell counts were determined by plating on LBG media. Tn5 selective cell counts were determined by plating on LBG-Kanamycin agar. The pTOM selective cell counts were determined by dilution plating on phenol-kanamycin-HCMM2 low carbon agar plates.

Comparison of the control and test reactor total cell counts demonstrates the extent of toxicity incurred by the 17616-pTOM$_{31c}$ cells by prolonged exposure to TCE. Differences between the test reactor's total counts and selective counts indicates the number 17616-pTOM$_{31c}$ cells injured as a result of TCE exposure. Finally, the occurrence of significant numbers of TFMP-negative LBG-kanamycin plates would symbolize that the plasmid pTOM$_{31c}$ may have been recombined as a result of structural instability and would insinuate that the kanamycin resistance marker either had been incorporated into the cells' chromosomal DNA or remained active while the TOM pathway had been deemed inactive.

4.8.2 Non-selective TCE exposure studies

A non-selective fed batch reactor was run under the same conditions as the selective reactors, except kanamycin was not provided in either the test or control reactors. Analysis of the non-selective test and control reactors included: protein content, pTOM specific activity, and $pTOM_{31c}$ selective direct colony transfers to determine if TCE exposure resulted in increased plasmid loss. Results from this experiment also give information on the toxicity and degree of selection that TCE has on 17616-$pTOM_{31c}$ cells.

4.9 Analytical Methods and Protocols

4.9.1 Protein assay - Protein content was determined using the enhanced BA Protein assay (Pierce Co.) This protein assay was used in both the suspended and biofilm culture TFMP assays. Suspended protein determinations were usually made using the appropriate A_{600} versus protein calibration curve. The calibration curves were determined for each specific microbial strain and medium (Appendix E). Direct protein measurements (no calibration curve) were determined for all biofilm packing and suspended phenol growth samples. All absorbance measurements were taken using a Milton Roy Spectronic 601 photospectrometer.

4.9.2 Phenol assay

Phenol concentrations were determined using a colorimetric phenol assay which involved the addition and thorough mixing of 50ul of 2 N NH_4OH and 25 ul of 2% 4-aminoantipyrene (Aldrich Chemical Co., Inc., Milwaukee Wisc.) to a 1ml phenol sample. After mixing, 25 ul of 8% $K_3Fe(CN)_6$ (Sigma Chemical Co., ST. Louis, Mo.) was added, and the contents were then mixed again and centrifuged (14,000 x g) for 2 minutes. A_{500} of the centrifuged sample supernatant was measured. Phenol concentrations were calculated by reference to a standard curve (Appendix F).

4.9.3 Phthalate analysis

Phthalate was analyzed using a Hewlitt Packard-1050 High Pressure Liquid Chromatograph equipped with variable wavelength and multiple wavelength detectors using a HP, OD Hypersil, 5um, 100x2.1 mm analytical column and a HP OD Hypersil, 5um, 20 X 2.1 mm guard column. A calibration curve for the IC phthalate analysis is shown in Appendix G.

4.9.4 TCE analysis

Vapor and liquid phase TCE samples were analyzed using a Schimadzu GC9A gas chromatograph equipped with an electron capture detector (Shimadzu Scientific Instruments, Inc., Columbia, MD) using a 30 m, 0.53mm ID Vocal mega-bore column (Supelco, Inc., Bellefonte, PA.). The TCE method was isothermal (110° Celsius oven temp., 200° C detector and injector temp.) with a carrier gas flow of 5 ml/min and a make-up gas flow of 50 ml/min. Ultra-high purity nitrogen was used for both the carrier and make-up gases. Since the method was isothermal using nitrogen, there were two linear calibration curves, one for high TCE concentrations (~10 nM to 10uM, detector range set at 2, current set at 1) and one for low TCE concentrations (~10 uM to 1000 uM, detector range set at 1, current set at 2).

Vapor TCE samples were injected directly. Liquid TCE samples were extracted using an equal volume of pentane, and a 2 ul sample of the pentane TCE extraction was analyzed. Liquid TCE standards were made with high grade TCE (Sigma Chemical Co.) in HPLC grade methanol or pentane. Vapor TCE was supplied from a 700 uM TCE-ultra zero air balance compressed gas bottle (Air Liquide, La Porte, Texas). A representative calibration curve for the G.C. TCE analysis is presented in Appendix H.

4.9.5 Acetate analysis

Acetate was analyzed using a Dionex ion chomatograph (Model AI-450; Dionex Co., San Francisco) with a pulse electrochemical detector (Model DX300) using a IonpacAS10 column(4mm). Calibration information is presented in Appendix I.

4.9.6 $pTOM_{31c}$ selective direct colony transfer method (PSDCT)

The PSDCT method was used to determine total cell counts and the fraction (.01 to 1) of plasmid-bearing and plasmid-free cells in a given population. The method involved the following steps:

1) Dilution plating of sample on general growth LBG agar media to determine total cell counts.
2) 80 to 100 LBG colonies were transferred from the LBG plates directly to selective 2mM phenol-kanamycin low carbon agar plates via a sterile toothpick.
3) Selective plates were incubated for 2 days at 30° C and all colonies were counted.
4) A TFMP colony assay was performed on the selective plates, and the number of TFMP positive colonies (plasmid bearing and active colonies) was determined.

The PSDCT method was performed in triplicate to give three values for total cell counts, and plasmid-free and plasmid-bearing cell fractions for each sample.

4.9.7 Cell enumeration techniques

Selective and non-selective plating techniques were used to determine total viable heterotrophic cell concentration, fractions of p(+) or p(-) cells with respect to the total viable heterotrophic cell concentration, percent toxicity, and injured cell concentration. Table 6 lists the plating methods used and what cell type each method enumerates.

Toxicity fractions

Toxicity fractions are determined by the ratio of total viable cell concentration from a reactor that has grown under TCE exposure to the total viable cell concentration from a reactor that has not been operated under TCE selection (control).

Injured cell fractions

The fraction of injured cells in a given population is the ratio of cells that cannot grow on phenol-Km to those that grow on general rich medium (LBG). If the cells are taken from a reactor where they have been growing under selection (phenol or TCE exposure) and they do not grow on selective medium they are defined as injured.

p(+) and p(-) cell fractions

The PSDCT method determines if the cell possess an active pTOM plasmid and whether or not the cell is injured or not. The p(+) cell concentration is determined by multiplying the total viable cell concentration by the fraction of PSDCT cells that grow on phenol-km plates and are TFMP positive.

Table 6 - Plating Methods for Cell Enumerations

Cell Type	Enumeration Method	Medium
1) Total Heterotrophic cell concentration.	Growth on carbon rich agar medium	#1 - LBG plates or Acetate-HCMM2 plates
2) Kanamycin resistant cell concentration	Growth on carbon rich agar amended with Km	#2 - LGB-kanamycin or Acetate-HCMM2-Km
3) Healthy and active plasmid-bearing cell concentration.	Growth on plasmid (pTOM) selective medium.	#3 - Phenol-HCMM2 or Toluene vapor
3) Plasmid-free cell concentration	Negative growth upon transfer from rich carbon(#1) media to selective medium (#3).	LBG plate and phenol-HCMM2-Km low-carbon agar medium.
4) Plasmid-bearing cell concentration	Positive growth upon transfer from rich carbon (#1) medium to selective medium (#3) and TFMP positive (PSDCT method).	LBG plate and phenol-HCMM2-Km low-carbon agar medium.
5) Injured cell fractions	No-growth on selective medium (#3), but growth on rich carbon medium (#1) with subsequent growth on selective medium (#3).	LBG plates and phenol-HCMM2-Km low-carbon agar medium.

4.9.8 Biofilm culture suspension (BCS) method

The first step in the BCS method involves removing a slide from the annular rotating reactor via a pair of sterile needle-nose pliers. The biofilm is then scraped from the slide using a flat edged sterile Teflon spatula into a petri dish containing 40 mls of HCMM2 buffer media, which is also used to rinse all of the biofilm off the slide. Once the slide is thoroughly scraped clean, the 40ml HCMM2 solution is then placed in a sterile 100 ml beaker. Finally the solution is homogenized using a Tekmar Tissuizer (Tekmar Co., Cincinnati, OH) at medium-high speed for 2 minutes to disperse the biofilm cells. The BCS method provides a bacterial sample with minimal cell clumps, which may result in poor quality cell counts.

4.9.9 Temperature and pH.

All lab experiments were run at room temperature which was measured to be 23 ± 2 ° Celsius. The pH of all media and influents and effluents were monitored throughout the studies.

Chapter 5

Results

5.1 Results from Initial Lab and Field Studies Using PR1-pTOM$_{23c}$

Suspended batch culture and chemostat studies, plus biofilm column studies were performed to determine the feasibility of using *Burkholderia cepacia* PR1-pTOM$_{23c}$ within a TCE biofilm reactor. Research involved both lab- and field-scale studies. Suspended culture lab studies determined the growth kinetics of PR1-pTOM$_{23c}$ in continuous culture using non-inhibitory, non-competitive phthalate medium. In addition, batch culture lab studies determined the TCE specific activity of *B. cepacia* PR1-pTOM$_{23c}$. Laboratory column studies were performed to ascertain the ability of PR1-pTOM$_{23c}$ to persist and thrive as a biofilm culture grown on crushed oystershell packing using both selective and non-selective media (see Figure 8). Finally, a field-scale study, applying PR1-pTOM$_{23c}$ to a 120 liter vapor phase TCE reactor (see Figure 9), was carried out at the Hanscom Airforce Base outside of Boston, Massachusetts.

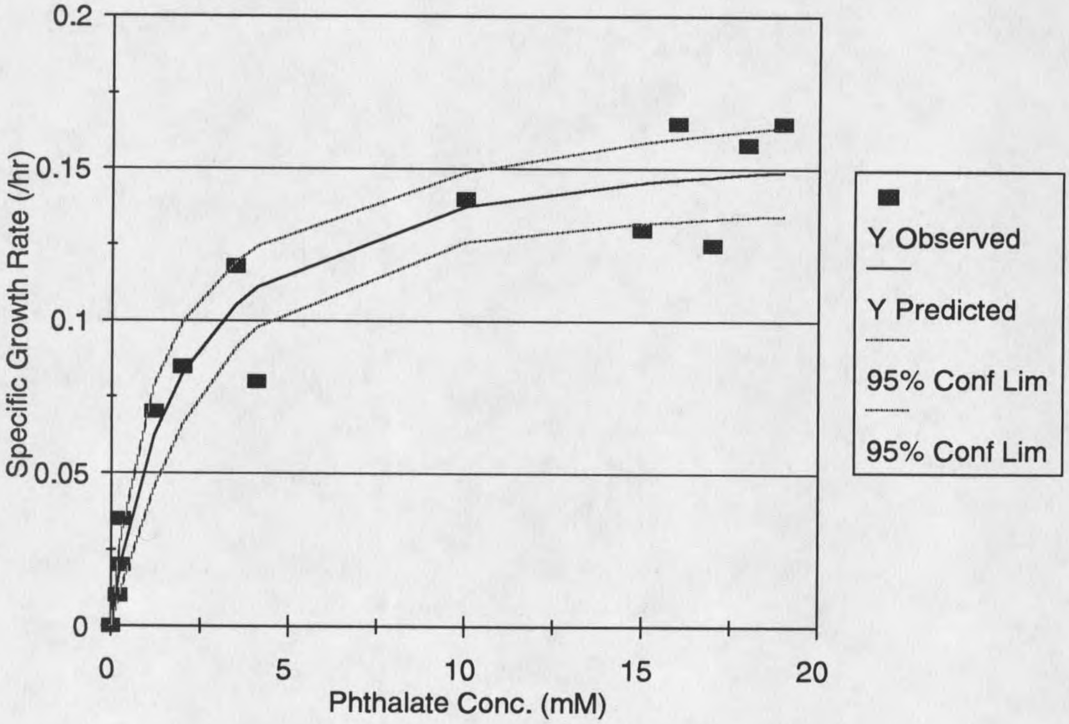
5.1.1 Initial lab studies

Growth of PR1-pTOM$_{23}$ on phthalate

A series of continuous culture experiments to determine the growth kinetics of PR1-pTOM$_{23c}$ on phthalate were performed. Phthalate was chosen as the growth source for PR1-pTOM$_{23c}$ because it does not competitively inhibit TCE degradation. However, phthalate does not select for pTOM$_{23c}$ bearing cells. Figure 13 shows the relationship between growth rate and phthalate concentration obtained from the continuous culture studies. Using a non-linear regression spread method to fit the single substrate Monod expression (Eqn. 3.2), the values of maximum growth rate and the Monod half-saturation constant (μ_{max} and Ks) were determined to be 0.16/hr and 3.2 mM respectively. The Monod expression with the appropriate kinetic values is shown in Equation 5.1. Overnight TCE disappearance assays performed on effluent samples from the continuous culture experiments were all positive and demonstrated that the phthalate grown PR1-pTOM$_{23c}$ cultures were actively expressing pTOM and could readily degrade TCE.

$$\boxed{\mu(hr^{-1}) = \frac{0.16\ S}{(3.2 + S)}} \qquad \text{Eqn. 5.1}$$

Figure 13 - Monod Growth Kinetics for PR1-pTOM$_{23c}$ on Non-Selective Phthalate Mediu. Where: μ_{max} = 0.16/hr and Ks = 3.2 mM phthalate. Carried out in aerated BSM medium at a pH of 7.2 and a temperature of 25 °C.



PR1-pTOM$_{23c}$ TCE degradation kinetics

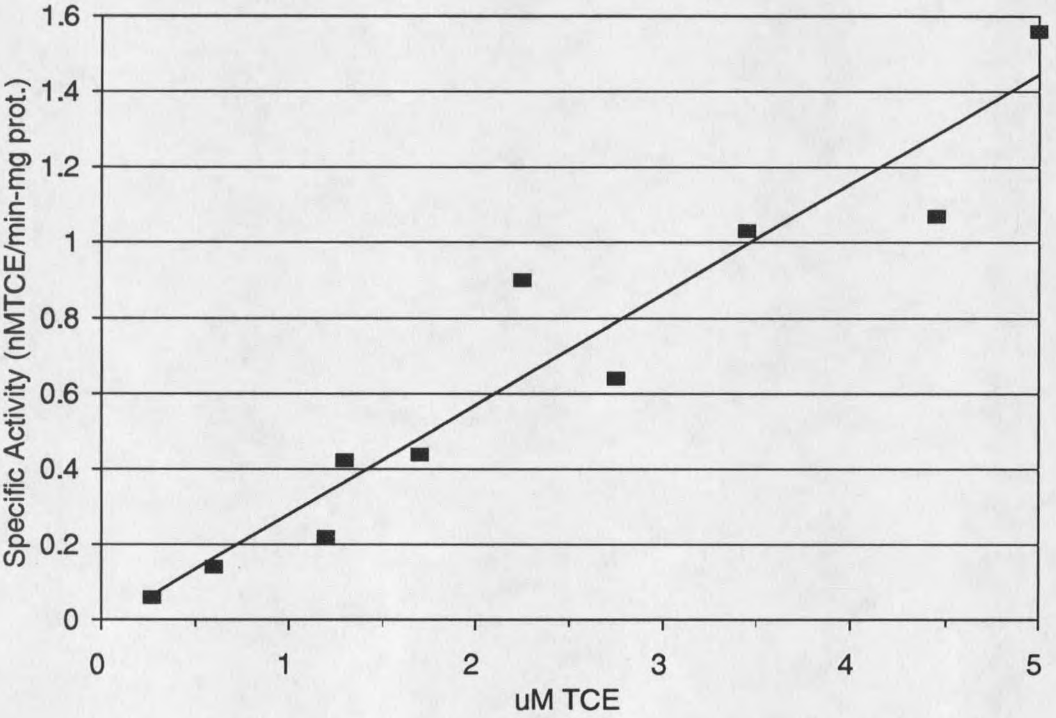
TCE degradation kinetics for PR1-pTOM$_{23c}$ were determined at relatively low concentrations of TCE using head-space batch reactor studies. Inocula for the suspended cell batch cultures were harvested from a phenol chemostat operating at a steady-state dilution rate of 0.1/hr. The range of initial TCE concentrations used in the batch experiments were equivalent to the average TCE concentrations to be treated at the field study site. All liquid TCE concentrations were determined from TCE head-space concentrations using a dimensionless Henry's Law constant of 0.4 (Folsom et al 90). Results from the TCE kinetic studies are shown in Figure 14. TCE specific activity could be described as a linear function of the TCE concentrations tested, as shown in Equation 5.2.

TCE Specific activity (nMoles TCE/min-mg.prot.)= 1.96 [TCE] Eqn. 5.2

Lab column studies using PR1-pTOM$_{23c}$:

Column studies to determine the ability of PR1-pTOM$_{23c}$ to produce and persist in a biofilm when grown on crushed oystershell support were carried out using phthalate as the primary growth. The column system used is shown in Figure 8. After inoculation with a liter of PR1-pTOM$_{23c}$ cells at an O.D.of 1, the

Figure 14 - Degradation of TCE by PR1-$pTOM_{23c}$: Note the linear relationship between TCE specific activity and TCE concentration indicative of Monod kinetics when substrate concentration is much less than Km (S<<Km). Using the following equation - (Specific Activity) = k*[TCE]^n where n = 0.95, and k = 1.96, with units of mg-prot^{-1}- min^{-1}, R^2 = .92.



reactor was run in total recycle mode to promote attachment and biofilm growth. Phthalate-HCMM2 media was replaced every two days.

Biofilm and suspended cells counts and phthalate concentrations were determined every day. Figure 15 shows the daily concentration of phthalate in the reactor effluent during the experiment. Eight days into the operation, most of the 20 mM phthalate batch feeds were utilized completely. Complete removal of the phthalate was also observed after the phthalate concentration was increased to 27 mM. However, Figure 16 shows that the microbiology of the column involved mixed microbial population, with at least one culture other than PR1-$pTOM_{23c}$ capable of utilizing phthalate. PR1-$pTOM_{23c}$ could be maintained in the reactor only when periodic pulses of kanamycin were introduced. Without kanamycin, PR1-$pTOM_{23c}$ could not establish itself in the system due to its inability to both form a significant biofilm and compete with the other phthalate utilizing microbial populations present in the reactor. Further investigation suggested that the other phthalate utilizing microbial populations originated from the crushed oystershell. Attempts to sterilize the oystershell by autoclaving were unsuccessful.

Figure 15 - Phthalate Concentrations in the Lab Scale Biofilm Column Reactor Over Time. Note that the recycled medium was replaced every two days and the phthalate analysis performed on the feed days was done prior to the medium replacement.

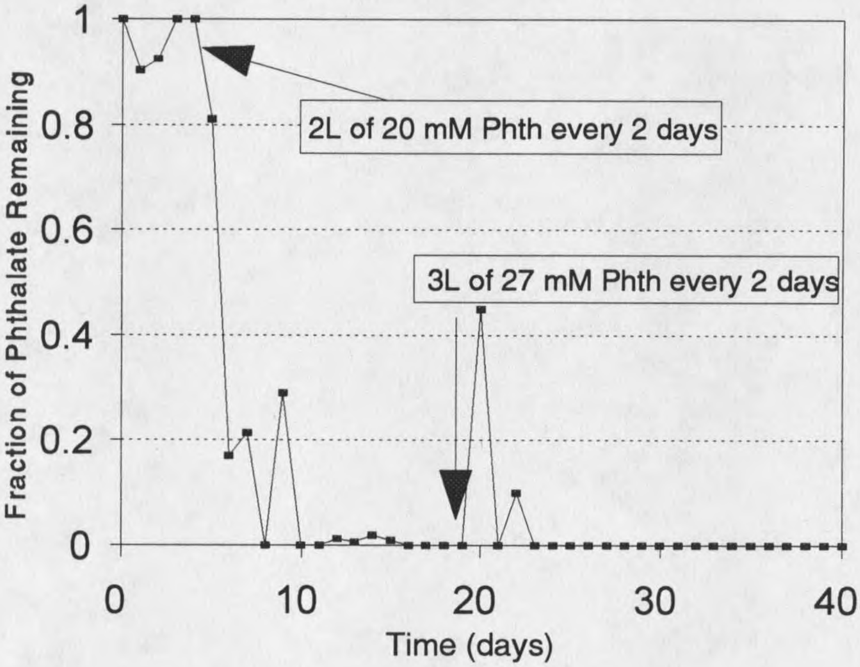
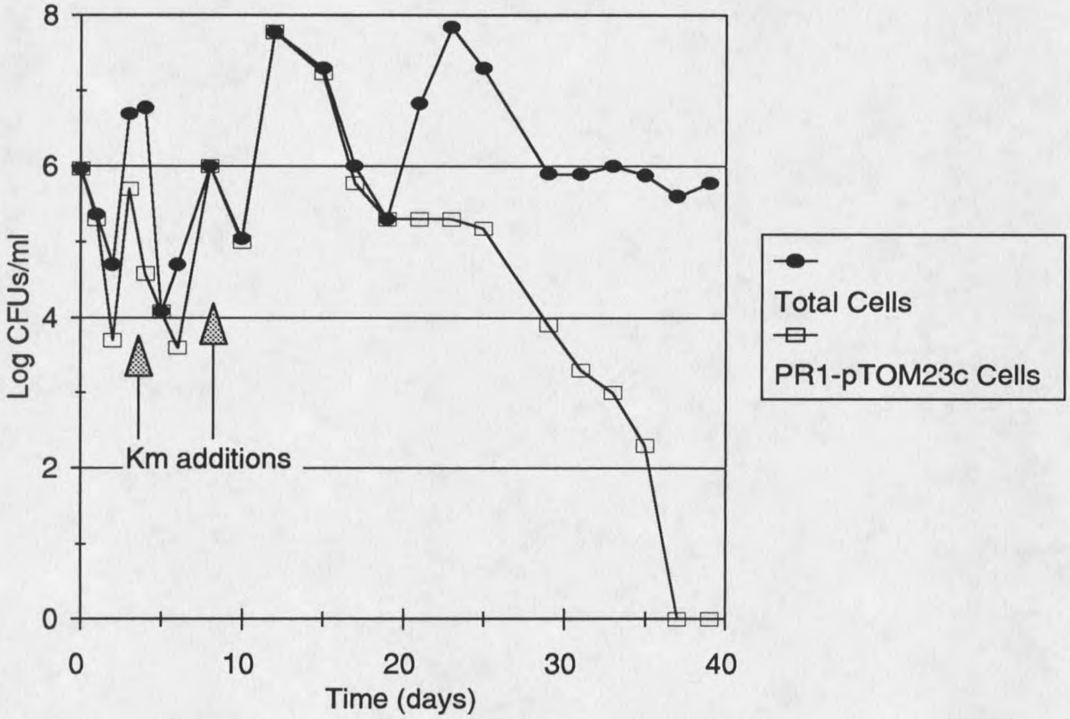


Figure 16 - Total Heterotrophic Cell Counts and PR1-pTOM$_{23c}$ Cell Counts in the Lab Biofilm Reactor Column. Note, two additions of 50 mg/l kanamycin were made at days 4 and 8 of column operation.



5.1.2 Field studies using PR1-pTOM$_{23c}$ in a VPBR

A TCE vapor phase bioreactor (VPBR) field study was conducted at Hanscom Airforce Base in Massachusetts. The goal of the field study was assess the ability of PR1-pTOM$_{23c}$ in a VPBR to treat TCE laden vapor-phase effluent from a TCE concentrator that was being operated at the site to remediate a TCE polluted groundwater aquifer.

VPBR start-up:

Toluene was used as a selective carbon source to start-up and colonize the 120 liter column reactor. Toluene was used in place of phthalate because toluene is selective for pTOM and previous experiments using phthalate as the primary carbon source resulted in poor PR1 biofilm production and eventual washout of the PR1-pTOM cells. Toluene vapor, at a gas phase concentration of 8 mg/l, was fed to the system from the bottom of the reactor at an average volumetric flow rate of 28 liters/hr (28 degrees C and 1 atm). BSM liquid medium was introduced at the top of the column at a rate of 10 liters/day. The system was inoculated with the effluent of a phthalate-Km fed PR1-pTOM$_{23c}$ continuous culture operating at a steady-state dilution rate of 0.1/hr. The liquid medium and inocula were removed from the reactor via the sump at the bottom of the VPBR.

Microbial activity of VPBRs:

Figure 17 shows the number of total heterotrophic bacteria found on the oystershell packing throughout the test column during the first 30 days of start-up. The protein content of the biomass throughout the test column is shown in Figure 18. Figures 19 and 20 show that most of the total pTOM activity and pTOM specific activity was found at the top of the test column where inoculation took place. It can be seen that 20 to 28 days after startinmg inoculation, the top of the column had the highest cell number, protein content, pTOM activity, and pTOM specific activity.

Although the cell numbers, protein contents, and pTOM activities were highest at the top of the test column, their numbers were surprisingly low demonstrating an apparent difficulty in colonizing the large reactor with PR1-$pTOM_{23c}$.

After considerable effort trying to attain an effective PR1-$pTOM_{23c}$ biofilm population within the reactor, a number of inoculations were repeated. The additional inoculations resulted in only minor increases in pTOM positive and total heterotrophic cell counts. Many of the same heterotrophs found in the test column also appeared in the control column, suggesting that the bacteria associated with the oystershell survived well under the selective toluene vapor feed.

Figure 17 - Total Heterotrophic Cell Counts in the Field Scale Test VPBR Column.

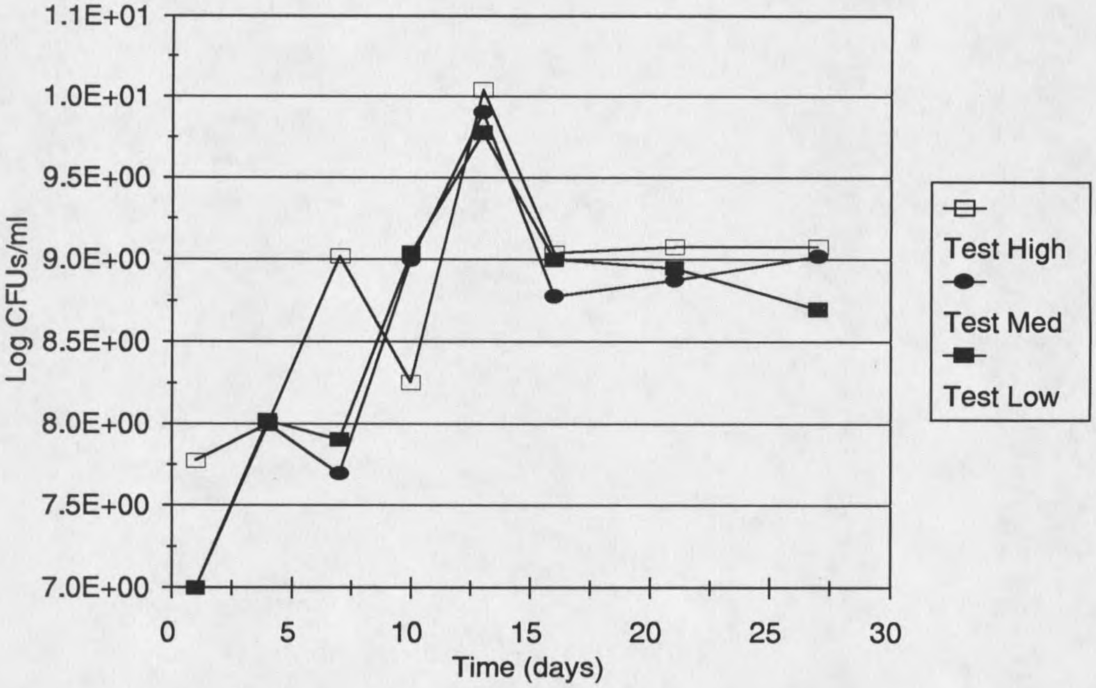


Figure 18 - Biofilm Protein Content in the Field Scale VPBR Column.

Note that the highest protein content in the column is observed at the top of the test column where the delivery of the inocula took place.