The Dissertation Committee for Claudia Kneller Gunsch Certifies that this is the approved version of the following dissertation:

Linking Gene Expression to Performance in a Fungal Vapor-Phase Bioreactor Treating Ethylbenzene

Committee:

Kerry A. Kinney, Supervisor

Mary Jo Kirisits

Gerald E. Speitel, Jr.

Paul J. Szaniszlo

Christian P. Whitman

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by

Claudia Kneller Gunsch, B.S., M.S.

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Dedication

To my husband Patrick

and my parents.

Thank you so much for all your love and support.

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Linking Gene Expression to Performance in a Fungal Vapor-Phase Bioreactor Treating Ethylbenzene

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Claudia Kneller Gunsch, Ph.D

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Supervisor: Kerry A. Kinney

Large quantities of volatile organic compounds (VOCs) are emitted from industrial sources into the atmosphere every year. Fungal biofiltration has been demonstrated to be an attractive treatment option for VOCs. In this technology, contaminated air is passed through a biologically active packed bed where the microorganisms degrade the pollutants to benign products. Most biofilter research conducted to date has relied solely on macroscale monitoring such as degradation profiles and nitrogen availability. While these macroscale parameters are important for system performance, they neglect molecular level biodegradation mechanisms in the biofilm. In this research, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify gene expression variations in the biofilm. The objective of this research was to assess the utility of the qRT-PCR tool for linking microscale gene expression to macroscale bioreactor performance and optimization. The model system used was a biofilter inoculated with the fungus *Exophiala lecanii-corni* treating ethylbenzene. A comparative threshold method was employed to quantify the relative

gene expression of the target gene to a housekeeping gene (*18S rRNA*). A portion of a key gene involved in ethylbenzene metabolism (*ElHDO* – homogentisate dioxygenase) was isolated and its gene expression monitored as a function of substrate feed, nutrient concentration and transient loading conditions. In batch experiments, qRT-PCR effectively described changes in relative gene target expression numbers (T_N) as a function of substrate mixtures and nutrient concentrations. In the biofilter, T_N was found to be a leading indicator of bioreactor failure when a repressor compound was introduced into the column feed. During the transient feed experiment, *ElHDO* expression slowly shutdown over a 24-hour time period when the ethylbenzene feed was discontinued, but rapidly recovered upon its re-introduction to the system. Overall, qRT-PCR provides valuable insights into the microscale phenomena occurring in the biofilm. However, this method may not be well suited to describe the effect of operational modifications which cause only small gene expression changes due to its low sensitivity under such conditions.

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Chapter 1: Introduction

Every year, large quantities of volatile organic compounds (VOCs) and hazardous air pollutants are released into our atmosphere. In Texas alone, over 100 million lbs of VOCs were emitted into the atmosphere in 2001 (USEPA, 2003). VOC emissions originate from a wide variety of industrial processes including petrochemical facilities and surface coating operations as well as other processes involving organic solvents. VOCs commonly emitted into the atmosphere include those found in gasoline (benzene, toluene, ethylbenzene and xylene), styrene, as well as chlorinated organic compounds (perchloroethylene, trichloroethylene and vinyl chloride). In the presence of sunlight, VOCs react with nitrogen oxides in the atmosphere to form ground level ozone - a very reactive oxidizing gas. Human exposure to high levels of ozone causes irreversible accelerated lung aging as well as severe eye, nose and throat irritation. In addition to their role in ozone formation, 188 VOCs have also been classified as hazardous air pollutants due to the adverse human health effects they cause. Benzene and vinyl chloride, for example, are known carcinogens, and ethylbenzene is classified as a possible carcinogen. As a result of their potential impacts on human health, regulations have been promulgated internationally to control VOC emissions.

For many years, physico-chemical technologies have been the primary treatment methods for VOC-contaminated waste gas streams. However, recent concerns over the rising cost of energy and the environmental impacts of abiotic control technologies have increased interest in the use of biological treatment alternatives. In vapor phase bioreactors (VPBs), such as biofilters, the VOC-contaminated gas to be treated is passed through a biologically-active packed bed. Microorganisms growing in the biofilm

attached to the packing can degrade many VOC pollutants in a cost effective and environmentally friendly manner. VPBs have been used successfully to treat a wide range of contaminated streams including BTEX, dimethyl sulfide, ethanol, methyl ethyl ketone and styrene.

Despite these successes, VPBs face several challenges that must be overcome for broader application of the technology. In particular, the elimination capacity (mass of contaminant removed per unit packing per unit time - EC) must be maximized to reduce the size and cost of biofiltration systems for high volumetric flow rate applications. In addition, a better understanding of how the biofilm responds to pollutant mixtures is needed to optimize removal of the VOC mixtures commonly found in waste gas streams. Fungal biofilters have the potential to deal with these challenges, but studies conducted to date have only examined macroscopic factors controlling EC and VOC mixture degradation in these systems. The metabolic response of the microbial population to changing environmental conditions in the bioreactor has not been monitored at the molecular scale. As a result, when the removal efficiency in the bioreactor declines or some other major perturbation occurs, it is often difficult to pinpoint exactly why these declines are occurring and how they have affected the process of most interest – the biodegradation process occurring in the biofilm. To monitor this effect, it is necessary to obtain molecular level information. Since enzymes are responsible for all biodegradation processes, it would be advantageous to monitor the activity of these specific enzymes and see how they relate to macroscale operational data. If the limitation is occurring at the enzymatic level, molecular information would be able to describe the impact of operating changes on these key processes and should allow more rapid optimization of the system.

1.1. PROBLEM DEFINITION

Most VPB research conducted to date has focused on monitoring macroscale operating parameters such as biomass accumulation, nutrient availability, pH and moisture content with the objective of linking bioreactor performance to these bulk parameters. While useful for some simple treatment applications, this approach neglects the molecular details of the biodegradation. Since these processes are ultimately responsible for the destruction of the pollutants in VPBs, a better understanding of these processes is needed to optimize performance, particularly when pollutant degradation is relatively slow and the waste gas stream contains a mixture of substrates. Batch kinetic studies of the microbial cultures found in the system are often used to delineate the processes occurring in the biofilm. While batch studies provide valuable information on maximum pollutant degradation rates and potential substrate interactions, they provide limited information about what is actually occurring in the biofilm at the molecular level in an operating biofilter. For instance, these studies cannot provide direct evidence of whether or not substrate inhibition is occurring as a result of transcriptional or translational regulation. Thus, while bioreactor operating data and batch studies can be used to paint a picture of a biofilter's operation, it would be useful to understand the biodegradation processes actually occurring in the biofilm at a molecular level.

Just as most of the research to date has focused on macroscale investigations of biofilter performance, most of the work has concentrated on bacterial degradation of VOCs in these systems. Although fungal systems present a number of advantages over bacterial systems, little research has focused on fungal biofiltration applications. Recently, Woertz et al. (1999) showed that the dimorphic fungus *Exophiala lecanii-corni*

can be used to treat several VOCs including toluene and ethylbenzene and can maintain its degradative capacity under much harsher operating conditions than bacteria. *E. lecanii-corni* was able to sustain degradation of toluene at low pH values and high contaminant concentrations both of which are inhibitory to bacteria. In addition, *E. lecanii-corni* regained its high elimination capacity more quickly and survived much longer after shutdown periods. While much work has been done to optimize toluene degradation in biofilters inoculated with *E. lecanii-corni*, the ability of *E. lecanii-corni* biofilters to treat other VOCs, as well as mixtures of VOCs, has not been studied. Ethylbenzene is of particular interest, because many studies have shown that substrate interactions occur between ethylbenzene, toluene and other substrates. In addition, ethylbenzene degradation rates are generally slower than those of toluene. Therefore, we would greatly benefit from understanding how ethylbenzene degradation is occurring at the molecular level and from determining if molecular monitoring of the VPB biofilm can be used to explain the macroscale bioreactor performance.

1.2. OBJECTIVES

The main objective of this research was to determine if gene expression studies can be used to delineate how substrate interactions and bioreactor operation affect pollutant degradation at the molecular level in a VPB. This study focused on a fungal biofiltration system degrading ethylbenzene. The effect of single substrate (ethylbenzene) and multiple substrates on ethylbenzene gene expression were determined. Similarly, the impact of two key operating parameters (nitrogen availability and shutdown/startup conditions) were delineated. Specific objectives were:

- Identify a gene in the ethylbenzene degradation pathway in *E. lecanii-corni* that can be used in gene expression studies;
- Optimize the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) technology to allow quantification of gene expression in a fungal biofilm degrading ethylbenzene only;
- Determine whether qRT-PCR can be used to understand how substrate mixtures, nitrogen availability and shutdown/restart conditions affect ethylbenzene degradation in a VPB; and
- Assess whether qRT-PCR accurately reflects macroscale pollutant degradation observed in a fungal VPB.

1.3. RESEARCH HYPOTHESIS AND APPROACH

This research investigated the use of qRT-PCR as a method to follow gene expression patterns for *E. lecanii-corni* degrading ethylbenzene. Specifically, an analysis was performed using a gene involved in ethylbenzene metabolism to determine if this method can provide some insight as to the relationship between macroscale bioreactor performance and molecular level information. In addition, because ethylbenzene is commonly found in contaminated air stream mixtures, it was of interest to determine the impact of other compounds on ethylbenzene degradation kinetics. qRT-PCR was used and its potential for *in situ* enzymatic regulation studies assessed.

As a first step, *E. lecanii-corni* ethylbenzene degradation was characterized in batch systems and in *E. lecanii-corni* VPBs. Next, experiments were performed to postulate a metabolic pathway for ethylbenzene degradation in *E. lecanii-corni* and to determine some of the regulatory mechanisms involved in its degradation. In addition,

this information was used to identify a gene of interest from the *E. lecanii-corni* genome that could be used in ethylbenzene qRT-PCR gene expression studies.

Once this gene was identified, the qRT-PCR system was optimized for a single substrate system. cDNA was synthesized from total RNA samples isolated from batch reactors, as well as from a VPB biofilm using ethylbenzene as the primary substrate. Relative gene expression quantification was then performed by normalizing the expression of the gene of interest to *18S rRNA*. After confirming that qRT-PCR could be used to monitor gene expression of ethylbenzene biodegradation in a single substrate system, its utility was evaluated for the interpretation of mixed substrate situations. *E. lecanii-corni* cultures growing on ethylbenzene were spiked in batch systems with varying concentrations of other substrates (e.g., phenyl acetate, styrene, methyl propyl ketone, and benzene) to determine which compounds act as repressors, inducers or have no effect on ethylbenzene kinetics. The effect of a regulatory substrate from each category (repressor, inducer and no effect) were analyzed in a continuously fed ethylbenzene bioreactor. In addition, the effect of nitrogen supply and shutdown/startup were analyzed. Pollutant degradation profiles were obtained for each treatment condition and compared to qRT-PCR results to determine if the molecular level gene expression information could be linked to overall VPB performance.

To date, very little research has been performed using qRT-PCR to monitor gene expression in environmental biofilm systems. This research uses this tool to quantify VPB gene expression and assess its utility as a complement to traditional methods for monitoring biological treatment systems such as biofilters.

Chapter 2: Background and Literature Review

Biofiltration has become an increasingly popular treatment method for VOCs. In the biofiltration process, VOCs from the waste gas diffuse into an aqueous biofilm where they are removed by biological oxidation. Numerous studies have shown that VPBs can be used to treat a variety of VOCs, as well as inorganic compounds such as H_2S. Many of these studies attempt to link macroscale level measurements to bioreactor performance without truly having an understanding of the mechanisms occurring at the molecular level.

This chapter provides an overview of methods that can be used to describe biodegradation performance in biofilters, emphasizing each method's advantages and drawbacks. The first section of this chapter describes the biodegradation processes that occur in VPBs and summarizes the common types of airstreams treated as well as microorganisms used to treat aromatic pollutants in these systems. The second section presents an overview and discussion of methods used to interpret bioreactor performance at the macroscale and at the molecular level. This section ends with a description of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and shows how this method can be used to describe gene expression in a VPB. Since the research focuses on VPBs degrading ethylbenzene, the last section summarizes the known biodegradation pathways for ethylbenzene.

2.1. VAPOR-PHASE BIOREACTORS

Biofilters have been used to remove hydrogen sulfide as well as a variety of VOCs from a wide range of processes. Biofiltration systems can be classified according to the mobility of the microorganisms and liquid-nutrient phase, as well as the packing media to which the microorganisms are attached. This section provides an overview of the different types of VPBs, their basic operational principles, the range of pollutants that they can treat, and their performance criteria.

2.1.1. Basic Types of VPBs

There are three types of VPBs. Each class is categorized based on the mobility of the liquid phase and the location of the microorganisms (i.e., suspended growth or biofilm). The three types are biofilters, bioscrubbers and biotrickling filters. These are described in the following paragraphs.

Biofilters are the simplest type of biological treatment reactor used for the treatment of air pollutants. As shown in Figure 2-1A, in this process, a contaminated airstream is passed through a packed bed. Microorganisms grow in the bed and create a biofilm into which the contaminant diffuses. The microorganisms use those chemicals as a source of carbon and energy and metabolize them to carbon dioxide, water and biomass. Additional nutrients, when necessary, are provided to ensure optimal operating

conditions. The packing bed usually contains bulking agents and natural materials that can provide large surface areas (van Groenestjin and Hesselink, 1994).

Bioscrubbers are similar to gas absorption systems, but are complemented by a separate activated sludge bioreactor as shown in Figure 2-1B. Contaminated airstreams are passed through a spray scrubber packed with inert media, and the pollutants are transferred from the airstream to the liquid phase. The liquid phase is then passed to another bioreactor containing activated sludge that completes the biotreatment of the pollutant to more benign compounds. Liquid with suspended microorganisms is continuously recirculated in the bioscrubber making it easier to control reaction conditions in the bioscrubber as compared to the biofilter (van Groenestjin and Hesselink, 1994).

Biotrickling filters are a variation of the biofilter technology. A schematic of a biotrickling filter can be seen in Figure 2-1C. Just as in a biofilter, microorganisms grow on the packing media and degrade the contaminants from the air stream. However, in biotrickling filters, synthetic inert packing media such as plastic pall rings, ceramic pellets and foam are used. As a result, biotrickling filters require an external source of nutrients. Also, as in bioscrubbers, a liquid stream is sprayed over the packed bed and continuously recirculated. However, unlike bioscrubbers, absorption and biodegradation occur in a single reactor. Reaction conditions in biotrickling filters are generally much easier to control than in biofilters (van Groenestjin and Hesselink, 1994).

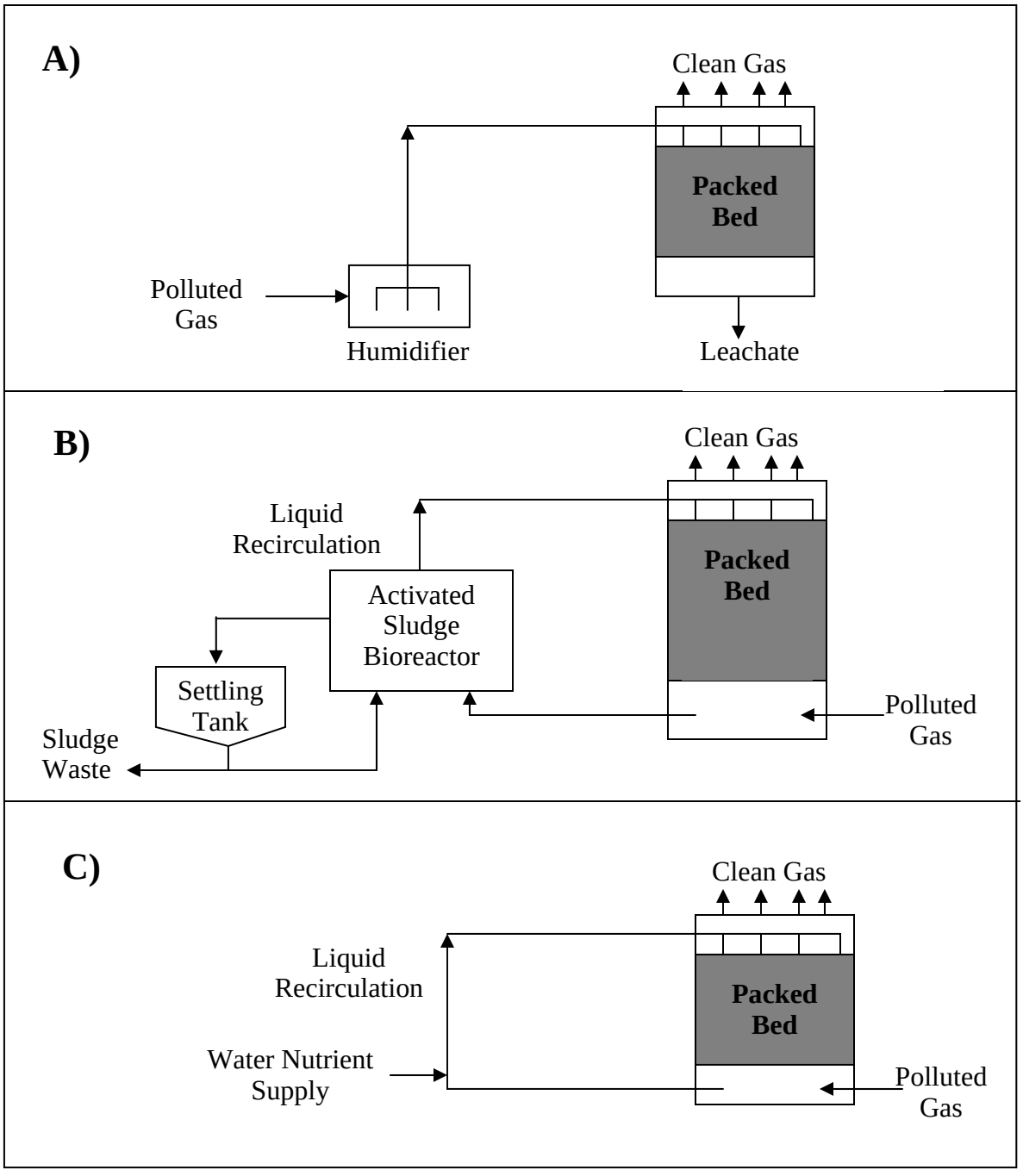

Figure 2-1: Schematic of the three types of VPBs. A) biofilter, B) bioscrubbers, C) biotrickling filter. (van Groenestjin and Hesselink, 1994; Kinney, 1996)

2.1.2. Basic Operational Principles of Biofiltration

For biodegradation to occur, the contaminant must first transfer into the biofilm. The pollutant biodegradation occurs in two steps. First, the contaminant is transferred from the gas phase to the liquid biofilm phase that contains the microorganisms. Second, microorganisms degrade the target pollutant within the biofilm using it as a carbon and energy source. Figure 2-2 illustrates this process.

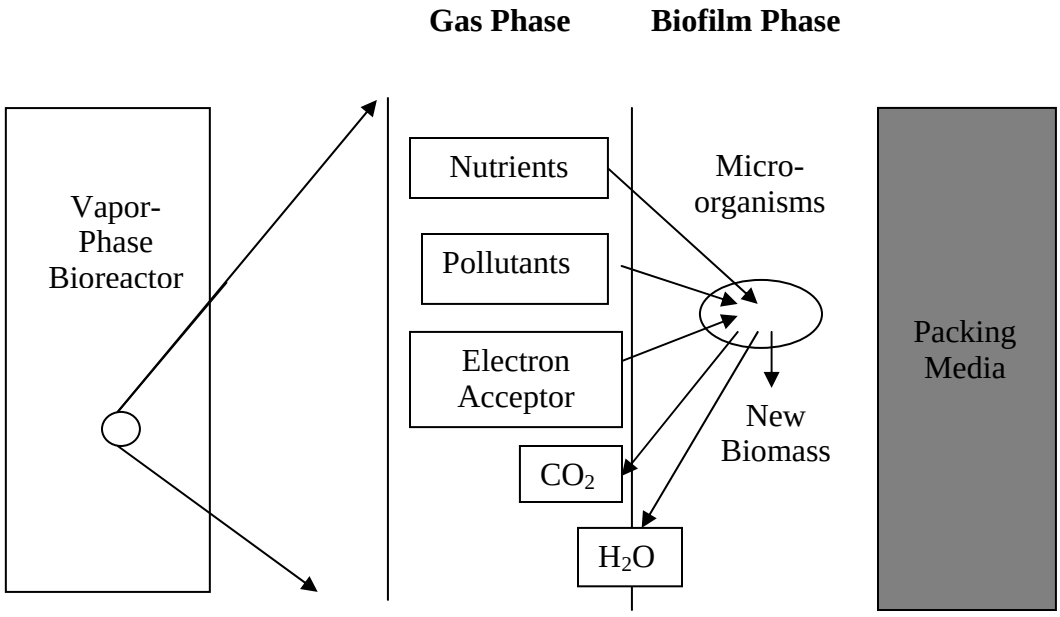

Figure 2-2: Microscale biological degradation of contaminants in VPBs

Henry's law is used to estimate equilibrium pollutant concentrations between the liquid and gas phases. In VPBs, however, biofilms are rarely under equilibrium conditions since VOCs are continuously degraded across the biofilm depth. Therefore, it

is common to assume that the VOC concentration at the gas/biofilm interface is in equilibrium with the bulk VOC concentration in the gas phase and that the VOCs are transferred into the biofilm phase, driven by a concentration gradient. Pollutant mass transfer can be described by the general equation shown below:

$$\frac{dC_i}{dt} = K_L a\,(C^* - C_i) \qquad \text{(Equation 2-1)}$$

where K_L is the mass transfer coefficient (m/hr), a is the interfacial area per unit volume (m^2/m^3), C_i is the concentration of pollutant in the liquid phase (mg/L) and C* is the concentration of pollutant in the gas phase at equilibrium with the liquid phase (mg/L).

K_L is dependent on the gas flow characteristics (diffusion and dispersion) while the wetted interfacial area depends on the structural form of the biofilm (pore shape and particle size). Therefore, mass transfer rates and ultimately pollutant removal rates are greatly impacted by the configuration of the biofilm and the physical/chemical properties of the pollutant. Once VOCs are transferred into the biofilm phase, biodegradation is controlled by the kinetics of the microorganisms capable of degrading the pollutant of interest. Several groups of organisms, including bacteria, fungi and some higher organisms, are used for VOC biodegradation in VPBs. A description of the types of airstreams that can be treated by VPBs, as well as a summary of the species used in these studies will be presented in the next two sections.

2.1.3. Applications

Biofiltration is used to provide odor control from wastewater treatment plants and industrial processes and the treatment of VOC-contaminated industrial airstreams (Ottengraf et al., 1986; van Groenestijin and Hesselink, 1994; Swanson and Loehr, 1997). Typically, industries emitting large volumes with low concentrations of target compounds can use biofiltration for the treatment of their waste gas streams. A summary of the industries that have used biofilters can be found in Table 2-1.

Table 2-1: Industries using biofilters

Chemical Operations	Composting Facilities	Cocoa Roasting
Film Coating	Slaughter Houses	Flavor and Fragrances
Print Shops	Waste Oil Recycling	Coffee Roasting
Chemical Storage	Landfill Gas Extraction	Fish Frying
Paint Spray Booths	Investment Foundries	Tobacco Processing
Pet Food Manufacturing	Industrial Wastewater Treatment	Municipal Wastewater Treatment

(Leson, 1991; Friedrich et al., 2003)

In general, VPBs cannot be used to treat extremely high concentrations of air pollutants because of toxicity effects on microbial growth. In addition, under high loading conditions, VPBs have a high potential for clogging, and large bioreactor sizes are required. However, overally, they tend to have lower costs and less secondary waste stream problems as compared to traditional physical/chemical treatments (Bohn, 1996;

Lu et al., 2000; Deshusses, 1997). Biofiltration can be used to remove VOCs and reduced sulfur or nitrogen compounds. A list of the chemicals that have been treated by biofiltration is shown in Table 2-2.

Table 2-2: List of chemicals commonly treated by biofiltration

Acetate	Diethyl amine	Methyl ethyl ketone
Acetone	Dimethyl disulfide	Methyl propyl ketone
Ammonia	Ethanol	Methyl mercaptan
Benzene	Ethylbenzene	Nitrogen oxide
Butanol	2-Ethyl hexanol	Nitrogen dioxide
Butylaldehyde	Hexane	Pentane
Butyl acetate	Hydrogen sulfide	Scatole
Carbon monoxide	Indole	Styrene
Monochloromethane	Isopropanol	Toluene
Dichloromethane	Methane	Tetrachloroethylene
Trichloromethane	Methanol	Xylene

(Teran Perez et al., 2002; Lu et al., 2002; Shah et al., 2003; Ortiz et al., 2003; Fitch et al., 2002; Li and Moe, 2003; Chang and Lu, 2003; Qi et al., 2002; du Plessis et al., 1998; Demeestere et al., 2002; Kim et al., 2002; Zilli et al., 2003; Dupasquier et al., 2002; Darlington et al., 1998)

2.1.4. Microbial Species used in VPBs

Several studies have looked at degradation parameters for a number of aromatic compounds in single and mixed substrate situations. However, only results for toluene, ethylbenzene and styrene will be presented because of their relevance to this research. As shown in Table 2-3, the vast majority of these studies were performed with bacteria.

This section first presents applications using bacteria followed by a description of their fungal counterparts.

Many studies have been performed using single bacterial species as well as mixtures of bacteria for the degradation of monoaromatic hydrocarbons. These studies are summarized in Table 2-3. As shown below, these studies were performed in batch reactors, fibrous bed reactors and biofilters.

Table 2-3: Bacterial degradation of aromatic VOCs

VOC(s) Treated	**Bacterial Culture**	**Reactor Type (Packing)**	**Reference**
BTEX Mixture	*Pseudomonas putida* and *Pseudomonas fluorescens*	Fibrous Bed Reactor	Shim et al., 1999
BTEX Mixture	Mixed Culture	Biofilter (Compost)	Namkoong et al., 2003
Toluene, Ethylbenzene and Xylene	Mixed Culture	Biofilter (living botanical matter packing)	Darlington et al., 2000 and 2001
Toluene	Mixed Culture	Biofilter (Peat)	Morales et al., 2003
Toluene	Mixed Culture	Biofilter (polyurethane foam)	Moe and Irvine, 2001
BTEX	Engineered *Pseudomonas putida* F1	Batch Reactor	Choi et al., 2003
Benzene, Toluene and Ethylbenzene	Mixed Culture	Batch Reactor	Yerushalmi and Guiot, 1998
Styrene	*Rhodococcus rhodochrous* AL	Biofilter (Peat)	Zilli et al., 2003
Styrene	Mixed Culture	Biofilter (Compost)	Juneson et al., 2001
Styrene	Mixed Culture	Biofilter (Coal)	Lu et al., 2001
Toluene	*Pseudomonas putida* F1	Biofilter (Celite)	Woo et al., 2000
Toluene	Mixed Culture	Biofilter (Celite)	du Plessis et al., 1997
Toluene	Mixed Culture	Biofilter (Peat moss and chicken manure)	Juteau et al., 1999
Toluene	Mixed Culture	Biofilter (Peat)	Acuna et al., 1999
Chlorobenzene and o-dichlorobenzene	Mixed Culture	Biofilter (Perlite)	Oh and Bartha, 1994
Toluene and Styrene	*Acinetobacter* sp. and *Rhodococcus rhodochrous* AL	Biofilter (Peat and Glass Bead)	Zilli et al., 2003
Toluene, Ethylbenzene and o-xylene	Mixed Culture	Biofilter (Perlite)	Veiga et al., 1999
BTEX	Mixed Culture	Biofilter (Pine bark packing)	du Plessis et al., 2001
Toluene, Ethylbenzene and o-xylene	Mixed Culture	Biofilter (Perlite)	Veiga and Kennes, 2001

As shown below (Table 2-4), several biofiltration studies have also been conducted with fungi. Fungal species have several advantages over bacteria. They can withstand much higher concentrations of VOCs, as well as lower pH and moisture conditions than their bacterial counterparts. In addition, they regain their degradation capacity much faster after shutdown periods. One drawback of fungal systems is the potential for a higher pressure drop along the column as compared to bacterial systems caused by biomass clogging.

Table 2-4: Summary of fungi used for VOC treatment in biofilters

VOC Treated	**Fungi**	**Reference**
Toluene	*Scedosporium* sp. and *Cladosporium* sp.	Auria et al., 2000
Toluene	*Exophiala lecanii-corni*	Woertz et al., 1999
Styrene	*Exophiala jeanselmei*	Cox et al., 1993 and 1997
Styrene	*Exophiala jeanselmei*	Kraakman et al., 1997
Toluene, Xylene and Ethylbenzene	Fungal Mixture	Kennes et al., 1996
Toluene	*Cladosporium spaerospermum*	Weber and Hartmans, 1996 and Weber et al., 1995
Styrene	Fungal Mixture	Van Groenestijn and Hesselink, 1994
Styrene	White Rot	Majcherczyk et al., 1989
Toluene	*Scedosporium apiospermum* TB1	Garcia-Pena et al., 2001

In addition to the studies performed in VPBs, aromatic VOC removal has also been demonstrated for several other fungi. Prenafeta-Boldu et al. (2002) showed that *Cladophialoaphora* sp. strain T1 grew on a mixture of BTEX compounds. It metabolized toluene, xylene and ethylbenzene and cometabolized benzene. Yadav and Reddy (1993) showed that the lignin-degrading basidomycete *Phanerochaete chrysosporium* degraded a BTEX mixture. All four compounds were mineralized, although benzene degradation kinetics were much slower. Qi et al. (2002) studied five different fungal species and their ability to grow on benzene, toluene, ethylbenzene and styrene in Petri dishes exposed to VOC-saturated air. *Cladosporium resinae* was able to grow on toluene and ethylbenzene. *Cladosporium sphaerospermum* grew on benzene, toluene, ethylbenzene and styrene. *E. lecanii-corni* grew on toluene, ethylbenzene and styrene. *Mucor rouxii* was not able to grow on any of the 4 substrates and *Phaenerochaete chrysosporium* did not grow on benzene, toluene and ethylbenzene. Even though fungi have certain advantages over bacteria, a review of the literature indicates that only a limited amount of research has focused on their use in engineered systems, especially in VPBs.

2.2. BIOREACTOR PERFORMANCE CRITERIA

Several performance criteria are used to compare VPB treatment efficacy. No single parameter, however, has been used which can adequately describe bioreactor performance. Several parameters are needed to clearly depict what is occurring in the biofilm and, therefore, the biodegradation process (Murphy et al., 1995). Generally, overall removal efficiency, elimination capacity and pressure drop are used in conjunction with other simple measurements such as biofilm thickness, total dry weight,

total cell counts and nutrient levels. However, as described in this section, however, these parameters often inaccurately reflect biofilm biodegradation mechanisms since VPB performance is not directly proportional to the total quantity of biomass, but rather is influenced by a variety of operational parameters. A description of the most commonly used macroscale measurements is presented in this section along with a discussion of the factors which affect biofilter performance.

2.2.1. Macroscale Measurements

Removal efficiency (RE) is described as the fraction of inlet pollutant removed in a VPB. Since RE varies with residence time, inlet concentration and microbial activity, this parameter is only applicable to a specific set of operating conditions. Therefore, this measure is only helpful when comparing VPB experimental results obtained under a given set of conditions.

Elimination capacity (EC) is another common measure that is used to assess VPB performance. EC is described as follows:

$$EC = \frac{Q\,(C_{in} - C_{out})}{V} \qquad \text{(Equation 2-2)}$$

where C_{in} is the inlet concentration (g/m^3), C_{out} is the outlet concentration (g/m^3), Q is the gas flow rate (m^3/hr) and V is the bioreactor packed bed volume. EC has units of g pollutant/m^3.hr. To determine the EC of a bioreactor, the inlet concentration is typically increased in a stepwise fashion for several hours until the new steady state is reached at each concentration level. Several different loadings (mass of compound applied to the

column per volume per unit time) are applied to the VPB, and the corresponding ECs are calculated using Equation 2. For each loading, a depletion profile is generated and is used to determine the maximum EC as well as the critical loading. Maximum EC is defined as the point where an EC curve has its highest value, while critical loading is defined as the point at which the EC starts to deviate from the 100% removal line (Deshusses and Johnson, 2000).

Pressure drop is another parameter that is used as a measure of bioreactor performance. Excess biomass accumulation creates high pressure drops across bioreactor columns and can therefore hinder the operation of VPBs. Pressure drop measurements across the filter bed can be used to indicate excess biomass accumulation in pore space. Deront et al. (1998) studied the relationships between pressure drop and friction factor, Reynolds number and macroporosity. This study proposed that biofilm density and thickness could be estimated using the pressure drop measurements as a function of organic loading rates. However, high pressure drops and clogging in filter beds were not directly correlated to biomass accumulation unless biomass expansion significantly narrows down the flow passage. In a study correlating pressure drop and characteristic clogging time to critical voidage in a biotrickling filter, Okkerse et al. (1999) found that pressure drop increased steeply only after the voidage of the filter bed dropped below a critical value. Therefore, a rising pressure drop across a bioreactor column indicates that excess biomass has already blocked a significant portion of the total void space between in the bed.

While EC, RE and pressure drop provide valuable information about the bioreactor performance, they do not explicitly take into consideration the quantity of microorganisms in the VPB that are actively degrading pollutants. Rather, they only provide a bulk measure of performance. For instance, EC will vary with time of operation and is dependent on biomass quantity and activity, nutrient supply as well as other operating parameters. Therefore, when using these macroscale parameters to determine their correlation to VPB performance, it is important to carefully compare their values between experiments especially when the quantity of biomass varies significantly over the operational period. The following section will describe some of the parameters that may affect the quantity of the active biomass fraction and therefore the overall VPB performance.

2.2.2. Factors Affecting Biofilter Performance

Several parameters have been shown to play an important role in VPB performance. These include substrate mixtures, length of reactor shutdown periods, nutrient concentrations, pH and moisture content. However, while each of these parameters has been shown to affect biofilter performance through the macroscale measurements described in the previous section, little information has been obtained about the molecular changes responsible for the differences in overall VPB performance. The effect of each of these parameters is described in the following paragraphs.

2.2.2.1. Substrate Mixtures

The presence of additional substrate can have a strong effect on the degradation capacity of a culture for a specific compound. For instance, the presence of an easily degradable compound could increase degradation rates because it enables faster biomass accumulation. On the other hand, it may decrease the degradation rate by decreasing the metabolic flux (rate at which material is processed through a metabolic pathway). The following section discusses some studies that have analyzed the effect of adding substrates on the overall metabolic degradation rates.

In batch systems, Bhattacharaya et al. (2001) found that the presence of ethanol increased o-dichlorobenzene degradation rates, and Lovanh et al. (2002) found that it increased BTEX removal rates. At low concentrations, ethanol had the effect of initially

decreasing the toluene degradation rates; its presence was solely linked to increased biomass growth. Eventually, however, the biomass increase resulted in faster toluene degradation rates. This was also the case for benzene. At high ethanol concentrations, however, the opposite was found to occur. The authors suggested that this was caused by the oxygen limitations caused by the ethanol linked metabolism. Deeb et al. (2001) found that the presence of MTBE decreased benzene and toluene degradation rates in mixtures. They postulate that this is caused by the preferential degradation of MTBE over benzene and toluene

Mohseni and Allen (2000) found that the presence of methanol totally suppressed the growth of a α- pinene-degrading community in a batch reactor. In a biofilter, the presence of methanol reduced the α-pinene removal rates, but did not completely supress it. α-pinene removal rates gradually decreased as methanol loadings were increased, indicating a definite inhibition on metabolism. Methanol degradation rates remained constant regardless of α- pinene loading. Similar results have been observed in biofilters treating toluene and ethyl acetate. High loadings of ethyl acetate inhibited the removal of toluene (Mohseni et al., 1999).

As demonstrated in the studies summarized above, substrate mixture interactions are especially difficult to define because they vary for each situation. It is dependent on the microbial species used in the biofilter, as well as on the compound being treated and the corresponding loading. However, while these studies have shown that these interactions undoubtedly occur and affect the overall VPB performance, the macroscale

measurements do not provide any explanation for why and how these inhibitions are occurring in the biofilm.

2.2.2.2. Nutrient Supply

Nutrient composition has also been shown to significantly impact both the structure and the physicochemical nature of the biofilm (Moller et al., 1997). As a result, nutrient availability has a direct influence on pollutant EC and bioreactor performance. Pedersen et al. (1997) suggest that nutrient availability may cause limitations on biodegradation activity and microbial growth. Grady and Lim (1980) suggest that total carbon to nitrogen and phosphorous (C:N:P) ratios of at least 100:5:1 are generally necessary to maintain microbial activity without serious nutrient limitation. In another study, Cherry and Thompson (1997) showed that the addition of a commercially available fertilizer (N-P-K) helped a compost biofilter regain microbial activity and removal efficiency when the reaction performance of the biofilter dropped after long-term hexane degradation.

Nitrogen plays an important role in microbial growth. Since nitrogen is one of the major cell constituents (approximately 8-13% of dry cell weight), it has the potential to limit cellular growth if adequate amounts are not available, as demonstrated in several studies. For example, Corsi and Seed (1995) found that total available nitrogen levels in excess of 200 mg/kg were required to obtain high pollutant removal efficiency in biofilters packed with various compost materials. Gribbins and Loehr (1998) also found that nitrogen affected biofilter performance. In this study, they suggest that soluble

nitrogen concentrations greater than 1000 mg N/ kg dry weight is required to maintain stable bioreactor performance. Nutrient supply has also been shown to affect degradation kinetics. Pedersen et al. (1996) showed that a species of *Pseudomonas putida* exhibited maximum growth rates in a nitrogen-rich environment when degrading toluene.

2.2.2.3. Transient Loading

Another parameter that affects VPB performance is dynamic feed conditions. Some VPB applications include frequent startup and shutdown periods which can have a detrimental effect on biomass maintenance. Several studies have shown that a substantial decrease in biomass activity occurs under carbon-deprived conditions (van Groenistjin, 1994). Re-acclimation periods ranging from several hours to days are often required to reestablish degradation capacities similar to that prior to the carbon-deprived period (Martin et al., 1996; Wani et al., 1998; Tang et al., 1996).

Microbial systems also tend to respond poorly under non-steady state operating conditions. Choi et al. (1998) found that as long as the shutdown periods were short (on the order of hours), there was no effect on biotrickling filter operation. However, Tang et al. (1996) found that in a VPB system, when operating conditions were modified, more than three days were required to reach a new steady-state.

In general, the longer the shutdown period, the longer the re-acclimation period required to return to high removal efficiency. Park and Kinney (2001) showed that the re-acclimation time can be reduced by using a slip feed system. The introduction of a

low flow surrogate slip gas stream to the bioreactor during 3 and 7 days shutdown periods reduced the re-acclimation time by as much as 70% in these slip feed systems. This suggests that the enzymes necessary to process the contaminants were expressed earlier in the slip feed systems as demonstrated by the reduction in re-acclimation time. This needs to be confirmed by performing microscale level studies.

2.2.2.4. pH and Moisture Content

pH and moisture content also affect VPB performance. Optimal pH for bacterial biofilters generally range from 6 to 9, while fungal biofilters can sustain growth at lower pH values (Leson, 1991; Madigan et al., 2000). Because the microbial metabolism occurring in biofilters often leads to the production of acidic products, such as sulfuric acid, nitric acid and hydrochloric acid, buffers are often required to maintain pH levels in the optimal range for a specific biofiltration process (Sanchez-Pena et al., 2000; van Groenistjin, 1994).

Moisture content also affects biofilter performance. Non-optimal moisture content can lead to compaction, breakthrough of incompletely treated waste gas and the formation of anaerobic zones that emit odorous compounds (Leson, 1991). Deshusses and Johnson (1999) showed that poor humidity control leads to drying and poor pollutant elimination capacity. Rapid and nearly complete loss of biological activity was observed upon drying in a VPB treating ethyl acetate. Moisture contents of 40-60% by weight should be maintained for optimal biofilter operation in bacterial systems (Ottengraf,

1986). Fungal VPBs can maintain high removal efficiencies at much lower moisture contents (20-30%). However, if they drop much lower than 20%, their degradation efficiciency declines (Woertz et al., 2001).

As described in this section, substrate mixtures, nutrient supply, transient loadings, pH and moisture content have all been shown to affect the RE in biofilters. While it is intuitive that these parameters act to modify the microbial activity in the biofilm and by extension the corresponding EC, few studies have linked the effect of changes in these parameters to biofilm activity. The following section describes methods that are used to measure biofilm activity.

2.3. BIOFILM ACTIVITY

While bioreactor performance and elimination capacity are strongly dependent on microbial activity, relatively little attention has been given to biomass activity in VPBs (Moller et al., 1997; Mirpuri et al., 1997; Song and Kinney, 2000; Shingelton et al., 2001; Song and Kinney, 2001). Some analytical techniques are available to measure general and specific microbial activity. Most methods have been developed for planktonic cells and require some adjustment for application to biofilms. The two general types of techniques used to estimate biofilm activity are microelectrode and biochemical/molecular tests.

2.3.1. Microelectrode Methods

Microelectrodes can be used to measure concentration profiles of reactants across the biofilm depth. These concentration profiles can in turn be used to determine microbial activity of that biofilm. One of the advantages of this method is that it provides insight into micro-environments, micro-kinetics and mass transfer resistance within the biofilm. It is particularly useful for studies of multi-species biofilms. This method has been used to estimate the activity of nitrifying bacteria by measuring fluxes of ammonia, oxygen, nitrate and pH (de Beer et al., 1993). It has also been used to determine competition among microbial species for substrate and space within different types of biofilms (Zhang et al., 1995).

However, even though this method is used to estimate microbial activity, it can lead to flawed results if used on its own to depict what is occurring in the biofilm, because of the limited coverage of the microelectrodes. In addition, its application is generally limited to that of a simple flat-plate biofilm system. It is not conducive to a more complex system with more realistic geometric features and/or older and thicker biofilms.

2.3.2. Biochemical, DNA and rRNA based Molecular Biology Tools

Oxygen uptake rates (OUR) and CO_2 evolution are common measurements used to describe respiratory activity. Their measurement, however, is extremely sensitive to experimental conditions and it can be difficult to separate uptake rates resulting from substrate degradation from uptake rates due to endogenous respiration. Substrate uptake ratios have been used to measure respiratory activity by using ^{14}C-labeled toluene. In this study, the specific pollutant-degrading activity was normalized by toluene-degrading cell counts. Mirpuri et al. (1997) concluded that the specific activity of suspended cells was the same as that for cells in biofilms.

Dehydrogenase activity has also been used to estimate microbial activity in a variety of biomass samples (Lazarova and Manem, 1995). Since all respiring microorganisms have an active electron transport system that consists of several dehydrogenases, their activity should be directly linked to overall microbial activity. This activity is measured by determining the ability of microorganisms to reduce an oxidoreduction dye as an indicator under defined conditions. Tetrazolium salts such as 2,3,5-tri-phenyltetrazolium chloride (TTC), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) are commonly used as indicators of dehydrogenase activity (Anderson et al., 1988; Blenkinsopp and Lock, 1990). After reduction, these indicators are transformed to distinct, insoluble crystals that are easily quantified by spectrophotometry. The greatest disadvantage of this method is that it targets all microorganisms in the system. Thus, one

cannot separate out a specific organism's activity or look at the activity related to a specific pollutant's degradation.

More recently, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) measurements have been used. Since RNA and DNA molecules are essential components of microorganisms and their synthesis is a function of cell growth rate and activity, they are good candidates for providing a more direct measure of biomass quantity and to some degree biomass activity. Most work to date in biofilms has only dealt with using DNA to identify specific microorganisms in a system. This approach does not provide a correlation to activity but rather offers information about the presence and organization of specific microbial populations. The two most common DNA-based techniques are denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH). Both these methods make use of the 16S rDNA gene. DGGE uses polymerase chain reaction (PCR) to amplify a specific region from the 16S rDNA gene. These fragments are then separated on a denaturing polyacrylamide gel using formamide and urea based on the various melting points of each fragment. While this method can be used to determine the diversity of microorganisms over time in a system, it does not provide any information about the activity of each species or even of the whole consortium.

FISH also makes use of 16S rDNA by using probes that are fluorescently labeled. Cells are treated with reagents that cause them to become permeable, allowing the fluorescent probe to penetrate the cell. Following hybridization of the probe directly to the rRNA in the cell's ribosome, the cells become uniformly fluorescent and can be

observed under a fluorescent microscope. This technique can be applied directly to cells in culture or in a natural environment (Madigan et al., 2000). While this method only targets active cells, it does not indicate the level of activity of each cell within the system, it only indicates their presence (Amann et al., 1995). In addition, while this method can be applied to determine if a broad class of organisms is present (i.e., Bacteria vs. Eukarya) if one wants to detect a specific organism, a 16S rDNA sequence for that organism must be known. One advantage of this method is that it is *in situ* and therefore gives a more accurate depiction of actual conditions.

More recently, microarray DNA chips have been used to study the presence of a specific organism in a system (Denef et al., 2003). In this method, several hundreds of known genes are affixed to a nylon or glass microarray plate and complementary DNA (cDNA) hybridizes to them and fluoresces. The pattern and extent of fluorescence provides information on the presence as well as the quantity of each target gene present in the mixture. This work is also being extended to RNA which is a much better indicator of actual activity in a system. The following section describes qRT-PCR, a promising method which can be applied to quantify biofilm levels of gene expression.

2.3.3. mRNA based Molecular Biology Tools

mRNA molecules carry the information necessary for cells to build the enzymes required for pollutant metabolism. In contrast to cellular DNA levels, which remain constant, mRNA levels vary as a response to cellular requirements. When environmental

conditions are optimal, a greater quantity of mRNA will be transcribed causing a greater amount of the corresponding enzyme to be built which in turn allows the pollutant degradation process to be enhanced. In addition, since only active biomass will produce the enzyme, measurements of enzymatic levels should be linked to VPB pollutant degradation to a much higher degree than overall biomass quantity. Therefore, since mRNA levels dictate enzymatic production, it follows that mRNA levels should be linked to pollutant degradation and that gene expression should be linked to VPB performance.

One advantage of mRNA-based molecular tools is that they provide an *in situ* measurement of enzyme production. Since mRNA can be isolated directly from the source without any additional culturing requirements, they provide a much more accurate representation of the system than do conventional methods. While molecular-based research is very promising, most research of this type performed in environmental engineering systems has dealt with organisms involved in nitrogen metabolism in wastewater treatment. However, because relatively few other genes have been sequenced that are relevant to environmental engineering systems, very little molecular based research has been performed in this area. mRNA can be used with microarrays in a similar fashion to that described for DNA in the previous section. However, many genes must be known to justify making a microarray plate that can hold hundreds of genes. In contrast, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) can be used to obtain similar information for one gene at a time. This method can be used to quantify the levels of expression for specific genes, and, therefore, qRT-PCR can be used to compare mRNA levels under various operating conditions. As previously mentioned, mRNA is a much better indicator than DNA of cell activity and should be

more directly linked to VPB performance. The remainder of this section will provide an overview of the qRT-PCR technique.

qRT-PCR correlates the level of mRNA for a specific gene to the specific activity of that gene and is based on the fact that there should be a relationship between the amount of PCR amplicon and the amount of template originally present in the sample. The real-time part of this method stems from the fact that the amount of amplicon is measured as it is being produced by fluorescence. Figure 2-3 shows a schematic of this reaction. First, forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes (Taqman® probe) anneals to the gene sequence between the two primers. Second, as the polymerase extends the primer, the probe is displaced. Third, an inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. Last, after release of the reporter dye from the quencher, a fluorescent signal is generated (ABRF, 2003).

A TaqMan® probe is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) at any T position or at the 3' end (Livak et al., 1995). The probe is designed to have a higher melting temperature than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as both fluorochromes are on the probe, the quencher molecule prevents fluorescence by the reporter. However, as the *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter

fluorochrome (Holland et al., 1991). The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

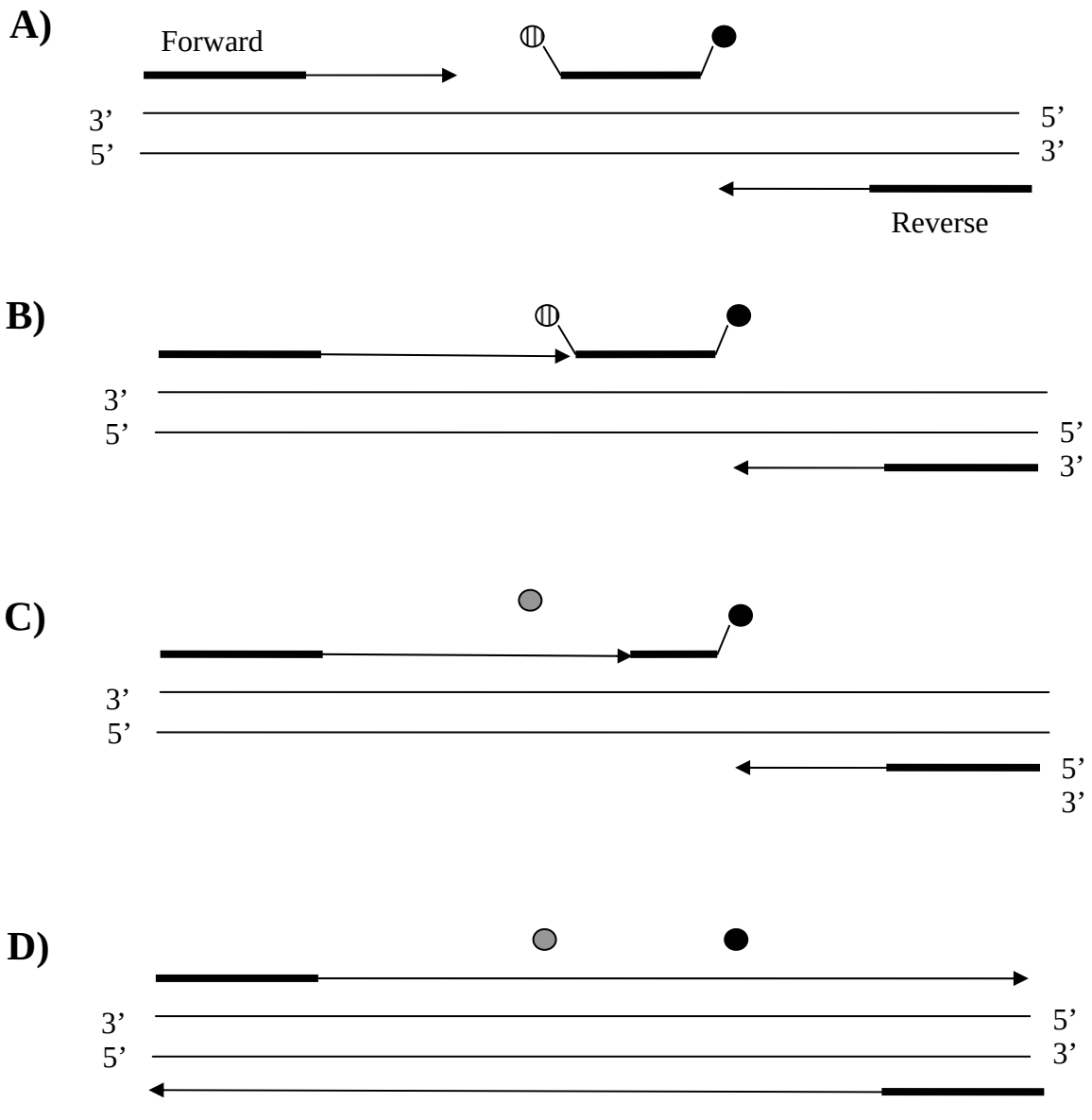

Figure 2-3: qRT-PCR Schematic. (a) Polymerization, (b) Strand Displacement, (c) Cleavage, (d) Polymerization Completed. ◍ , ● and ● represent the non-fluorescing reporter, fluorescing reporter and quencher respectively. (Source: ABRF, 2003)

The greatest advantages to quantifying gene sequences by qRT-PCR are its precision and specificity of the process. The precision exists because quantitation of the target gene is determined by the threshold cycle (C_t), which is calculated during the exponential phase of the reaction. This value is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise to a threshold fluorescence level. The C_t is determined at the exponential phase of the reaction and is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold cycle measured. High specificity is conferred by the requirement of three oligonucleotides to anneal to the DNA before any data are collected. In addition, data acquisition and analysis by qRT-PCR is extremely short as compared to other quantitative PCR methods. This method requires approximately 3 to 4 hours as compared to several days for other methods such as competitive PCR (ABRF, 2003).

In environmental engineering, qPCR can be used for DNA based analysis in a similar fashion to DGGE and FISH to quantify the number of organisms present which carry a specific gene. By extension, qRT-PCR can be used to measure the expression of a specific gene in a sample, and gene expression can be compared across various samples. In VPBs, parameters such as nutrient supply and substrate mixtures can be modified and the effect on gene expression and pollutant degradation can be compared. Gene expression is measured after extraction of total RNA and preparation of cDNA by a reverse transcription (RT) step. Similar to microarrays, one of the main advantages of this technology is that it provides a direct measure of *in situ* system characteristics. A total RNA sample can be isolated directly from the cells without any prior subculturing.

While this method has been used to quantify *amo* genes involved in nitrification, aromatic oxygenase genes in groundwater remediation systems, and the *tceA* genes involved in chloroethylene dechlorination, it has not been applied to environmental biofilm systems (Harms et al., 2003; Baldwin et al., 2003; Rahm et al., 2003).

Thus, molecular level investigations in VPBs can help to answer fundamental questions that have been left unanswered by the application of conventional tools. qRT-PCR has the potential to be used to demonstrate which portion of the biomass is truly active and is producing the enzymes responsible for pollutant degradation. These results can then be used to relate the quantity of pollutant degraded to enzyme activity rather than to overall biomass quantity.

In this research, qRT-PCR was used to directly determine how substrate mixtures, nutrient supply and shutdown periods affected pollutant degradation. Research to date has focused on bulk measurement of degradation capacity as a function of biomass quantity without any knowledge of biofilm specific activity. These data, while useful for generalizations, do not provide details about biofilm level occurrences. This dissertation took this line of research one step further by linking the performance of the biofilter to the actual activity of a gene involved in the degradation of the pollutant of interest under a variety of substrate mixtures and during shutdown/startup periods. For any of these studies to be performed, however, information about genes involved in the pollutant of interest's degradation pathways had to first be obtained.

2.4. ETHYLBENZENE DEGRADATION

Since this dissertation deals with ethylbenzene degradation by the fungus *E. lecanii-corni*, a gene involved in this pathway was required to perform this research. This section outlines the currently known degradation pathways for ethylbenzene as well as their regulation mechanisms.

Most ethylbenzene metabolic research has been performed with bacteria, predominantly in *Pseudomonas* and *Rhodococcus* spp. By contrast, very little work has been done with fungi, especially for the initial reactions of these pathways. As described later, some studies have been performed with fungi *Aspergillus nidulans* and *E. jeanselmei* to determine specific enzymes that are analogous to those involved in bacterial ethylbenzene metabolism. This work, however, was not very extensive and was not directed toward investigating the ethylbenzene degradation pathway specifically. Because the research in this dissertation incorporates substrate mixtures, the latter part of this section reviews some results from research dealing with regulation in pathways related to bacterial ethylbenzene metabolism.

2.4.1. Ethylbenzene Degradation Pathways

In general, aromatic compound degradation can be separated into two stages. The upper pathway consists of initial oxidation of the ring by either a monooxygenase or a dioxygenase and subsequent transformation of the compound to a common intermediate

such as catechol, protocatechuate, gentisate and homogentisate. In the lower pathway, the ring is cleaved by incorporating two oxygen atoms into the substrate. This reaction is generally mediated by a dioxygenase (Jenkins and Dalton, 1985; Overhage et al., 1999; O'Leary et al., 2002; Jindrova et al., 2002).

To date, ethylbenzene degradation in bacteria has been described to proceed by three different pathways. The first pathway proceeds through 2,3-dihydroxyethylbenzene; the second pathway proceeds through styrene; the third pathway proceeds through acetophenone. These pathways are described further in the following sections.

2.4.1.1. 2,3-dihydroxyethylbenzene Pathway

In this first pathway, the initial steps of ethylbenzene degradation is mediated by ethylbenzene dioxygenase and cis-ethylbenzene glycol dehydrogenase were demonstrated to occur in *Pseudomonas putida* by Gibson et al. (1973). The next step is mediated by the 2-hydroxy-6-oxo-octa-2,4-dienoate hydrolase (Smith and Rutledge, 1989). The final steps leading to acetaldehyde and pyruvate are carried out by 2-hydroxypenta-2,4-dienoate hydratase and the 4-hydroxy-2-oxovalerate aldolase (Lau et al., 1994; Sakai et al., 2003). Acetaldehyde and pyruvate can both be further broken down in the Krebs cycle.

2.4.1.2. Styrene Pathway

In this pathway, ethylbenzene is initially converted to styrene by naphthalene-1,2-dioxygenase as shown in Figure 2-4 (Lee and Gibson, 1996). This reaction was discovered in *Pseudomonas* sp. strain NCIB 9816-4. Styrene is then further degraded along two pathways as shown in Figures 2-5 and 2-6.

The first styrene pathway was demonstrated in *Rhodococcus rhodochrous* strain NCIMB 13259 and *Pseudomonas putida* strain CA-3 (Warhurst et al., 1994). As shown in Figure 2-5, styrene is first transformed to phenyl acetate by a styrene monooxygenase, styrene oxide isomerase and phenylacetaldehyde dehydrogenase (O'Connor et al., 1997). These reactions have also been shown to occur in the fungus *E. jeanselmei* (Cox et al., 1996). Phenyl acetate is further processed to homogentisate by a phenyl acetate hydroxylase and 2-hydroxyphenol-acetate hydroxylase (Olivera et al., 1994). Homogentisate is further degraded to 4-maleylacetoacetate by a homogentisate-1,2-dioxygenase. This reaction has also been shown to occur in the fungus *Aspergillus nidulans* (Fernandez-Canon and Penalva, 1993). Maleylacetoacetate isomerase is the next enzyme that transforms the maleylacetoacetate to fumarylacetoacetate which in turn is converted to acetoacetate and fumarate (Crawford, 1976). Fumarate and acetoacetate can both enter the Krebs cycle.

The second styrene pathway was also demonstrated in *Rhodococcus rhodochrous* NCIMB 13259. As shown in Figure 2-6, styrene is converted to acrylate and 2-hydroxypenta-2,3-dienoate through reactions mediated by styrene dioxygenase, cis-

glycol dehydrogenase, catechol-2,3-dioxygenase and 2-hydroxymuconate semialdehyde hydrolase (Warhurst et al., 1994). 2-hydroxypenta-2,4-dienoate is further broken down to acetaldehyde and pyruvate by 2-hydroxypenta-2,4-dienoate hydratase and 4-hydroxy-2-oxovalerate aldolase (Lau et al., 1994). Acetaldehyde and pyruvate enter the glycolysis cycle.

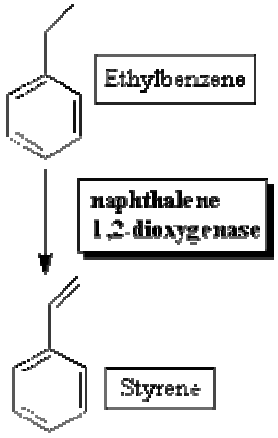

Figure 2-4: Ethylbenzene conversion to styrene (UMBDD, 2003)

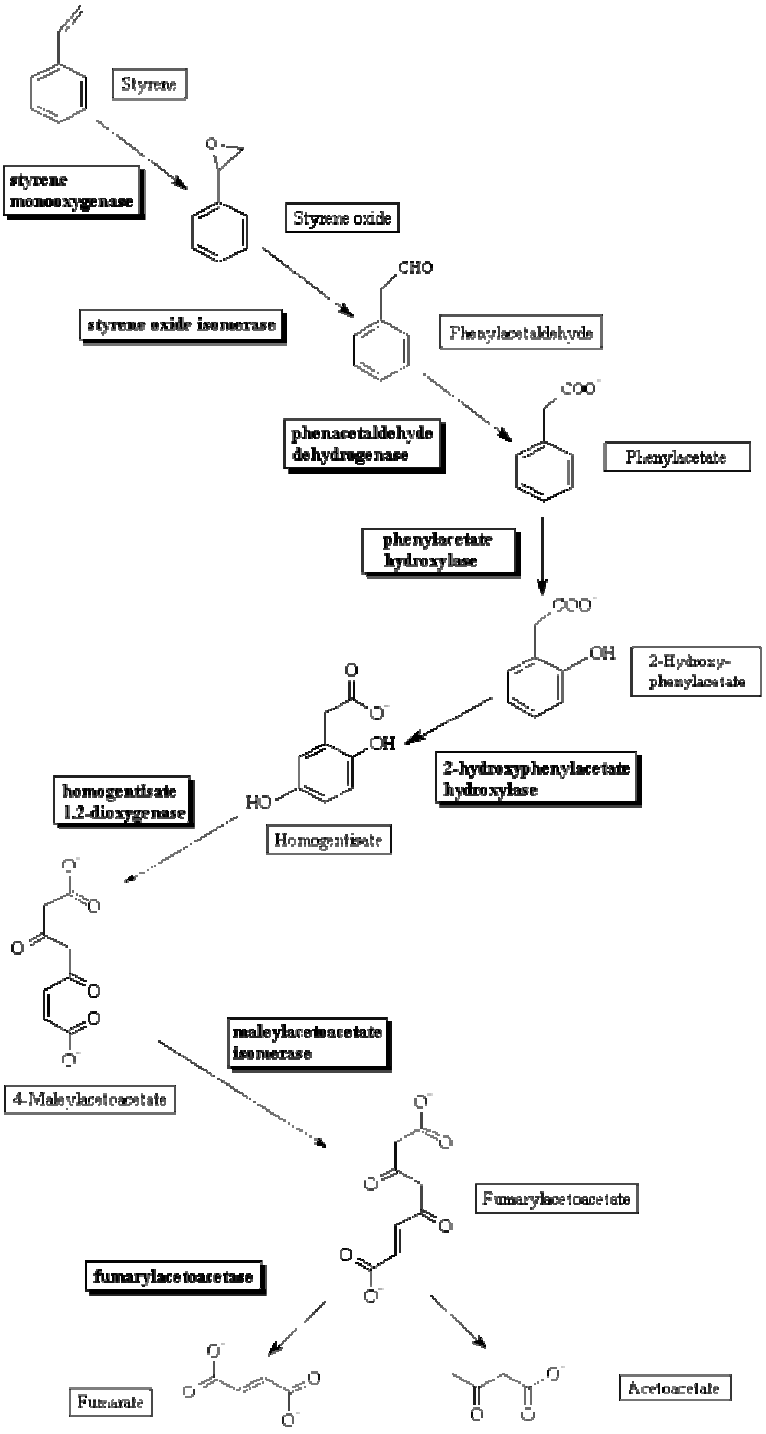

Figure 2-5: First styrene pathway (UMBDD, 2003)

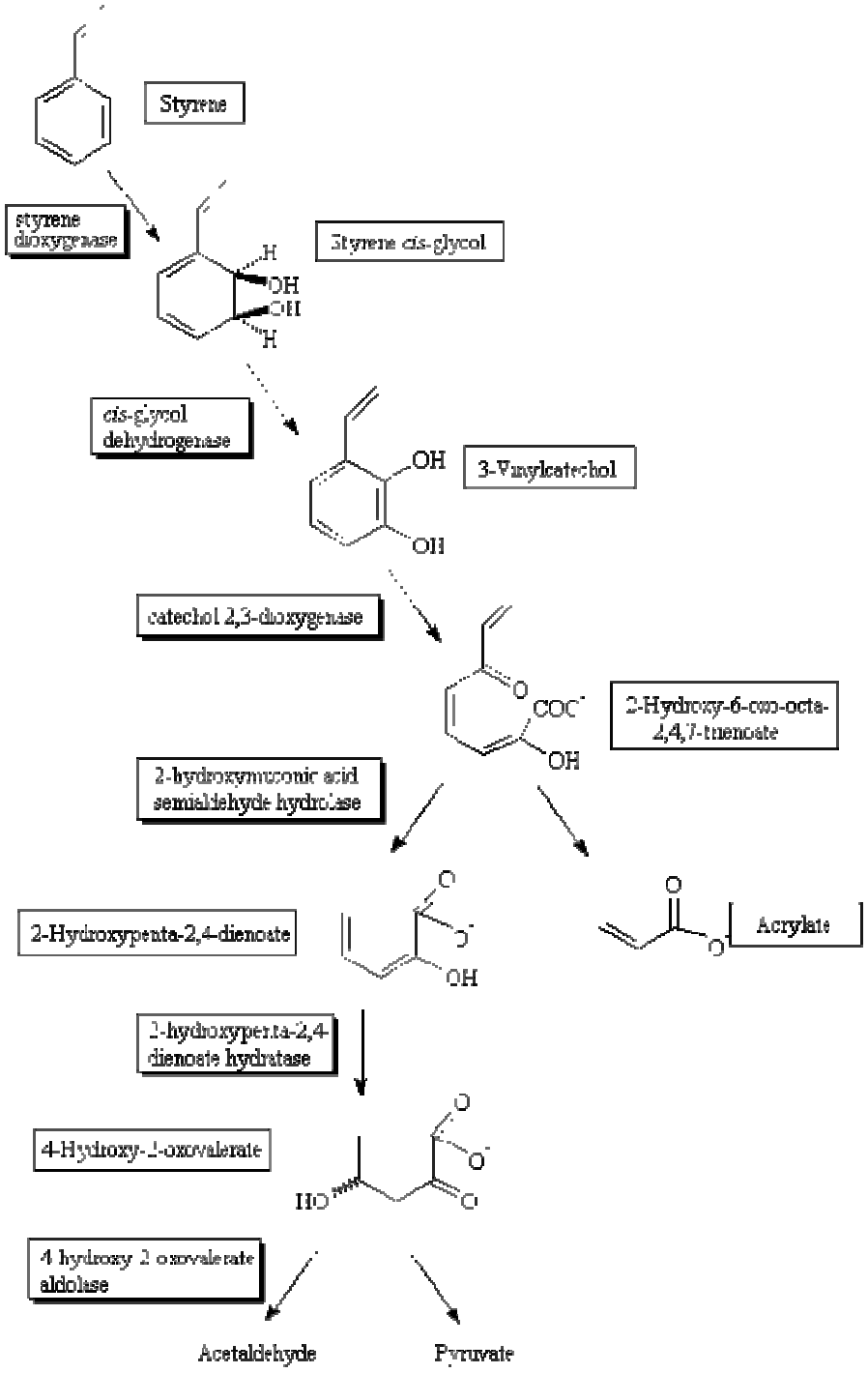

Figure 2-6: Second styrene pathway (UMBDD, 2003)

2.4.1.3. Acetophenone Pathway

The final ethylbenzene pathway was demonstrated in *Pseudomonas putida* strain NCIB 9816-4. In this pathway, ethylbenzene is transformed to (S)-1-phenethyl alcohol, acetophenone and 2-hydroxy-acetophenone by naphthalene-1,2-dioxygenase (Lee and Gibson, 1996).

2.4.2. Ethylbenzene Degradation Enzymatic Control

While several microorganisms have been found to metabolize ethylbenzene, very few studies have focused on the regulation of these degradative pathways. Several *Pseudomonas* spp. have been characterized, and their enzymatic regulation is discussed below. To date, no such studies have focused on the regulation of fungal ethylbenzene degradation pathways.

2.4.2.1. Pseudomonas fluorescens strain CA-4

Extensive studies have been performed on *Pseudomonas fluorescens* strain CA-4 to determine its enzymatic control mechanisms. *P. fluorescens* was initially found to degrade ethylbenzene via side chain oxidation to 2-phenylethanol and phenylacetic acid. Corkery et al. (1994) showed that ethylbenzene is the major inducer of this pathway. This is similar to data for *Pseudomonas putida* 01G3 (Chablain et al., 2001). In addition, Corkery et al. (1994) found that glutamate represses ethylbenzene degradation in this pathway whereas glucose and citrate do not.

A secondary ethylbenzene degradation pathway was later discovered for this strain which involves the ring dioxygenation by ethylbenzene dioxygenase (Corkery and Dobson, 1998). The genes involved in this reaction are very similar to those involved in isopropyl degradation in *Pseudomonas* sp. strain JR1 and *P. fluorescens* strain IP01. Corkery and Dobson (1998) postulate that this is the primary ethylbenzene degradation pathway. In this study, they showed that the expression of these genes is affected by the presence of second carbon substrates and is mediated at the level of transcription.

2.4.2.2. Effect of Nutrient Limitations

O'Leary et al. (2002) studied the effects of various nutrient-limiting conditions on the expression of the *sty* operon which degrades styrene via phenylacetic acid in *Pseudomonas putida* strain CA-3. The two genes that were followed in this study were those that encoded styrene monooxygenase and phenylacetyl coenzyme A ligase, which can be thought of as indicators of upper and lower pathway regulation, respectively. O'Leary et al. (2002) showed that the control mechanism in this case is at the level of gene transcription. Limiting the quantity of phenylacetic acid present increased the expression of the lower pathway enzyme, phenylacetyl coenzyme A ligase, which was accompanied by an increase in the expression of the upper pathway enzyme, styrene monooxygenase.

In other studies, O'Connor et al. (1995 and 1996) looked at the effect of limiting nitrogen, sulfur and phosphorus on styrene degradation in *P. putida* CA-3 in the presence and absence of carbon sources. Their results showed that ammonia and sulfate

limitations caused dramatic reductions in styrene degradation enzyme activity. In contrast, styrene and phenylacetic acid limitations had a much smaller effect, indicating that nutrient conditions may play an important role in environmental systems.

Hendrickx et al. (2002) showed that an increase in phosphorus favored bacterial over fungal growth in a fixed biofilm process. In this study, they studied phosphorous concentrations ranging from 0 to 5 mg/L and demonstrated that fungi can remove greater quantities of organic compounds at low phosphorous concentrations.

Amor et al. (2001) showed that the presence of heavy metals is highly inhibitory to monoaromatic hydrocarbon degradation. This study focused on a *Bacillus* sp. grown on alkylated derivatives of benzene (ethylbenzene as well as ortho-, meta- and para-xylenes) in the presence of cadmium, nickel and zinc.

2.4.2.3. Effect of Ethanol on BTEX Degradation

Lovanh et al. (2002) showed that the presence of ethanol directly affects BTEX degradation kinetic rates in mixed cultures. As discussed earlier, the presence of ethanol decreases the metabolic flux of toluene. However, this effect was countered by the increase in biomass caused by the presence of ethanol in the samples. The presence of low concentrations of ethanol had a positive effect on benzene degradation, ultimately resulting in an increase in benzene removal efficiency.

Chapter 3: Pathway Investigation and Homogentisate Dioxygenase Fragment Isolation

The main goal of this research project was to determine if gene expression studies could be linked to the macroscale performance of a fungal vapor-phase bioreactor degrading ethylbenzene. To accomplish this, ethylbenzene degradation in *E. lecanii-corni* was first characterized. A portion of a gene relevant to ethylbenzene degradation was also isolated in this phase of the research. The remainder of this section outlines the protocols used and the results that were obtained.

3.1. Comparison of Ethylbenzene and Toluene Degradation in Bottle Studies

Ethylbenzene is commonly found in mixtures treated by VPBs. Because the previous work with *E. lecanii-corni* dealt mostly with toluene, ethylbenzene degradation was characterized and compared to the toluene parameters to establish a benchmark for this study.

3.1.1. Methods

Depletion curves for ethylbenzene and toluene were performed in 250-mL glass bottles sealed with Teflon tape and Minninert caps. Toluene and ethylbenzene were aseptically added as neat liquid in the amounts of 1, 2 or 4 μL. Headspace samples were collected over time using a Hewlett Packard Model 5890 Gas Chromatograph (GC) equipped with a flame-ionization detector (FID) and a Restek RTX-624 capillary column

(State College, PA). Helium was used as the carrier gas. The make-up gas flow to the detector consisted of He, H_2 and zero grade air. An isothermal program was used to operate the column at 60 ^oC. The injector and detector were maintained at 250 and 275 ^oC respectively. The GC was calibrated using five bottles containing a known amount of each chemical to be analyzed. A 0.5-mL headspace sample of each standard was injected onto the GC, and a five-point calibration curve was produced. Gas and liquid concentrations were calculated using Henry's constant. The toluene Henry's constant used was 0.209 at 22°C (Peng and Wan, 1997). The ethylbenzene Henry's constant used was 0.345 at 22°C (Mackay et al., 1973).

In all cases, triplicate sets of bottles were inoculated with *E. lecanii-corni* spores (asexual fungal reproductive cells which develops into an adult cell after a state of dormancy) to reach an initial cell concentration of 10^5 cells/mL as determined by hemacytometer count. An additional triplicate set of killed autoclaved cells, to which 2 μL of each VOC had been added was used to measure abiotic losses. Basal medium (20 mL) was added to each bottle. The basal medium was composed of 5 g/L $(NH_4)_2.SO_4$, 0.25 g/L $MgSO_4.7H_2O$, 1.01 mg/L $MnSO_4.H_2O$, 0.24 mg/L $Na_2MoO_4.2H_2O$, 0.02 g/L $CaCl_2.2H_2O$, 0.10 mg/L $NiCl_2.6H_2O$, 0.17 mg/L $CuCl_2.2H_2O$, 1.36 mg/L $FeSO_4.7H_2O$, 0.24 mg/L $CoCl_2.6H_2O$ and 0.58 mg/L $ZnSO_4.7H_2O$. The bottles were placed on a shaker at 22°C after addition of ethylbenzene and toluene, and headspace samples were collected regularly. Specific substrate utilization rates were determined during these experiments according to Equation 3-1.

$$\text{Specific Utilization Rate} = \frac{\text{Total Quantity of VOC Consumed (g)}}{\text{(Total Incubation Time – Lag Time) (hr)} \cdot \text{Cell Mass (g dry weight)}} \quad \text{(Equation 3-1)}$$

For dry weight measurements of the biomass produced in each incubation bottle, aluminum planchettes and Whatman filter papers (0.2 μm pore size) were dried at 105 °C for 4 hours, cooled to room temperature in a desiccator and weighed on a four-place balance. Each culture was individually filtered by vacuum through the dried filter paper. The paper was then replaced in the planchette and dried overnight at 105 °C. The planchette was then placed in a desiccator until it reached room temperature and weighed on a four-place balance.

3.1.2. Results

Specific substrate utilization rates for ethylbenzene and toluene were determined after complete degradation of the second injection of toluene or ethylbenzene using Equation 3-1. Degradation profiles can be found in Appendix A (Figures A1-A6). The specific ethylbenzene utilization rate was approximately 38% lower, on average, than that for toluene (Table 3-1).

Table 3-1: Specific Substrate Utilization Rates

Carbon Source	Average Specific Substrate Utilization Rate (g substrate/g-cells/hr)	Standard Deviation
Ethylbenzene	78.22	4.78
Toluene	125.32	7.97

3.2. ETHYLBENZENE PATHWAY INVESTIGATION - STYRENE AND PHENYL ACETATE DEGRADATION

E. lecanii-corni was hypothesized to degrade ethylbenzene through a pathway similar to that in the bacterial pathway described in Section 2.4.1.2. To further confirm this, depletion tests were performed using styrene and phenyl acetate as the sole carbon source.

3.2.1. Methods

Neat liquid was added aseptically to each bottle that was sealed with Teflon tape and a Minninert cap. Headspace samples were regularly collected using a Hewlett Packard Model 6890 Gas Chromatograph (GC) equipped with a flame-ionization detector (FID) and a HP-5 capillary column. Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The make-up gas flow to the detector consisted of He (28.4 mL/min), H_2 (35 mL/min) and zero grade air (350 mL/min). The column temperature started at 75 ^{o}C for 1 min, increased at a rate of 15 ^{o}C/min to 130 ^{o}C and was held at 130 ^{o}C for 1 min. The

injector and detector temperatures were maintained at 250 ^{o}C. The GC was calibrated using five standards as described previously.

A range of concentrations was used in these depletion tests for both compounds. The liquid phase concentration range was 60-200 mg/L for styrene and 50-400 mg/L for phenyl acetate. For styrene, triplicate sets of bottles were used and for phenyl acetate, =duplicate sets of bottles were used. In both cases, the initial cell concentration used was $5x10^4$ cells/mL as determined by hemacytometer count. An additional triplicate set of killed cells was used to measure abiotic losses.

3.2.2. Results

As shown in Figures 3-1 and 3-2 respectively, styrene and phenyl acetate concentrations decreased over a period of 2-20 days as compared to killed controls. In addition, a visible biomass increase was observed, indicating that *E. lecanii-corni* is capable of using either styrene or phenyl acetate as a sole carbon source. Both of these compounds are intermediates in the bacterial ethylbenzene degradation pathways proceeding via styrene, suggesting that *E. lecanii-corni* could degrade ethylbenzene through the same pathway as presented in Section 2.4.1.2.

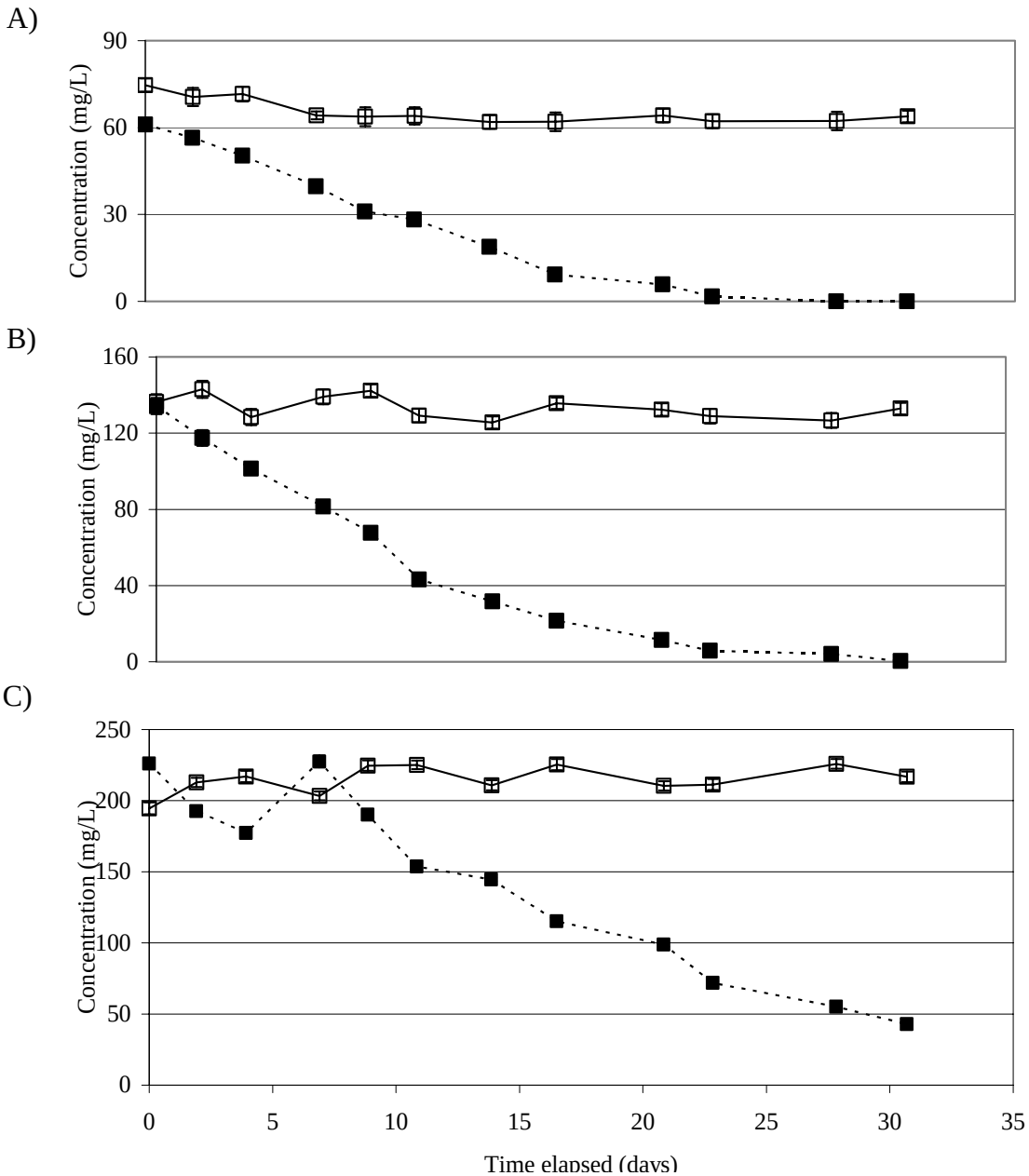

Figure 3-1: Styrene Depletion Curves by *E. lecanii-corni* (■) at various initial concentrations: (A) 60 mg/L, (B) 140 mg/L and (C) 225 mg/L. Solid lines represent killed controls. Error bars represent one standard deviation.

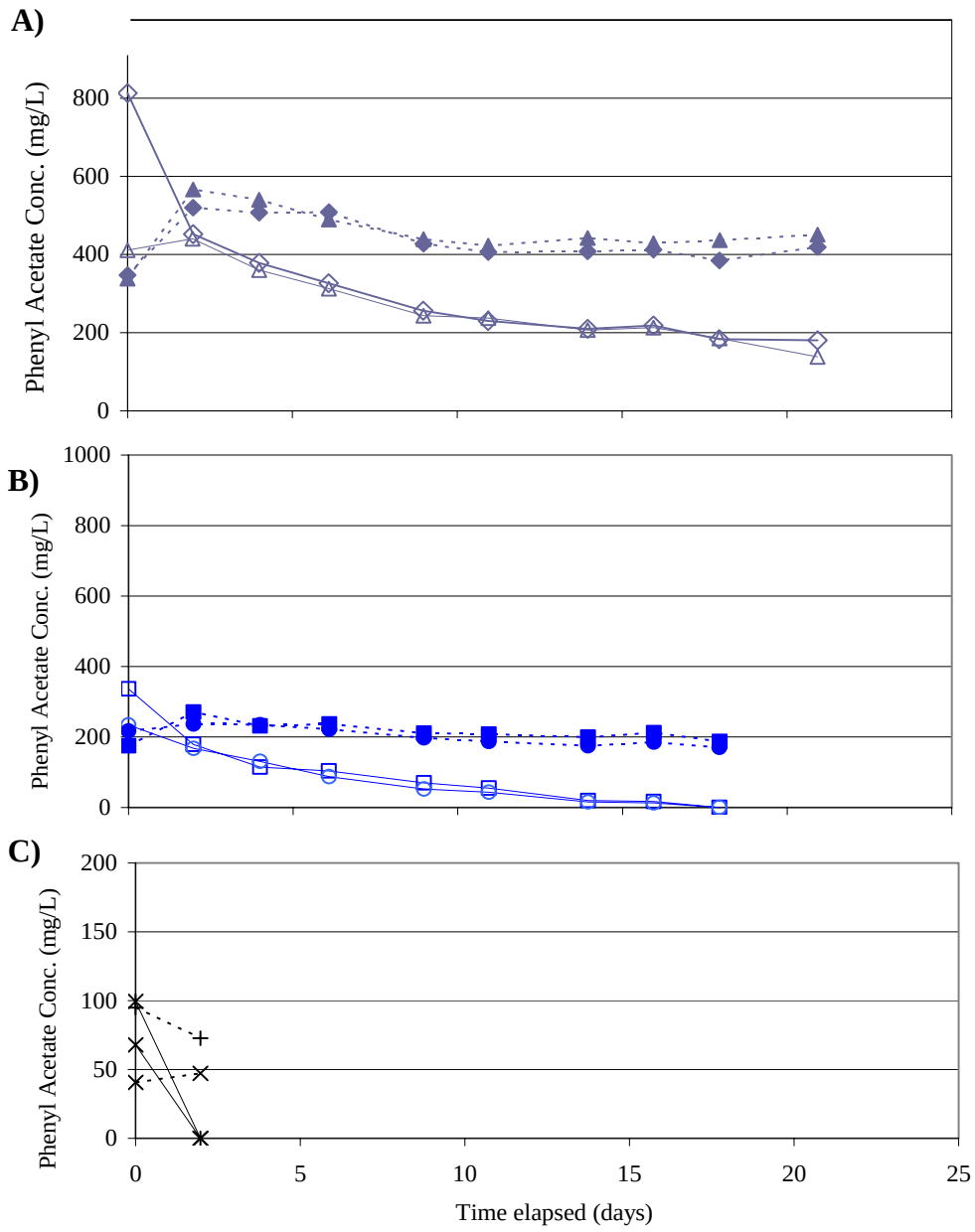

Figure 3-2: Phenyl Acetate Depletion Curves by *E. lecanii-corni* (solid lines) at various initial concentrations: A) 400 mg/L, B) 200 mg/L and C) 50 mg/L. Dashed lines represent killed controls. Error bars represent one standard deviation.

3.3. HOMOGENTISATE DIOXYGENASE (*ELHDO*) FRAGMENT ISOLATION AND EXPRESSION

Based on the results presented in Section 3.2, it appeared that *E. lecanii-corni* degraded ethylbenzene through styrene and phenyl acetate. Comparing these results to the bacterial ethylbenzene pathways presented in Section 2.4., it was hypothesized that the ethylbenzene ring cleavage was mediated by homogentisate-1,2-dioxygenase (see Figure 2-5). To further confirm that ethylbenzene degradation proceeds through styrene and phenyl acetate, degenerate polymerase chain reaction primers were designed to amplify a portion of this gene, so that it could be used as a probe in ethylbenzene expression studies and to confirm the involvement of the homogentisate-1,2-dioxygenase in ethylbenzene metabolism.

3.3.1. *ElHDO* PCR

To obtain a DNA fragment from the *E. lecanii-corni* homogentisate-1,2-dioxygenase gene, DNA was extracted and amplified using degenerate PCR primers according to a method developed by Qiang Cheng (personal communications). The resulting fragment was then cloned and sequenced to verify its identity. The following paragraphs describe the experimental protocol used for DNA extraction, PCR and fragment cloning.

3.3.1.1. Methods

DNA was extracted from *E. lecanii-corni* cells in the late log phase using a modified standard method (Ausubel et al., 1991; Cheng et al., 2003). Late log phase cultures of *E. lecanii-corni* were collected and cells centrifuged at 1200-1800 g for 5 minutes. The supernatant was removed and 0.5 ml of deionized water was added to the pelleted cells. The cells were centrifuged again at 1200-1800 g for 5 minutes, and all remaining supernatant was removed. Cells were resuspended in 200 µl breaking buffer and transferred to a 1.5 ml microtube. Phenol:chloroform:isoamyl alcohol (25:24:1) in the amount of 200 µl was added along with 0.3 g of 1-mm glass beads. The microtube cap was closed tightly, placed in a microtube rack and vortexed at full speed for 5 minutes with a VWR multitube vortexer. The microtubes were opened and 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) added. Tubes were flicked to mix and then centrifuged at full speed for 5 minutes. The top layer was transferred to a clean microtube, and 1 ml of 100% ethanol was added. Tubes were inverted to mix and then centrifuged at full speed for 3 minutes. The pellet was suspended in 200 µl TE buffer and 15 µl RNAse A was added. The microtubes were incubated at 37°C for 1 hour. Twenty µl of 3 M sodium acetate and 0.5 ml of 100% ethanol were added. Tubes were inverted, mixed and centrifuged at full speed for 3 minutes. Supernatant was removed and the pellet was dried using a lyophilizer. The DNA pellet was resuspended in 30-50 µl deionized water and stored at -20°C.

Published conserved regions of homogentisate dioxygenase gene sequences were aligned using DIALIGN, and sections with high sequence identity were used as templates

to design degenerate primers. The homogentisate dioxygenase genes used in this alignment were *Aspergillus nidulans* (NCBI Accession Number U30797), *Xanthomonas campestris* (NCBI Accession Number NC003902), *Pseudomonas putida* (NCBI Accession Number AE016791) and *Vibrio vulnificus* (NCBI Accession Number NC004459). The forward primer sequence used was 5'-GAT GGC GAY TTC YTG ATY GT-3' and the reverse primer sequence was 5'-CCA KCG YCG YGG AAA RAT RAC A-3' where Y, K and R represent C/T, G/T and A/G nucleotides respectively.

PCR was performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). Reactions in the amount of 50 μL were used containing 1 μL of 50-500 ng/μL DNA, 4 μL of 25 mM magnesium chloride, 5 μL of 10X Buffer, 0.2 μL of Taq polymerase (5 units/μL), 1 μL of 50 mM dNTPs, 1 μL of forward and reverse primers and 36.5 μL of water. The program used was 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes followed by a final extension at 72°C for 7 minutes. The samples were held at 4°C until they were removed from the PCR machine.

A PCR product, approximately 500 base pairs in length, was obtained and cloned using a pGEM-T Easy Vector System (Promega, Madison, WI). The PCR reaction performed in the previous step used Taq polymerase. One of the advantages of using this polymerase is the creation of TA tails on the end of the PCR fragments. These, in turn, can be used to clone the resulting fragments. A ligation reaction was set up consisting of 5 μL of the 2X Rapid Ligation Buffer, 1 μL of the pGEM-T Easy vector, 3 μL of the PCR product and 1 μL of T4 DNA Ligase (3 Weiss units/μL). The reagents were mixed by pipetting and the microfuge tubes were incubated at 4°C overnight. XlBlue

Escherichia coli cells were transformed by heat shock using 2 μL of the ligation reaction. The heat shock competent cells were prepared as follows. 100 μL of an overnight culture was used to inoculate 25 mL of Luria-Bertani medium. They were grown at 37°C until the optical density at 600 nm reached 0.4. They were incubated on ice for 15 minutes and pelleted at 3000 rpm for 10 minutes at 4°C. The cells were gently resuspended in 10 mL of TFBI (30 mM potassium acetate, 100 mM rubium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% glycerol, pH adjusted to 5.8 with 0.1 M acetic acid, filter sterilized). Cells were incubated on ice for 15 minutes and centrifuged at 3000 rpm for 10 minutes at 4°C. Cells were resuspended in TFBII (10 mM MOPS, 75 mM calcium chloride, 20 mM rubium chloride and 15% glycerol. pH was adjusted to 6.5 with acetic acid and the entire solution was filter sterilized). Cells were then incubated on ice for 15 minutes and aliquoted into 50 μL volumes. 2 μL from the ligation reaction was mixed with 50 μL of the competent cells and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 60 seconds. The cells were then placed on ice for 60 seconds and transferred to 1 mL of LB media. The mixture was incubated at 37°C for 45 minutes. The cells were pelleted and plated on LB agar plates containing 100μg/mL ampicillin. Ligation efficiency was determined by adding 4 μL of 0.5 M IPTG and 40 μL of 0.25 g/L Xgal to the plate and looking for the ratio of white and blue colonies. Blue colonies are colonies which do not contain an insert and therefore are able to express the lacZ gene.

White colonies were selected and plasmids isolated using the Qiagen QiaPrep® Miniprep (Valencia, CA). Following this protocol, 2 mL of cells that had been grown overnight, were transferred to a microfuge tube and pelleted. The supernatant was removed by pipetting. The pelleted bacterial cells were resuspended in 250 μL of Buffer

P1 and transferred to another microfuge tube. 250 μL of Buffer P2 was added, and the tube was gently inverted 5 times. 350 μL of Buffer N3 was added, and the tube was gently inverted 5 times. The microfuge tube was then centrifuged for 10 minutes at 12,000 rpm. The supernatant was applied to a Qiaprep column and centrifuged for 1 minute. The flow through was discarded, and 1 mL of Buffer PE was applied to the column. The column was transferred to a clean microfuge tube and 100 μL of elution buffer was applied to the center of the column. After waiting 1 minute, the tube was centrifuged for 1 minute to elute the plasmid from the column. The fragment contained in the isolated plasmid was then sequenced using the pGEM-T Easy T7 and SP6 primer sites. Homology to other genes was determined using BLAST (NCBI). The genes showing homology to the PCR fragment were aligned using DIALIGN.

3.3.1.2. Results

A 360-bp portion from the PCR fragment showed 83% similarity to the *Aspergillus nidulans* homogentisate dioxygenase gene at the amino acid level. Alignments at the protein level are shown in Figure 3-3.

ELC PVRGYIMELYQGHFKLPELGPIGSNGLANARDFQTPVADFIEDHENTE

PVRGYI ELYQGH++LPELGPIGSNGLANARDFQ PVA F ++ TE

AN PVRGYICELYQGHYQLPELGPIGSNGLANARDFQAPVAAFDDEEGPTE

ELC WTLYGKFGGNLFAAKQSHTPFDIVAWHGTYYPYKYDLGRFNPVGSIS

+ LY KF +LF+A +Q HTPFDIVAWHG YYPYKYDLGRFN +GS+S

AN YRLYSKFNNHLFSARQDHTPFDIVAWHGNYYPYKYDLGRFNTMGSVS

ELC YDHPDPSIFTVLTAPSIPHGAGTA

+DHPDPSI+TVLT PS G A

AN FDHPDPSIYTVLTGPSDHVGTAIA

Figure 3-3: Alignment of amino acid sequences between *E. lecanii-corni* (*ELC*) and *Aspergillus nidulans* (*AN*) homogentisate dioxygenase. + represent amino acids which have similar chemical characteristics but are not identical.

3.3.2. *ElHDO* Northern Blotting

Northern blots are used to compare the level of expression of a specific gene under different growth conditions. To further confirm the involvement of the homogentisate dioxygenase (*ElHDO*) gene in *E. lecanii-corni* ethylbenzene metabolism, mRNA was isolated and compared from cells grown using ethylbenzene and toluene as their sole carbon source. The *ElHDO* fragment isolated by degenerate PCR was used as a probe in this study. The following paragraphs describe the procedures used in these experiments as well as the results obtained.

3.3.2.1. Methods

mRNA was isolated using a Poly(A)Pure kit (Ambion, Austin, TX). Cells were grown in 2 L of basal medium with ethylbenzene supplied as the sole carbon source. *E. lecanii-corni* spores were inoculated at a final concentration of $5x10^4$ cells/mL using the bubbler system shown in Figure 3-4. When the cell density reached approximately 10^8 cells/mL, as determined by hemacytometer count, cells were harvested. Cells were pelleted by centrifugation at 8,000 x g at a temperature of 4°C. The cells were washed twice with ice cold water and rinsed with phosphate buffered saline solution. The cells were transferred into a porcelain crucible and ground in liquid nitrogen. Ground cells (1 g wet weight) was transferred into a sterile 50-mL conical tube and added to 10 mL of lysis solution. Cells were homogenized by vigorous vortexing. After adding dilution buffer (30 mL) to the lysate, the sample was mixed by shaking for 10 seconds. Samples were centrifuged at 4500 x g for 30 minutes at room temperature to remove any debris. Oligo(dT) cellulose (100 mg) was added to the sample and mixed by inversion to resuspend the resin. The tubes were gently rocked at room temperature for 60 minutes. The Oligo(dT) cellulose was centrifuged at 4500 x g for 10 minutes. Supernatant was removed by aspiration. Binding Buffer (10 mL) was added to the sample 3 times to wash the Oligo(dT) cellulose resin to remove nonspecifically bound material and ribosomal RNA. Between each step the sample was centrifuged at 4500 x g for 10 minutes at room temperature and the supernatant removed. The resin was washed 3 times using 10 mL of wash buffer. The resin was then resuspended in 0.6 mL of wash buffer, transferred into a spin column and microfuged for 10 seconds. After the flow-through was discarded, 0.5 mL was added to the top of the spin column and gently mixed into the resin using the tip

of the pipette tip and then microfuged. This wash step was repeated twice or until the absorbance of the flow-through at 260 nm was less than 0.05. The RNA was then eluted from the resin by placing the spin column into a new microfuge tube and adding 200 μL of elution buffer (previously warmed to 65°C). This step was repeated twice. 40 μL of 5 M ammonium acetate, 2 μL glycogen and 1.1 mL of 100% ethanol was added to the eluted sample, and the RNA was precipitated at -80°C for 1 hour. The RNA sample was centrifuged at 18,500 x g for 30 minutes, and the supernatant carefully removed with a fine-tipped pipette. The mRNA was resuspended in 30 μL diethyl pyrocarbonate treated water and stored at -80°C.

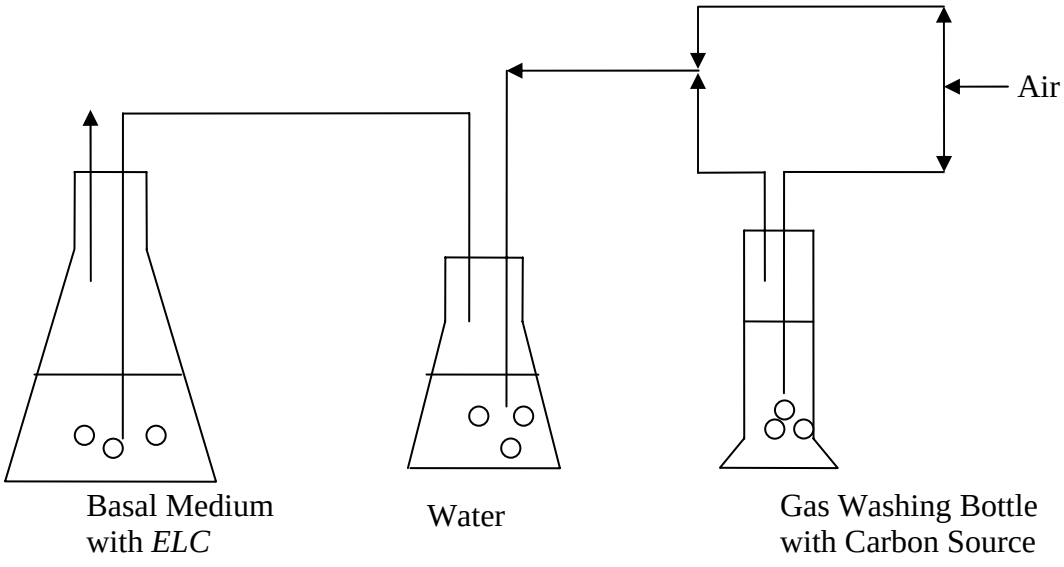

Figure 3-4: Bubbler system used to grow *E. lecanii-corni*

mRNA concentrations from cells grown on ethylbenzene and toluene were determined spectrometrically. Equal amounts of mRNA (0.5 and 1 μg) were transferred onto nylon membranes using a BioRad Slot Blot apparatus (Hercules, CA). This

apparatus consists of a vacuum manifold attached to tubing and a flow valve controlling the vacuum applied to the apparatus, a gasket support plate, a sealing gasket and a sample template with attached sealing screws. Three sheets of filter paper were placed over the sealing gasket. A nylon membrane was prewetted and placed over the filter papers. mRNA samples were denatured in 500 µl of ice-cold 10 mM NaOH/1 mM EDTA. The denatured samples were applied with a pipette to each slot and pulled through by applying a gentle vacuum. The wells were rinsed with 500 µl of ice-cold 10 mM NaOH/1 mM EDTA. A vacuum was applied until the sample wells were dry. The apparatus was disassembled and the membrane was rinsed in 2X SSC/0.1% SDS and dried at room temperature. Once dried, the membrane was placed in an oven at 80°C for 20 minutes and the mRNA was fixed onto the membrane by UV transillumination at 254 nm for 3 minutes.

The resulting nylon membrane was prehybridized for 1 hour at 68°C using PerfectHyb Plus (Sigma-Aldrich, Saint Louis, MO). The probe was added and the membrane was further hybridized 2-3 hours at 68°C. The membrane was removed from the hybridization tube and placed in a dish to be washed at room temperature in 2X SSC/0.1% SDS twice for 5 minutes. The membrane was placed back in the hybridization oven with 30 ml 0.5X SSC/0.1% SDS twice for 20 minutes. The final two washes were performed with 30 ml 0.1X SSC/0.1% SDS. The membrane was wrapped in plastic wrap and placed in an exposure cassette overnight. The film was developed the next morning.

The Ambion DECAprimeII Random Priming DNA Labeling Kit (Austin, TX) was used for probe preparation. First, 25 ng of the linearized DNA probe was denatured by heating in a water bath (95-100°C) with 2.5µl of 10X Decamer Solution. The reaction

was snap frozen in ice. Next, 5µl of 5X dCTP reaction buffer, 5µl [α-^{32}P]dCTP (10mCi/ml) and 1 µl Exonuclease-free Klenow were added to the mix. The whole mixture was gently mixed and the tube was incubated at 37°C for 30 minutes. To stop the reaction, 25 µl of 40 mM EDTA was added to the mixture. Unincorporated nucleotides were removed by filtering the 50 µl solution through a glass wool G50 column. Finally, the filtrate was boiled for 3 minutes and placed on ice for 3 minutes. At this stage, the probe was ready to be used for hybridization.

3.3.2.2. Results

As shown in Figure 3-5, a small amount of homogentisate dioxygenase appears to be constitutively expressed. However, the enzyme does seem to be more highly induced in the presence of ethylbenzene. This result, in addition to the styrene and phenyl acetate degradation results previously discussed, further suggests that ethylbenzene degradation occurs via the same pathway as that in bacteria discussed in Chapter 2.

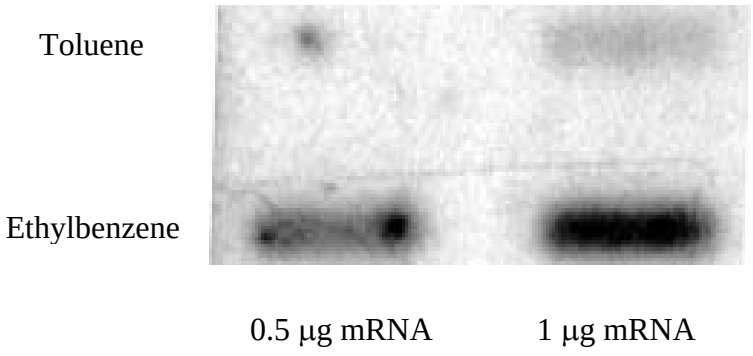

Figure 3-5: Northern blot showing *ElHDO* expression levels for *E. lecanii-corni* cells grown on toluene and ethylbenzene

Chapter 4: qRT-PCR Optimization

To assess if qRT-PCR can be used to study gene expression patterns in VPBs, this technology must first be optimized for use in biofiltration. The specific objectives of this phase of the research were as follows:

1) Determine if the *ElHDO*/*18S rRNA* system could be used to study variations in gene expression levels;
2) Identify the optimal biomass extraction method for the biofilter system and;
3) Validate the qRT-PCR method by using it to quantify *ElHDO* expression in toluene and ethylbenzene grown cells.

In the following paragraphs, the chosen methods are first described followed by an explanation and discussion of the results.

4.1. METHODS

4.1.1. Biomass Extraction

Because RNA is quite fragile and many biomass extraction techniques can be somewhat destructive, a study was performed to determine how the biomass extraction method employed affected qRT-PCR results. Three packing samples consisting of 3 packing pellets were removed from the inlet section of the bioreactor and placed in a 15-mL plastic tube containing 3 mL of sterile deionized water. All samples were collected at the same time and were subjected to one of the following extraction methods:

- 10 seconds vortex;
- 60 seconds vortex;
- 3 minutes vortexing followed by 3 minutes sonication; and
- scraping using a razor blade.

Packing pellets were then removed from the plastic tube using sterile tweezers, and the suspended cells were transferred to microfuge tubes. Cells were centrifuged for 1 minute at 15,500 x g and the supernatant was removed. This process was repeated until all the supernatant was removed. The cell pellet obtained was used for RNA extraction.

4.1.2. RNA Extraction

Cells were first disrupted by freezing and grinding in liquid nitrogen for 1 minute and were subjected to the total RNA isolation RNAqueous kit (Ambion, Austin, TX). The protocol is summarized in this paragraph. Cells were homogenized in 300 μL Lysis/Binding Solution by vortexing. An equal volume of 64% ethanol was added to the lysate and the entire mixture was applied to an RNAqueous filter cartridge, which was placed in a collection tube. The tube was centrifuged at 15,500 x g for 1 minute. The flow-through was discarded, and the sample on the filter cartridge was washed with 700 μL of wash solution #1 followed by 1 minute centrifugation at 15,500 x g. The sample was washed twice with 500 μL wash solution #2/3 followed by 1 minute centrifugation at 15,500 x g. After all the flow-through had been discarded, the sample was further centrifuged for 15 seconds at 15,500 x g to remove the last traces of wash solution. Elution Buffer (40 μL) preheated to 75°C was added to the center of the filter, and the eluate was recovered by centrifugation at 15,500 x g for 30 seconds. A second elution step was performed with 10 μL of elution buffer.

RNA concentration and quality were verified spectrophotometrically. A conversion factor of one absorbance unit at 260 nm corresponding to 40 μg RNA/mL was used. In addition, the A_{260}/A_{280} ratio was measured (Ausubel et al., 1991). A pure RNA sample should have a ratio greater than 1.8 (Ausubel et al., 1991). RNA samples were stored at -80°C until further processing.

4.1.3. DNase Treatment

To determine if a DNase treatment step was necessary, DNase treatment was performed on several samples prior to cDNA synthesis and the results were compared to samples that had not been treated with DNase. This step was necessary to assess whether the RNAqueous extraction previously described yielded total RNA sufficiently free from DNA. DNA-free™ Ambion (Austin, TX) reagents were used. The RNA sample was mixed with 10% total volume of 10X DNaseI buffer and 1 μL DNase (2 units/μL). The sample was gently mixed and incubated at 37°C for 20 minutes. DNase inactivation reagent (5 μL) was added. The sample was mixed and incubated at room temperature for 2 minutes. The tube was centrifuged at 10,000 rpm for 1 minute to pellet the DNase inactivation reagent. The RNA sample was transferred to another microfuge tube and stored at -80°C.

4.1.4. First-Strand cDNA Synthesis

Once an acceptable total RNA sample had been obtained, reverse transcription was performed using New England Biolab reagents (Beverly, MA). The following mixture was used: 0.25 pg – 1 μg total RNA, 2 μL of 15μM random nonamers (dN_9), 4 μL of 10 mM dNTPs and water to a final reaction volume of 16 μL. The sample was heated to 70°C for 5 minutes, and the following reagents were added to the tube: 2 μL of 10X reverse transcriptase buffer, 1 μL of RNase inhibitor (10 units) and 1 μL of M-MuLV reverse transcriptase (25 units). Samples were placed in a PCR Thermal Cycler and heated at 42°C for 1 hour. The enzyme was inactivated by heating to 95°C for 5 minutes and stored at 4°C until removal from the thermal cycler. Samples were diluted to 50 μL by addition of 30 μL sterile water and placed in a -20°C freezer until later use.

4.1.5. Real-Time PCR

In order to quantitatively measure the expression of the *ElHDO* gene, amplification of an endogenous control (i.e., "housekeeping") gene was performed simultaneously, and the relative expression between *ElHDO* and endogenous control gene was assessed. The *18S rRNA* was used as the housekeeping gene in this assay. An Applied Biosystem 7700 Real Time PCR machine (Foster City, CA) was used to perform the quantitative expression study.

Two different sequence detection chemistries can be used with this system. The first one, SYBR Green I dye, is a highly specific, double-stranded DNA binding dye that

detects a PCR product as it accumulates during PCR cycle. The biggest problem with this technology is that it will detect all double-stranded DNA, including non-specific reaction products. The second methodology consists of using Taqman® probes as discussed in Chapter 2. Using this system, PCR products are generated using *ElHDO* primers and the fluorogenic signal is only generated by the presence of a sequence homologous to the Taqman® homogentisate dioxygenase probe in the amplification product. Thus, the Taqman® probes reduce the possibility of a false-positive result. In addition, Taqman® minor groove binding (MGB) probes were used rather than the conventional Taqman® probes containing FAM dye as the quencher dye. The advantage of these probes is that a nonfluorescent quencher is located at the 3' end of the probe and therefore the quencher cannot fluoresce. Consequently, the MGB probes exhibit greater differences in melting temperature values between matched and mistmatched probes making the results even more accurate.

Each reaction was setup in triplicate. Each sample consisted of 18 μL from the cDNA reaction previously described, 20 μL 2X Taqman® Universal PCR Master Mix and 2 μL of 20X primer/probe mixture. The reaction protocol used consisted of one cycle at 50°C for 2 minutes, followed by one cycle at 95°C for 5 minutes, followed by 40 cycles of a 15-second denaturation step at 95°C followed by a 1-minute annealing/extension step at 60°C.

The primer/probe combinations used were as follows:

- *18S rRNA*:
  Forward Primer Sequence: 5'-CGGCGATGGTTCATTCAAATTTCTG-3'
  Reverse Primer Sequence: 5'-CCTTGGATGTGGTAGCCGTTT-3'
  Probe: 5'-CTCAGGCTCCCTCTCCG-3'

- *ElHDO*:
  Forward Primer Sequence: 5'-GGTGTGTGCGACTGTTTGG-3'
  Reverse Primer Sequence: 5'-TTCATCGAAGACCACGAGAATACC-3'
  Probe: 5'-CTCTACGGCAAGTTTG-3'

The primer/probe combinations were designed by Applied Biosystems (Foster City, CA) through their Assays-by-Design^{SM} service using their Primer-Express™ assay development software package. Probes were synthesized using a 6-FAM™ dye label.

4.1.6. Real-Time PCR Data Analysis

In order to quantify *ElHDO* gene expression, the comparative threshold cycle (C_T) method was used for relative quantitation of the gene of interest (*ElHDO*) with respect to an endogenous control (*18S rRNA*) (Livak and Schmittgen, 2001; Pfaffl and Hageleit, 2001; Schmittgen and Zakrajsek, 2000; Semighini et al., 2002; ABI, 2000). This method uses arithmetic formulas that are derived in this section.

The exponential amplification of PCR can be described by Equation 4-1 where X_n represents the number of target molecules at cycle n, X_o is the initial number of target molecules, E_x is the efficiency of target amplification and n is the number of cycles. In this research, the target molecule is *ELHDO*.

$$X_n = X_o \cdot (1 + E_x)^n \qquad \text{(Equation 4-1)}$$

C_T represents the number of cycles at which the amount of amplified target reaches a fixed threshold and can be used to derive Equation 4-2, where X_T is the

threshold number of target molecules, $C_{T,X}$ is the threshold cycle for target amplification and K_X is a constant.

$$X_T = X_o \cdot (1 + E_x)^{C_{T,x}} = K_X \qquad \text{(Equation 4-2)}$$

A similar equation can be written for the endogenous control (*18S rRNA*) gene and is described in Equation 4-3, where R_T represents the threshold number of reference molecules, R_o is the initial number of reference molecules, E_R is the efficiency of reference amplification, $C_{T,R}$ is the threshold cycle for reference amplification and K_R is a constant.

$$R_T = R_o \cdot (1 + E_x)^{C_{T,R}} = K_R \qquad \text{(Equation 4-3)}$$

A new constant, K, can be obtained by dividing X_T (threshold *ElHDO* molecule number) by R_T (threshold *18S rRNA* molecule number) as shown in Equation 4-4.

$$\frac{X_T}{R_T} = \frac{X_o \cdot (1 + E_x)^{C_{T,X}}}{R_o \cdot (1 + E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K \qquad \text{(Equation 4-4)}$$

The constant K is dependent on many factors and therefore does not have to be equal to one. Some of these parameters include reporter dye used in the probe, sequence context effects on the fluorescence properties of the probe, efficiency of probe cleavage, purity of the probe, and setting of the fluorescence threshold. If the efficiencies of the target and the endogenous control are assumed to be equal (Equation 4-5), then Equations 4-6 and 4-7 can be derived, where X_N represents the normalized amount of

target (X_o/R_o) and ΔC_T represents the difference in threshold cycles for target and reference ($C_{T,X}$-$C_{T,R}$).

$$E_X = E_R = E \qquad \text{(Equation 4-5)}$$

$$X_N \cdot (1 + E)^{\Delta C_T} = K \qquad \text{(Equation 4-6)}$$

$$\frac{X_o}{R_o} \cdot (1 + E)^{C_{T,X}-C_{T,R}} = \frac{K_X}{K_R} = K \qquad \text{(Equation 4-7)}$$

Equation 4-8 can be obtained by rearranging Equation 4-6.

$$X_N = K \cdot (1 + E)^{-\Delta C_T} \qquad \text{(Equation 4-8)}$$

The final step consists of dividing the X_N for any sample q by the X_N for the calibrator sample (cb); this is shown in Equation 4-9, where $\Delta\Delta C_T$ represents the difference between $\Delta C_{T,q}$ and $\Delta C_{T,cb}$. The calibrator sample was chosen as one that represented *E1HDO* expression levels at a baseline condition. For the VPB studies, the calibrator sample used was the sample collected at the beginning of each experiment. For the batch studies, the calibrator sample was collected from one of the bottles in which ethylbenzene was provided as the only substrate. These samples allow $X_{N,q}$ (*ElHDO* gene expression in sample q normalized to the reference (*18S rRNA*) gene expression) to be compared to the normalized gene expression in the calibrator sample, $X_{N,cb}$. This method allows one to directly quantify the effect on gene expression of a treatment condition in a given sample as compared to gene expression in the calibrator sample (Schmittgen and Zakrajsek, 2000).

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K\cdot(1+E)^{-\Delta C_{T,q}}}{K\cdot(1+E)^{-\Delta C_{T,cb}}} = (1+E)^{-\Delta\Delta C_T} \qquad \text{(Equation 4-9)}$$

If the amplicon design is properly optimized, efficiencies close to one should be obtained. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator is given by $2^{-\Delta\Delta C_T}$. For convenience, this unitless value will be referred to as T_N, the relative target gene expression number. Samples with T_N values greater than 1 have gene expression levels higher than the calibrator, and those with T_N values lower than 1 have expression levels lower than the calibrator. However, for these calculations to be valid, efficiencies of the target and endogenous amplifications must be approximately equal. To ensure that this was the case, standard curves were made to see how ΔC_T varies with total RNA dilution. Standard curves covering 0.25 pg to 1 μg total RNA were used for this purpose.

4.1.7. Gene Expression Comparison for *E. lecanii-corni* Grown on Toluene and Ethylbenzene

E. lecanii-corni cells were grown in a bubbler system as described in Chapter 3 using ethylbenzene and toluene as their sole carbon sources at a concentration of 1000 ppm_v. Cells were pelleted by centrifugation at 7800 x g at a temperature of 4°C. The cells were washed twice with ice-cold water and rinsed with phosphate buffered saline solution. The cells were transferred into a porcelain crucible and ground in liquid nitrogen. Approximately 100 μg of the ground cells were transferred to a microfuge

tube, and total RNA was isolated using the method previously described in this section. 250 ng of total RNA were used for cDNA synthesis.

4.2. RESULTS AND DISCUSSION

In this chapter, the methods necessary for the remaining experiments of this dissertation were developed and verified. A description of the important results is presented in the following section along with a discussion.

4.2.1. *ElHDO* and *18S rRNA* Standards

Relative quantification has been used in a number of studies (Livak and Schmittgen, 2001; Pfaffl and Hageleit, 2001; Schmittgen and Zakrajsek, 2000; Semighini et al., 2002). The most common housekeeping genes used in these studies include β-actin, glyceraldeyde-3-phosphate dehydrogenase and *18S rRNA*. Despite reservations regarding changes in expression levels and potential imbalances in rRNA and mRNA fractions between different samples, *18S rRNA* has recently been validated for normalized expression levels by quantitative RT-PCR analysis under a number of experimental conditions (Goidin et al., 2001; Bustin, 2000; Schmittgen and Zakrajsek, 2000). Several research studies have shown that *18S rRNA* is more reliable than other housekeeping genes for normalization (Goidin et al., 2001; Bustin, 2000; Schmittgen and Zakrajsek, 2000). The most critical factor for successful relative quantitative analysis is the use of normalized RNA (i.e., known quantity of RNA) in the cDNA synthesis step rather than starting the whole procedure from a normalized number of cells (Schmittgen

and Zakrajsek, 2000). For these reasons, a normalized quantity of total RNA and the *18S rRNA* were used in the comparative gene expression level studies in this research.

Total RNA amounts ranging from 0.25 pg to 1 μg were used to synthesize cDNA and amplified using qRT-PCR. The C_T values determined in this experiment are summarized in Tables 4-1 and 4-2 for *18S rRNA* and *ElHDO*, respectively. Plots of these results were also generated and are shown in Figure 4-1. The coefficient of determination (R^2) values relating C_T to log [RNA] values were greater than 0.97 for both targets over the entire range tested. This result indicates that for the given qRT-PCR setup and cycling conditions, RNA amounts between 0.25 pg and 1 μg are acceptable for relative comparison of *ElHDO* and *18S rRNA* expression levels. Subsequent experiments were run using 250 ng total RNA to ensure that conditions always fell within the linear range.

Table 4-1: *18S rRNA* Standard Threshold Cycle Values

RNA Amount (ng)	**C_T Value 1[1]**	**C_T Value 2**	**C_T Value 3**	**Average C_T**	**Standard Deviation C_T**
0.00025	31.06	31.67	32.04	31.59	0.49
0.0025	27.95	28.78	27.5	28.08	0.65
0.025	26.35	25.9	25.44	25.90	0.46
0.25	23.68	22.5	22.95	23.04	0.60
2.5	21.02	20.98	21.5	21.17	0.29
25	19.23	19.63	20.56	19.81	0.68
250	17.21	17.8	17.46	17.49	0.30
1000	13.54	13.01	12.78	13.11	0.39

[1] Values 1, 2 and 3 correspond to the C_T values obtained for each of the triplicate samples.

Table 4-2: *ElHDO* Standard Threshold Cycle Values

RNA Amount (ng)	**C_T Value 1**	**C_T Value 2**	**C_T Value 3**	**Average C_T**	**Standard Deviation C_T**
0.00025	40	40	40	40.00	0.00
0.0025	35.46	35.07	35.67	35.40	0.30
0.025	33.2	34.26	33.96	33.81	0.55
0.25	31.38	32.48	31.84	31.90	0.55
2.5	29.37	28.29	29.02	28.89	0.55
25	27.15	28.38	28.25	27.93	0.68
250	25.67	24.98	26.02	25.56	0.53
1000	21.07	22.25	21.67	21.66	0.59

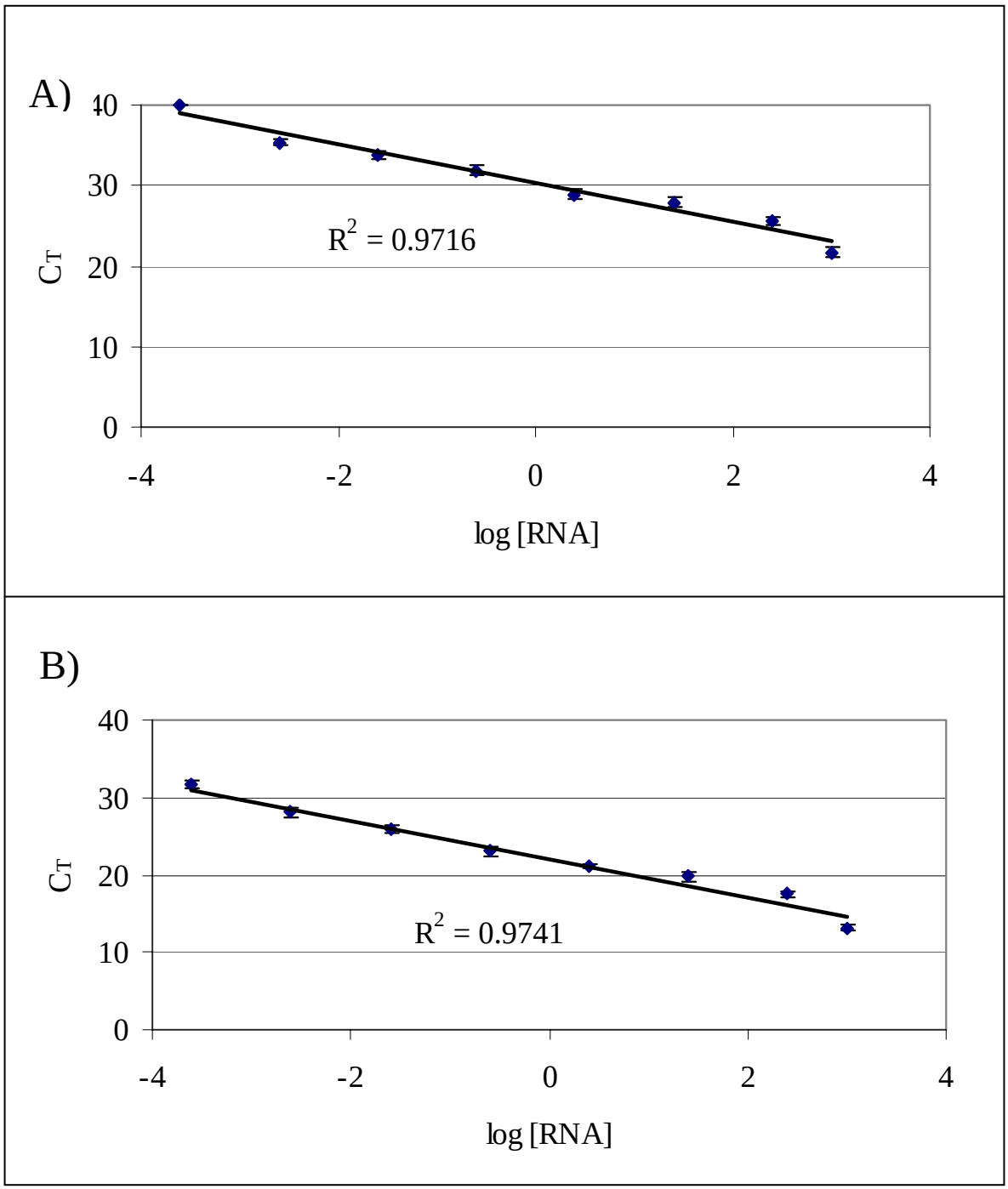

Figure 4-1: Analysis of 8-fold serial dilution of total RNA - a) *ElHDO* and b) *18S rRNA*. Error bars represent one standard deviation.

To be able to use the comparative threshold cycle analysis method, the amplification efficiencies for both targets must be similar over the RNA concentration range used. For this to be true, a plot depicting the difference between the C_T values for *ElHDO* and *18S rRNA* (ΔC_T) as a function of log [RNA] should have a slope of less than 0.1 (ABI, 2001). Figure 4-2 shows that, over the range analyzed (0.25 pg to 1 μg total RNA), ΔC_T values remain essentially constant. The slope of the curve is 0.0492, which is well below the required 0.1 value. This result indicates that the comparative threshold cycle analysis can be used to compare *ElHDO* and *18S rRNA* expression levels in *E. lecanii-corni*.

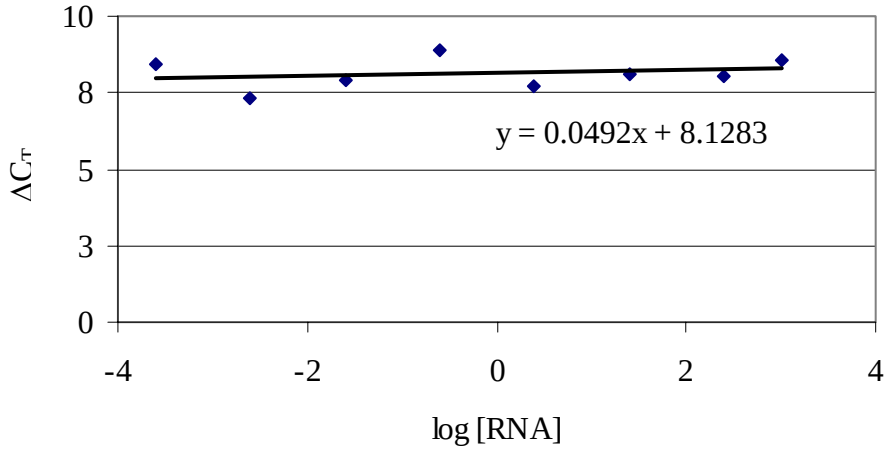

Figure 4-2: PCR Efficiency Comparison between *ElHDO* and *18S rRNA*

4.2.2. Biomass Extraction Method

The next set of experiments served to determine the quantity of biofilter packing needed for the qRT-PCR analysis as well as to determine the impact of the extraction method on qRT-PCR results. To extract a sufficient number of cells to isolate total RNA, at least 3 pellets were necessary to obtain enough cells to homogenize by grinding in liquid nitrogen and to use in the subsequent steps of the total RNA isolation protocol. Because of the large number of samples that needed to be collected over relatively short amounts of time, only one composite sample, consisting of 3 pellets, was collected from the inlet section of the biofilter for each time point for all remaining experiments. Because fresh packing must be added to the bioreactor to replenish packing used for analysis, collecting one composite sample per time point minimized the disturbance to the biofilter and ensured that the biofilm remained relatively stable throughout the experiment. As described in detail earlier in this chapter, total RNA was extracted from this composite sample, and cDNA synthesis/qRT-PCR amplification was performed in triplicate on each composite sample.

Four different extraction methods were evaluated for removing the fungal biofilm from the biofilter packing medium (Celite R-635, Celite Corp., Lompoc, CA). The four methods tested were (1) scraping, (2) 10 seconds vortexing, (3) 60 seconds vortexing, and (4) 3 minutes vortexing followed by 3 minutes sonication. For this comparison, samples of packing were obtained from the biofilter during the startup phase of operation (see Chapter 5 for more detailed information on VPB operation). Samples for each extraction method were collected simultaneously. Relative target expression numbers (T_N) for each

extraction method can be found in Table 4-3 and are shown graphically in Figure 4-3. All extraction methods were found to reflect similar T_N values. This result indicates that even if some of the extraction methods cause more RNA to shear, similar amounts of RNA were degraded for the two target genes yielding fairly constant T_N values. Because visual inspection indicated that the 10-second vortexing extraction method appeared to remove most of the biomass from the packing and the results presented in Table 4-3 indicate that it did not affect the T_N value, this technique was chosen as the preferred extraction method for the remaining experiments described in this dissertation.

Table 4-3: Analysis of the Impact of Biomass Extraction on T_N

Extraction Method	**C_T** ***ElHDO***	**C_T** ***18S rRNA***	**ΔC_T**	**$2^{-\Delta\Delta C_T}$**	**Average T_N**	**Standard Deviation T_N**	**t-test[2]**
10 second vortex	***22.35[3]***	***15.08***	***-7.27***	***1.00***	0.93	0.40	N/A
	21.95	15.05	-6.9	1.29			
	23.21	14.93	-8.28	0.50			
60 second vortex	22.96	15.07	-7.89	0.65	0.99	0.38	ns
	22.54	15.15	-7.39	0.92			
	22.05	15.27	-6.78	1.40			
3 minute vortex and sonication	22.34	14.69	-7.65	0.77	1.10	0.42	ns
	22.45	15.11	-7.34	0.95			
	21.97	15.35	-6.62	1.57			
Scraping	24.02	15.2	-8.82	0.34	0.46	0.28	ns
	23.88	14.67	-9.21	0.26			
	23.01	15.39	-7.62	0.78			

[2] t-test was performed by comparing each data set to 10-second vortex data. sig: significant, ns: non-significant, α=0.05

[3] This sample was used as the calibrator.

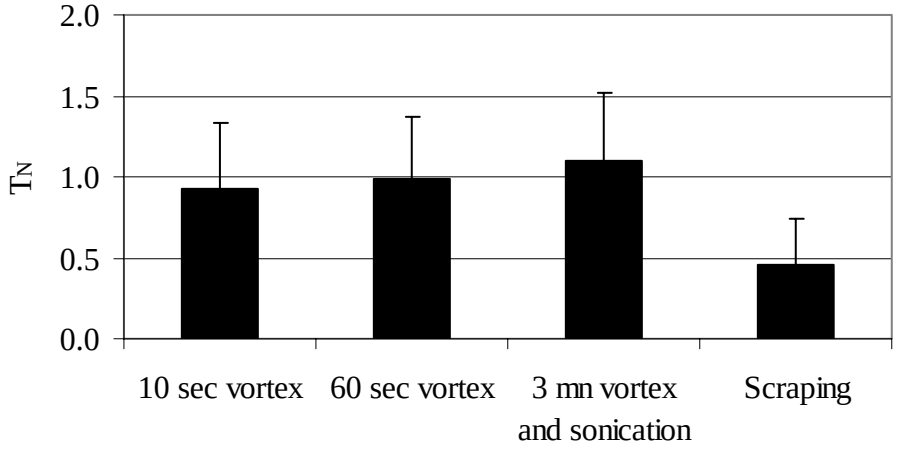

Figure 4-3: Biomass Extraction Method Comparison. Error bars represent one standard deviation.

4.2.3. Validation Experiment

To validate the qRT-PCR method, the gene expression study described in Chapter 3 was repeated using qRT-PCR rather than a Northern Blot to compare *ElHDO* expression for *E. lecanii-corni* cells grown on toluene and ethylbenzene. The experiment reported in Chapter 3 showed that using Northern Blot analysis, toluene-grown cells did not express *ElHDO* as highly as cells grown on ethylbenzene. In this experiment, the difference in the expression was analyzed using qRT-PCR to quantify this change and determine if in fact this method also yielded a lower *ElHDO* expression level for toluene-grown cells. Table 4-4 and Figure 4-4 summarize the results obtained for this comparison.

Table 4-4: Comparison of *ElHDO* and *18S rRNA* between *E. lecanii-corni* Cells Grown on Toluene and Ethylbenzene

Extraction Method	**C_T** ***ElHDO***	**C_T** ***18S rRNA***	**ΔC_T**	**$2^{-\Delta\Delta C_T}$**	**Average T_N**	**Standard Deviation T_N**	**t-test[4]**
Ethylbenzene with no DNase Treatment	***22.35[5]***	***14.08***	***-8.273***	***1.00***	1.15	0.17	N/A
	21.95	14.05	-7.901	1.29			
Ethylbenzene with DNase Treatment	22.21	13.93	-8.28	1.00			
	21.96	14.07	-7.891	1.30			
Toluene with no DNase Treatment	28.29	12.95	-15.337	0.02	0.03	0.22	sig
	28.38	13.18	-15.198	0.01			
Toluene with DNase Treatment	26.10	13.52	-12.579	0.05			
	27.01	13.95	-13.056	0.04			

[4] t-test was performed by comparing toluene to ethylbenzene data. sig: significant, ns: non-significant, α=0.05

[5] This sample was used as the calibrator.

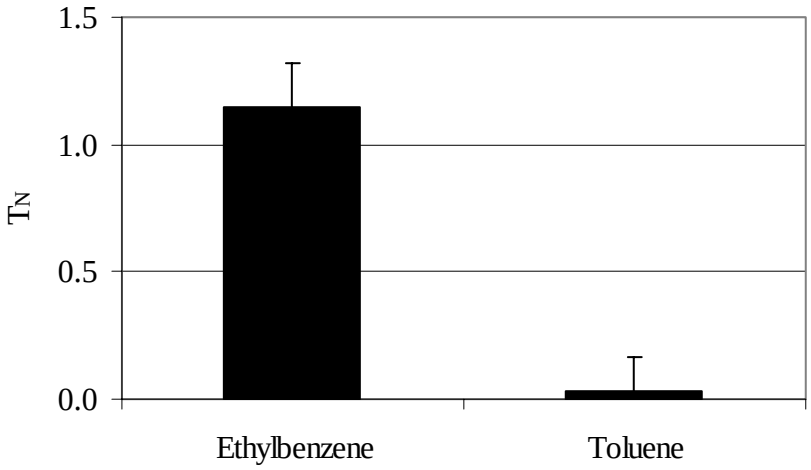

Figure 4-4: Relative Target Expression Numbers for Toluene and Ethylbenzene Grown Cells. Error bars represent one standard deviation.

As shown in Table 4-4, DNase treatment had little effect on T_N values indicating that there was negligible DNA contamination after the total RNA extractions using the Ambion RNAqueous kit. For this reason, a DNase treatment step was not included in the subsequent cDNA synthesis reactions for the remainder of the study. However, to ensure no subsequent DNA contamination occurred in subsequent RNA extractions, negative controls were included in all qRT-PCR experiments. These controls included the following:

1. no RNA-template but reverse transcriptase and polymerase provided;
2. RNA and polymerase provided but no reverse transcriptase; and
3. RNA, reverse transcriptase provided but no polymerase.

Similar to the Northern blot results presented in Chapter 3, qRT-PCR results show that *ElHDO* is induced at a much higher level in the presence of ethylbenzene as

compared to toluene. The amount of gene expression when ethylbenzene is not present is negligible and indicates that this gene is probably not constitutively induced or is constitutively induced at low amounts.

The results from this chapter indicate that the comparative threshold cycle method can be used to analyze *ElHDO* gene expression relative to *18S rRNA*. In addition, the sample preparation steps (number of packing pellets required, extraction method, total RNA preparation, cDNA synthesis and qRT-PCR amplification) were outlined. The remainder of this dissertation will use the methods developed and apply them to a variety of biofilter loading conditions to assess whether qRT-PCR can accurately reflect or predict macroscale pollutant degradation observed in a fungal VPB.

Chapter 5: Batch Reactor Substrate Mixture Study

Since one of the goals of this study was to quantify gene expression changes in a VPB as a function of the variation in substrate mixtures, it was necessary to find substrates that act as regulators of *ElHDO* expression. To this end, a repressor, an inducer and a compound that has no effect on ethylbenzene degradation were identified and their effects on *ElHDO* gene expression were analyzed. The first part of this chapter provides a summary of the batch reactor studies that were performed to determine which substrates had the desired regulatory effect on ethylbenzene degradation. The second part of this chapter describes the effect of each regulatory compound on *ElHDO* gene expression in batch reactors.

5.1. Identification of Ethylbenzene Regulatory Substrates

In this phase of the research, several substrates were tested to determine their regulatory effect on ethylbenzene degradation. To test the qRT-PCR gene expression system, compounds affecting ethylbenzene metabolism by induction and repression as well as a compound having no effect were identified.

5.1.1. Methods

Phenyl acetate was tested as the inducer, methyl propyl ketone as the repressor, and o-xylene as the no effect compound. All chemicals were obtained from Sigma-

Aldrich (Saint Louis, MO). Each substrate was tested at three different initial concentrations (10, 20 and 40 μM liquid phase concentration) in the presence of 20 μM ethylbenzene (liquid phase concentration) in batch reactors. Each reactor consisted of a 250-mL glass bottle containing 20 mL of basal medium. In the case of the repressor compound, the aforementioned protocol was modified because a diauxic degradation pattern was observed. Initially, ethylbenzene (20 μM) was added to each of the bottles. Once the ethylbenzene initially supplied to the reactor was degraded, the reactor was provided a second ethylbenzene feeding (20 μM liquid phase concentration). At the same time, the repressor substrate (methyl propyl ketone) was added at 10, 20 or 40 μM liquid phase concentration.

Each batch reactor described above was inoculated with $5x10^4$ spores/mL *E. lecanii-corni*. Twenty (20) mL of sterile basal medium was added to each 250-mL bottle and incubated on a shaker at room temperature (20-23°C). The basal medium recipe used was the same as that described in Chapter 3. The depletion of ethylbenzene and other substrates was monitored by gas chromatography under each growth condition as described in Chapter 3. Dry weight measurements were obtained at the end of each experiment following the same protocol presented in Chapter 3. Specific utilization rates for ethylbenzene were determined for each condition using Equation 5-1.

$$\text{Specific Utilization Rate} = \frac{\text{Total amount of EBZ Consumed (g)}}{(\text{Total Incubation Time} - \text{Lag Time})\ (\text{hr}) \cdot \text{Cell Mass (g dry weight)}} \quad \text{(Equation 5-1)}$$

5.1.2. Results

The greatest challenge in this part of the study was to decide which chemicals to test for a regulatory effect on ethylbenzene degradation by *E. lecanii-corni*. Compounds involved in the ethylbenzene degradation pathway were hypothesized to be inducers. Those that are not metabolized by *E. lecanii-corni* were hypothesized to have no effect and those that are easily degradable were hypothesized to be repressors of ethylbenzene degradation. Using these guidelines, three regulatory substrates were identified. Phenyl acetate, o-xylene and methyl propyl ketone were tested and found to be inducer, no effect and repressor compounds respectively. Specific utilization rates were calculated for ethylbenzene and are summarized in Table 5-1 and Figure 5-1. The actual substrate depletion curves are presented in Appendix A (Figures A7-A18).

Table 5-1: Specific utilization rates for methyl propyl ketone, phenyl acetate and o-xylene in the presence of ethylbenzene.

	Amount of Regulatory Substance Added	Specific Ethylbenzene Utilization Rate (g EBZ/g cells/hr)	Standard Deviation
Methyl Propyl Ketone	None	77.42	4.38
	Low[6]	71.03	1.88
	Medium	53.64	6.83
	High	42.78	0.41
Phenyl Acetate	None	66.61	1.22
	Low	76.83	1.03
	Medium	87.64	2.98
	High	112.58	2.07
o-Xylene	None	76.46	1.93
	Low	79.18	0.95
	Medium	78.84	0.84
	High	77.71	1.49

[6] Low, medium and high concentration represents 10, 20 and 40 μM of each regulatory substrate respectively.

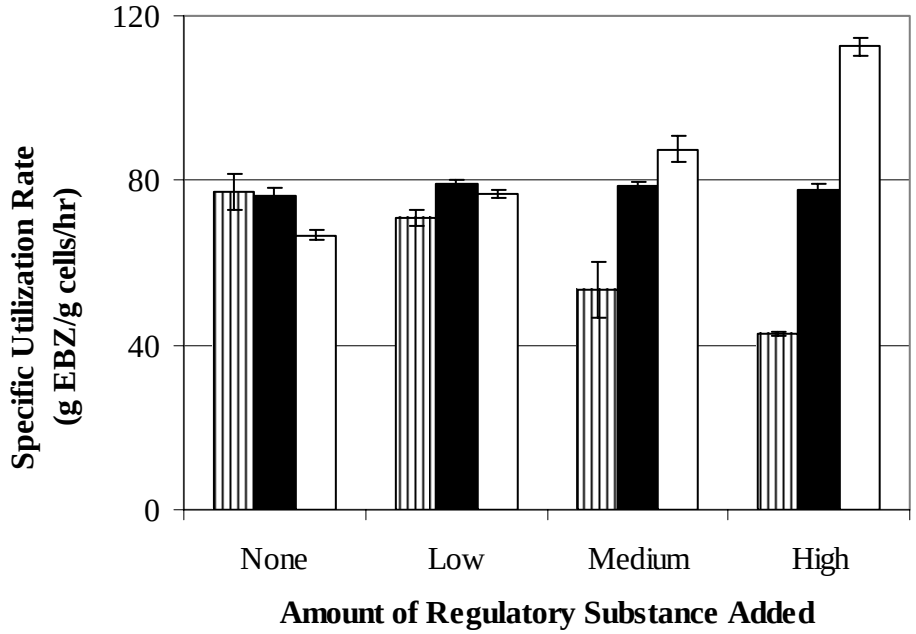

Figure 5-1: Ethylbenzene Specific Utilization Rate as a Function of Regulatory Substance[7]. (▥ , ■ and □ represent methyl propyl ketone, o-xylene and phenyl acetate respectively. Error bars represent one standard deviation.

A dramatic change in the specific substrate utilization rate was observed in the presence of the repressor. As expected, in the presence of methyl propyl ketone, the specific utilization rate of ethylbenzene decreased approximately 45% between cells that had not been exposed to methyl propyl ketone as compared to those exposed to the highest concentration tested (40 μM). As the concentration of the repressor was increased, the effect on the specific substrate utilization rate was amplified. In the presence of the inducer, phenyl acetate, the rate increased by 69% when comparing cells not exposed to phenyl acetate and those exposed to the highest concentration. In the presence of o-xylene, the ethylbenzene degradation rate remained relatively constant. These results indicate that these regulatory substrates are well suited for gene expression

[7] Low, medium and high concentrations refer to 10, 20 and 40 μM of the regulatory substance, respectively.

studies to follow the expression of a gene involved in ethylbenzene degradation such as *ElHDO*.

5.2. BATCH REACTOR GENE EXPRESSION STUDY

In the previous section, three compounds were identified that have a regulatory effect on ethylbenzene degradation at the macroscale. In this section, these substrates were used in batch reactor experiments to determine if the specific utilization rate observations can be correlated to gene expression data.

5.2.1. Methods

Four different sets of batch reactors were prepared using the same 250-mL bottles previously described: 1) ethylbenzene alone, 2) ethylbenzene with methyl propyl ketone, 3) ethylbenzene with o-xylene and 4) ethylbenzene with phenyl acetate. Batch reactors were prepared in triplicate. One set of killed controls was prepared to ensure substrate removal was not a result of abiotic reactions. Each bottle contained 20-mL of basal medium and an initial spore concentration of $5x10^4$ spores/mL. Each regulatory substrate was added at 30 μM (liquid phase concentration) in the presence of 20 μM ethylbenzene (liquid phase concentration). Headspace measurements were taken by gas-chromatography on the day the biomass was harvested to ensure both ethylbenzene and the regulatory substrate were still present. Cells were disrupted by freezing and grinding in liquid nitrogen. Total RNA and cDNA were obtained following the protocols described in Chapter 4.

5.2.2. Results

The results obtained are presented in Table 5-2 and Figure 5-2. As expected for cells grown only in the presence of ethylbenzene, the T_N values were close to 1.0 indicating that the expression levels were similar to that observed in the calibrator sample (also grown only in the presence of ethylbenzene). In the presence of the repressor, methyl propyl ketone, *ElHDO* expression was reduced. The average T_N value decreased by approximately 81%. In the presence of the inducer, phenyl acetate, the expression level increased by approximately 52%. Cells that were grown in the presence of ethylbenzene and o-xylene expressed *ElHDO* in quantities similar to those observed when only ethylbenzene was present. While the T_N value was slightly lower than with ethylbenzene alone, these two values are not statistically different when taking the standard deviation into consideration. The target expression numbers, therefore, follow the expected trends and mimic closely the results obtained in the specific ethylbenzene utilization rates determined in the previous section.

Table 5-2: Relative Quantitation of *ElHDO* Expression for the Batch Reactor Substrate Mixture Study. The sample which is marked in bold italics was used as the calibrator.

Growth Condition	**C_T** ***ElHDO***	**C_T** ***18S rRNA***	**ΔC_T**	**$2^{-\Delta\Delta C_T}$**	**Average T_N**	**Standard Deviation T_N**	**t-test[8]**
Ethylbenzene Alone	***22.38[9]***	***10.72***	***-11.66***	***1.00***	0.97	0.15	N/A
	22.85	10.88	-11.97	0.81			
	22.67	11.16	-11.51	1.11			
Ethylbenzene with Methyl Propyl Ketone	25.59	11.05	-14.54	0.14	0.18	0.05	sig
	25.82	12.02	-13.80	0.23			
	26.07	11.92	-14.15	0.18			
Ethylbenzene with o-Xylene	23.15	11.08	-12.07	0.75	0.85	0.12	ns
	23.17	11.20	-11.97	0.81			
	22.89	11.20	-11.69	0.98			
Ethylbenzene with Phenyl Acetate	22.38	11.14	-11.24	1.34	1.47	0.14	sig
	22.40	11.44	-10.96	1.62			
	22.40	11.27	-11.13	1.44			

[8] t-test was performed by comparing to ethylbenzene alone data. sig: significant, ns: non-significant, α=0.05

[9] This sample was used as the calibrator.

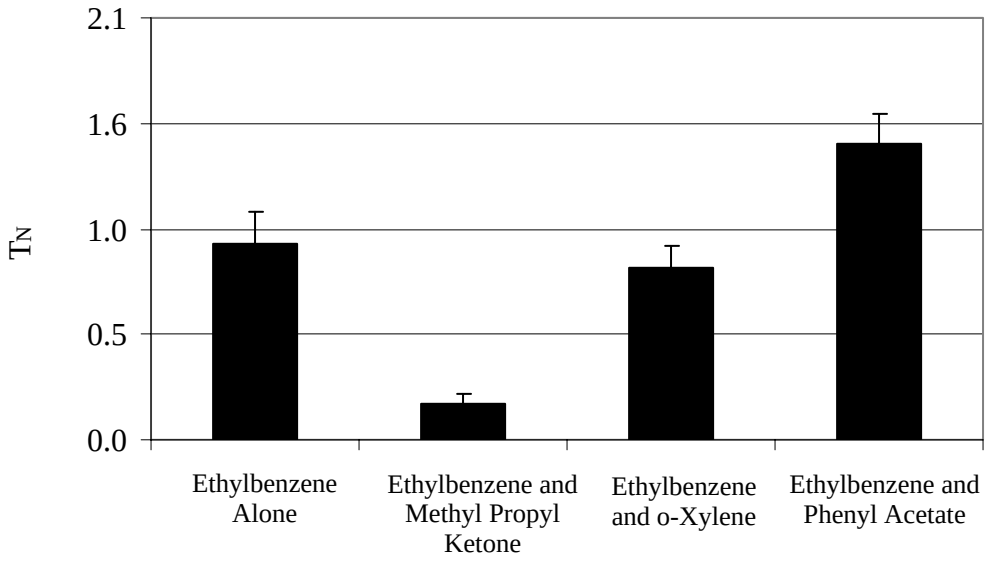

Figure 5-2: Normalized Target Expression Numbers for Ethylbenzene Alone (20 μM Initial Concentration) and for Ethylbenzene (20 μM Initial Concentration) in the Presence of Other Organic Substrates (30 μM Initial Concentration) in a Batch Reactor. Error bars represent one standard deviation.

5.3. DISCUSSION

The series of experiments presented in this chapter represent the first real-time based quantification of gene expression in an environmental engineering treatment system. Therefore, a comparison is warranted between the method used in this research and the competitive RT-PCR used in the one published quantification study with relevance to environmental engineering. First, the results obtained in this phase of the research will be discussed followed by a discussion comparing real-time qRT-PCR and competitive RT-PCR.

5.3.1. Substrate Mixture Study Using qRT-PCR

As described in Chapter 2, enzymatic levels are expected to vary according to a variety of parameters including the introduction of substrate mixtures. To coordinate their numerous metabolic activities, cells must regulate the catalytic activities of their enzymes to respond to changes in their environment. Several mechanisms are used in the cell to regulate enzymatic activity. The first method is to control the amount of available enzyme (Madigan et al., 2000). Since this amount is directly related to the rate of synthesis and degradation, the cell can directly control it by making modifications that affect these rates, such as through regulatory proteins binding to DNA sites (Alberts et al., 1994). The second method of enzymatic control is to directly control the activity. To this end, the catalytic activity of an existing enzyme can be altered by conformational or structural alterations to its active site. These changes will modify its substrate binding

affinity and therefore increase or decrease its activity depending on the modification. A common mode of metabolic regulation is feedback inhibition. This regulation is achieved through the presence of a biosynthetic pathway intermediate whose concentration directly controls the activity of an enzyme involved in that pathway. Therefore, if a higher concentration of this intermediate were present, either a higher amount of available enzyme would be present or its catalytic activity would be increased. In this manner, enzymes can be down-regulated by compounds that provide a better source of energy to cells and therefore are more easily degradable (Lodish et al., 1995). Those compounds that do not use similar enzymes and whose degradation is independent of the initial substrate should not impact its degradation. Using this rationale, three substrates with regulatory effects on ethylbenzene degradation were identified.

In this batch reactor study, several important factors were revealed. First, it appears that the qRT-PCR method is quite reproducible. Indeed, biomass was extracted independently from three separate incubation bottles and used in separate total RNA isolation and cDNA synthesis reactions. Despite the potential for variations between individual incubation bottles, the largest standard deviation was approximately 20%. This is an important result and implies that similar T_N values can be obtained for biomass samples that have received the same treatment. Because a greater number of samples were collected during the VPB experiments and because three pellets are required for each analysis (see Chapter 4), one composite sample rather than multiple individual samples were collected from the VPB at each sampling time.

The second important result obtained in this study is that T_N values did in fact follow the specific ethylbenzene utilization rate trends. T_N values and specific utilization

rates remained constant upon the introduction of o-xylene. In addition, they both decreased or increased in the presence of methyl propyl ketone or phenyl acetate, respectively. The increase in T_N relative to specific utilization rates however was not linear. Indeed, while T_N increased by 52% with phenyl acetate (comparing 0 and 30 μM phenyl acetate), the utilization rate increased by 69% (comparing 0 and 40 μM phenyl acetate). With methyl propyl ketone, T_N decreased by 81% (comparing 0 and 30 μM methyl propyl ketone) while the utilization rate decreased by 45% (comparing 0 and 40 μM methyl propyl ketone). Therefore, while these two parameters are related they are not always directly proportional.

The results obtained indicate that qRT-PCR can be used to describe the effect of regulatory substrates on *ElHDO* expression. In the following section, a comparison between qRT-PCR and competitive RT-PCR is presented.

5.3.2. Comparison of Competitive RT-PCR and Real-Time qRT-PCR

Although qRT-PCR has become increasingly popular for clinical applications, relatively few studies have been performed using this technique for environmental applications. Most research has focused on studying microbial ecology questions in an environmental system using the bacterial 16S rDNA gene or other functional genes such as the amoA gene or that encoding catechol-2,3-dioxygenase. This type of research has been applied to a wide range of environmental biological treatment applications including *in situ* bioremediation (Roling et al., 2002; Ralebits et al., 2002; Plaza et al., 2001), wastewater (La Para et al., 2001a; La Para et al., 2001b; Silvey et al., 2000) and biofiltration (Li and Moe, 2004). The studies that have incorporated the use of real-time

PCR have all been DNA-based experiments (Baldwin et al., 2003; Robinson et al., 2003; Mesearch et al., 2003; Dionisi et al., 2003; Rahm et al., 2003). DNA-based tools are very useful to determine if a specific microbial species is present in a given sample or to study how the community shifts over time. The main drawback of these techniques is that they do not provide any information about whether or not a specific species is active. Because 16S rDNA has been found to be quite stable in dead cells, the presence of a specific organism in a sample does not imply that it is actually responsible for any specific degradative activity (Lindahl, 1993).

By contrast, RNA-based tools specifically target the active fraction of biomass in a sample. RNA-based methods are those that need to be used to describe biofilm level activity. To date, only one environmentally-based study has been published that directly quantifies gene expression levels. Han and Semrau (2004) used competitive RT-PCR to quantify methanotrophic gene expression for *in situ* bioremediation. Quantification using competitive RT-PCR relies on the addition of a known amount of a standard to each sample. The standard is typically a genetically modified version of the target gene that is amplified in the study. This way, only one set of primers needs to be added during the amplification process. The expression level in each sample is then determined by comparing the quantities of unknown and known products after amplification.

Typically, slab gel electrophoresis has been used for quantification in competitive PCR (Martinelli et al., 1998). This method is difficult to automate and takes a relatively long amount of time. Therefore, its utility is limited. More recently capillary electrophoresis has become the method of choice for analyzing competitive PCR products. Han and Semrau (2004) used this method and were able to separate several

products in a column and quantified each relative to the RNA standards. Because the qRT-PCR method is automated, it should be less prone to error as compared to capillary electrophoresis.

Similar to the methods used in this research, competitive RT-PCR uses a normalized amount of total RNA to synthesize cDNA, and this product is then used in the amplification. One major disadavantage of the competitive RT-PCR method is the external addition of the RNA standard. Because such small amounts of RNA are added, even slight variations in pipetting could result in significantly different results. By contrast, this is not a concern in the qRT-PCR method employed in this research. If a different amount of RNA is added to each sample, the increase (or decrease) would be the same for both *18S rRNA* and *ElHDO* targets and therefore would not interfere with the results.

Another advantage of the comparative qRT-PCR method is the limited amount of sample manipulation required, which decreases the possibility of error as compared to a more labor intensive method. For instance, in competitive RT-PCR, the PCR products must be transferred from the PCR machine to the capillary electrophoresis machine. Even though capillary electrophoresis has parallel loading capabilities, the sample must still be transferred from the PCR tubes to the capillary electrophoresis apparatus adding one more step to the procedure. Using real-time qRT-PCR, results can be directly obtained after the PCR cycling. No additional manipulation is required.

In conclusion, comparative qRT-PCR was successful in linking microscale (relative gene expression) to macroscale (ethylbenzene substrate utilization rate) level

phenomena observed. In addition, qRT-PCR presents a certain number of advantages over competitive RT-PCR. First, no external RNA standards must be added to samples. All of the quantification is based on an internal RNA standard. Next, no additional manipulation is required after the PCR amplification step. Both of these factors should reduce errors and the time required for sample processing. In the following chapters, the comparative qRT-PCR method will be used to quantify gene expression changes in a more complex system, namely a VPB.

Chapter 6: VPB Substrate Mixtures Study

In Chapter 5, methyl propyl ketone, o-xylene and phenyl acetate were identified as having different regulatory effects on ethylbenzene degradation as demonstrated by their respective effects on specific ethylbenzene utilization rates and *ElHDO* expression levels in batch reactor systems. qRT-PCR was found to accurately reflect the macroscale ethylbenzene degradation occurring in these homogenous and well-mixed systems. In this chapter, the effect of these same three compounds is analyzed in a VPB, a more complex system, to determine if qRT-PCR can provide any insight into biofilm level gene expression.

6.1. Methods

6.1.1. VPB Operation

A biofilter was constructed and packed with Celite R-635 (Celite Corp., Lompoc, CA) in a manner similar to that used in previous toluene *E. lecanii-corni* studies (Woertz et al., 2001). The bioreactor design is shown in Figure 6-1. The packing media was presoaked for 24 hours in nutrient basal medium containing 5 g/L $(NH_4)_2.SO_4$ following the same recipe described in Chapter 3 (section 3.1.1). A humidified, synthetic waste gas stream was provided to the bioreactor by mixing two separate air streams. The first air stream was fed through a nebulizer (Vortran Medical Technology, Sacramento, CA)

filled with basal medium. This process created a fine, nutrient-laden aerosol that humidified the airstream. Approximately 750 mL of basal medium was aerosolized each day of operation. The second air stream continuously supplied ethylbenzene using a syringe pump (kd Scientific, Model KDS200, Holliston, MA). Ethylbenzene was provided to the column at a concentration of 100 ppm_v. The two air streams were mixed in a plastic mixing chamber prior to entering the VPB. A flow rate of 14.5 L/min and a VPB residence time of 1 minute were applied to the system corresponding to an ethylbenzene mass loading of 26.3 $g/m^3/h$. Regulatory substrates were mixed with the ethylbenzene and also provided to the column using the syringe pump. During each substrate mixture study, the concentration of the regulatory substrate being investigated was increased every 24-hour period as shown in Table 6-1. The column was operated at room temperature (20-23°C) in a downflow unidirectional fashion. *E. lecanii-corni* was inoculated into the VPB column by manually pouring 2-L of a suspended cell solution onto the packing in the column. The cells had been grown in a bubbler system (described in Chapter 3), which had been continuously supplied 1000 ppm_v ethylbenzene. The initial cell concentration in the bubbler system was 10^5 cells/mL. Cells were harvested after 3 weeks, at a cell concentration of approximately $5x10^8$ cells/mL. The VPB column retained approximately 90% of the inoculum solution when it was poured over the reactor packing media.

Table 6-1: Regulatory Substrate VPB Feeding Scheme

Time Period	Ethylbenzene Concentration	Regulatory Substrate Concentration
0-24 hour	100 ppm_v	50 ppm_v
24-48 hour	100 ppm_v	100 ppm_v
48-72 hour	100 ppm_v	200 ppm_v

Biofilter performance was monitored by checking effluent ethylbenzene and regulatory substrate concentrations from each of the bioreactor sections. Gas phase samples were collected from each section using a 0.5-mL gas tight syringe (Supelco, Baton Rouge, LA) and analyzed using a Hewlett Packard Model 5890 Gas Chromatograph (GC) equipped with a flame-ionization detector (FID) and a RTX-624 Restek capillary column (State College, PA) using the method described in Chapter 3.

Packing media samples were collected from each section along the biofilter for biomass and nutrient characterization. Biomass was removed from the packing media by placing packing pellets in 10 mL of deionized water, sonicating for 3 minutes and vortexing for 3 minutes. Biomass accumulation in each section was monitored by determining the chemical oxygen demand (COD) per unit packing weight.

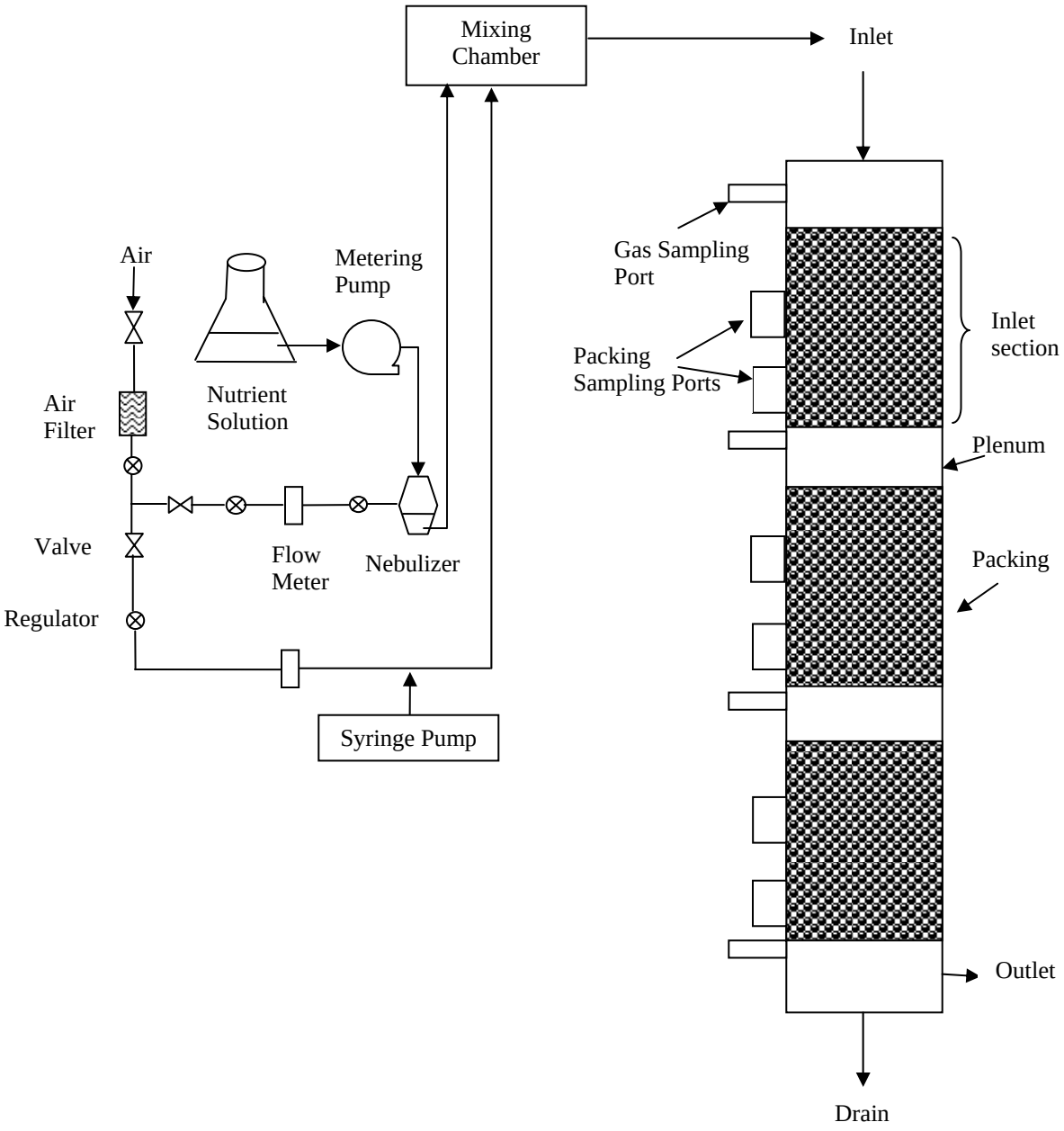

Figure 6-1: Vapor Phase Bioreactor System

Inorganic nitrogen (N) in the form of ammonium ($NH4^+$), nitrite (NO_2^-) and nitrate (NO_3^-) was also analyzed to estimate the nutrient levels in the bioreactor column. To determine the amount of ammonium and nitrate in the packing material, packing samples were taken from each section and suspended in 10 mL deionized water. Samples were vortexed for 3 minutes and sonicated for 3 minutes. 1-mL of this sample was added to 9-mL of deionized water. The 10-mL sample was then analyzed using an Orion ammonium/nitrate combination selective probe (Thermo Orion, Beverly, MA). Six-point calibration curves were prepared for ammonium and nitrate using NH_4Cl (10-1000 mg N/L) and KNO_3 (10-1000 mg N/L) respectively. The packing samples were dried overnight at 105°C to normalize the amount of ammonium and nitrate to the mass of dry packing.

Nitrite-nitrogen analysis was conducted according to the ferrous sulfate method (Hach, 1992). Nitriver®2 nitrite reagent powder pillows were added to a 1 mL suspended cell sample and 9 mL of deionized water. The suspended cell sample was obtained by removing biomass from the packing media by sonicating for 3 minutes and vortexing for 3 minutes. Samples were shaken to dissolve the reagent and incubated at room temperature for 15 minutes. A spectrophotometric reading at 585 nm was then taken. A 6-point calibration curve ranging from 2-250 mg/L NO_2^- was produced prior to each analysis.

The moisture content of the packing media was also determined periodically by comparing the weight of a packing media pellet sample before and after drying at 120°C overnight. The mass of volatile suspended solids was determined by placing packing samples in a furnace at 550°C overnight and comparing their weight before and after

drying. Samples were always allowed to cool to room temperature in a desiccator prior to weight measurements. The pressure drop across the column was measured by connecting a pressure gauge to the inlet and outlet sampling ports, and the pH of the leachate drained from the bioreactor column was monitored using a pH meter.

6.1.2. Gene Expression Study

Because most ethylbenzene degradation took place in the first VPB section, composite samples consisting of 3 pellets were collected only from the inlet section of the VPB at regular time intervals (time 0, 4, 12 and 24 hours for each feed condition). Time zero refers to the moment at which the regulatory substrate was introduced into the syringe pump feed at each of the tested concentrations (i.e. 50, 100 and 200 ppm_v) as outlined earlier in Table 6-1. The experiments were run in the following order: 1) methyl propyl ketone and ethylbenzene, 2) o-xylene and ethylbenzene, 3) phenyl acetate and ethylbenzene, 4) methyl propyl ketone, phenyl acetate and ethylbenzene. To minimize the disturbance to the biofilm in the bioreactor, only one composite sample of the packing was collected per time point. Biomass extraction, total RNA isolation, cDNA synthesis and qRT-PCR amplification were performed as previously described in Chapter 4.

6.2. Results

First, general macroscale operational data relating to VPB operation throughout this experiment are presented. Next, the microscale gene expression results are presented. A discussion follows the presentation of results.

6.2.1. VPB Operation

The biofilter was operated for approximately 70 days. The operation was divided into a startup phase followed by 7 experimental phases (Table 6-2). The ethylbenzene concentration profile in the biofilter's inlet section is shown in Figure 6-2. The startup phase lasted 18 days. During this time, the ethylbenzene removal varied significantly. The biofilm moisture content dropped slightly to 12% when measurements were taken on days 6 and 9 (Figure 6-3). It then increased and stabilized at levels greater than 20%.

Table 6-2: Experimental Phases

Phase	Days of Operation	Description
Startup	0-18	Startup (EBZ)
1	19-22	EBZ and MPK
2	25-28	EBZ and o-X
3	32-35	EBZ and PhAc
4	41-44	1-Day Shutdown (EBZ)
5	45-50	3-Day Shutdown (EBZ)
6	51-62	7-Day Shutdown (EBZ)
7	63-67	EBZ, MPK and PhAc

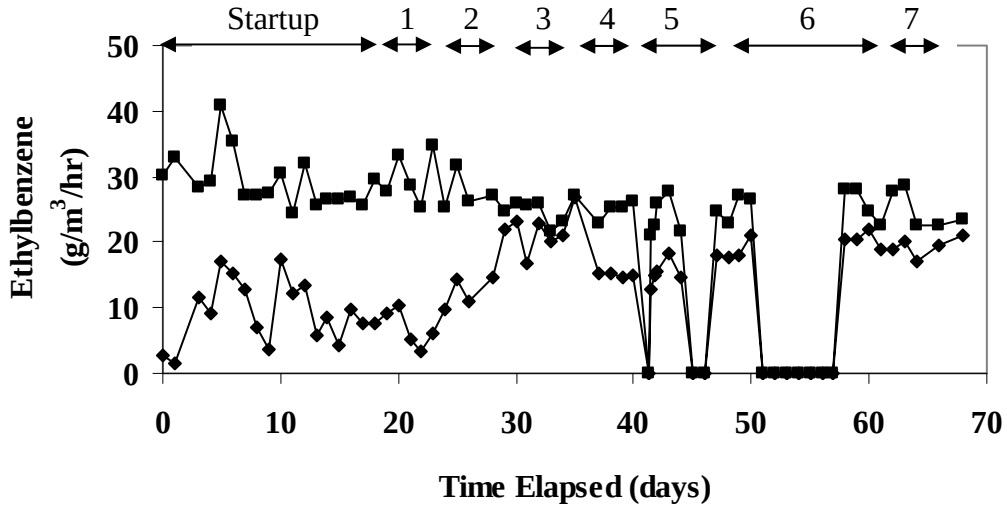

Figure 6-2: Ethylbenzene Loading (■) and Removal (◆) in the Inlet VPB Section

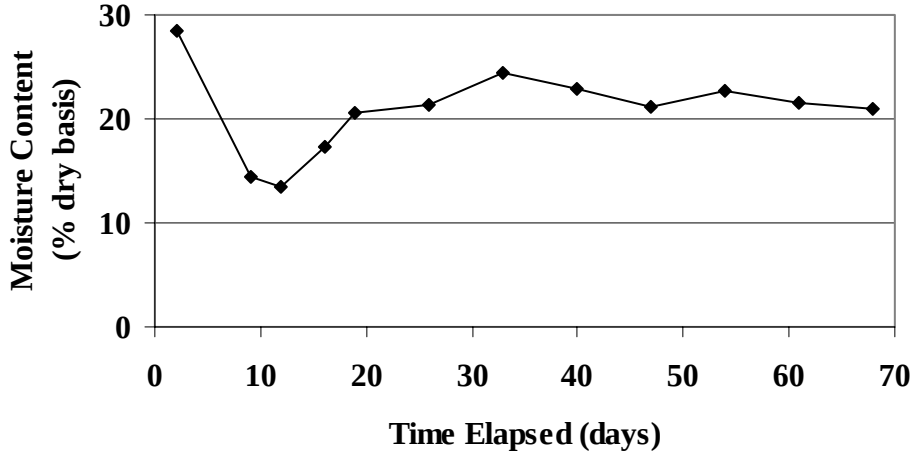

Figure 6-3: Moisture Content in Inlet Section

The pH of the VPB leachate remained at approximately 5 throughout the experiment. The pressure drop increased from 0.2 to 0.5 inches of water between the beginning and the end of the experiments. The nitrogen availability normalized to biomass present is shown in Figure 6-4. A high level of nitrogen was observed during the startup phase when biomass levels were low. As the biomass levels increased in the biofilter, nitrogen availability stabilized at approximately 0.04 g N/g COD. All available nitrogen detected in the bioreactor was in the form of ammonium. Nitrate levels were below the detection limit throughout the 70 day experiment and nitrite levels were insignificant as compared to the ammonium levels.

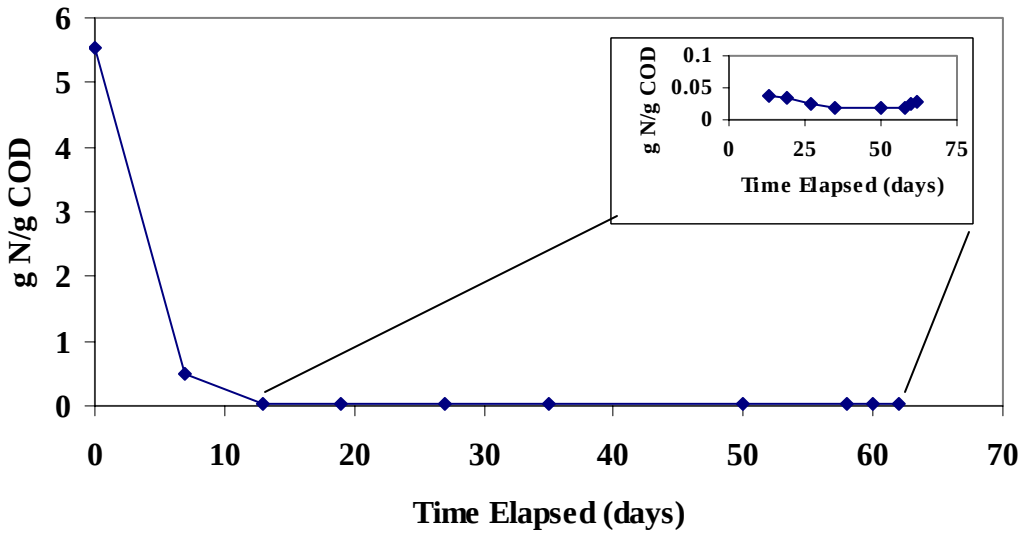

Figure 6-4: Nitrogen concentration as ammonium (◆) in the inlet biofilter section as a function of operating time.

6.2.2. Ethylbenzene and Methyl Propyl Ketone

Low levels of methyl propyl ketone (50 ppm_v) did not appear to impact overall ethylbenzene removal rates (Figure 6-5). However, as the concentration of methyl propyl ketone increased, a delayed response was observed in the VPB. For both the 100 and 200 ppm_v methyl propyl ketone loadings, a sharp decrease in the ethylbenzene removal rate (41 and 36%, respectively) was observed 24 hours after the loading change but was not observed during the first 12 hours of operation after the feed had been modified. Interestingly, at the microscale, the T_N values dropped off immediately upon the introduction of even low levels of methyl propyl ketone (Figure 6-6). *ElHDO* expression decreased by 73% during the first 24 hours of operation and then by another 65% four hours after the next incremental increase in the methyl propyl ketone feeding rate (Table

6-3). Beyond that point, however, it becomes difficult to draw any direct conclusions from the T_N values because the values are not statistically significantly different.

Table 6-3: Comparative Threshold Data for the VPB Treating Ethylbenzene and Methyl Propyl Ketone

Treatment Condition	Time Elapsed (hours)	C_T *ElHDO*	C_T 18S rDNA	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[10] Time zero / Previous time point
100 ppm_v EBZ + 50 ppm_v MPK	0[11]	***21.52***[12]	***17.56***	***-3.96***	***1.00***	0.98	0.36	N/A / N/A
		21.1	17.55	-3.55	1.33			
		20.99	16.32	-4.67	0.61			
	4	21.85	16.12	-5.73	0.29	0.72	0.37	ns / ns
		22.02	17.97	-4.05	0.94			
		22.24	18.17	-4.07	0.93			
	12	24.08	18.57	-5.51	0.34	0.41	0.06	sig / ns
		23.2	18.02	-5.18	0.43			
		23.72	18.62	-5.1	0.45			
	24	23	17.05	-5.95	0.25	0.26	0.02	sig / sig
		22.88	16.96	-5.92	0.26			
		23.09	17.31	-5.78	0.28			
100 ppm_v EBZ + 100 ppm_v MPK	28	23.47	16.47	-7	0.12	0.09	0.03	sig / sig
		23.12	15.31	-7.81	0.07			
		24.14	16.59	-7.55	0.08			
	36	23.56	16.57	-6.99	0.12	0.08	0.07	sig / ns
		23.52	12.48	-11.04	0.01			
		23.92	16.89	-7.03	0.12			
	48	25.45	19.53	-5.92	0.26	0.22	0.13	sig / ns
		25.31	17.54	-7.77	0.07			
		24.93	19.32	-5.61	0.32			
100 ppm_v EBZ + 200 ppm_v MPK	52	26.45	18.94	-7.51	0.09	0.04	0.04	sig / sig
		26.93	12.3	-14.63	0.00			
		26.39	17.33	-9.06	0.03			
	60	30.1	20.72	-9.38	0.02	0.05	0.05	sig / ns
		30.02	20.24	-9.78	0.02			
		29.9	22.82	-7.08	0.12			
	72	37.11	13.05	-24.06	0.00	0.10	0.17	sig / ns
		29.11	16.22	-12.89	0.00			
		29.3	23.58	-5.72	0.30			

[10] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 48 hours was collected to data collected at 36 hours). sig: significant, ns: non-significant, α=0.15

[11] Time zero refers to the moment when methyl propyl ketone was introduced into the bioreactor feed.

[12] This sample was used as the calibrator.

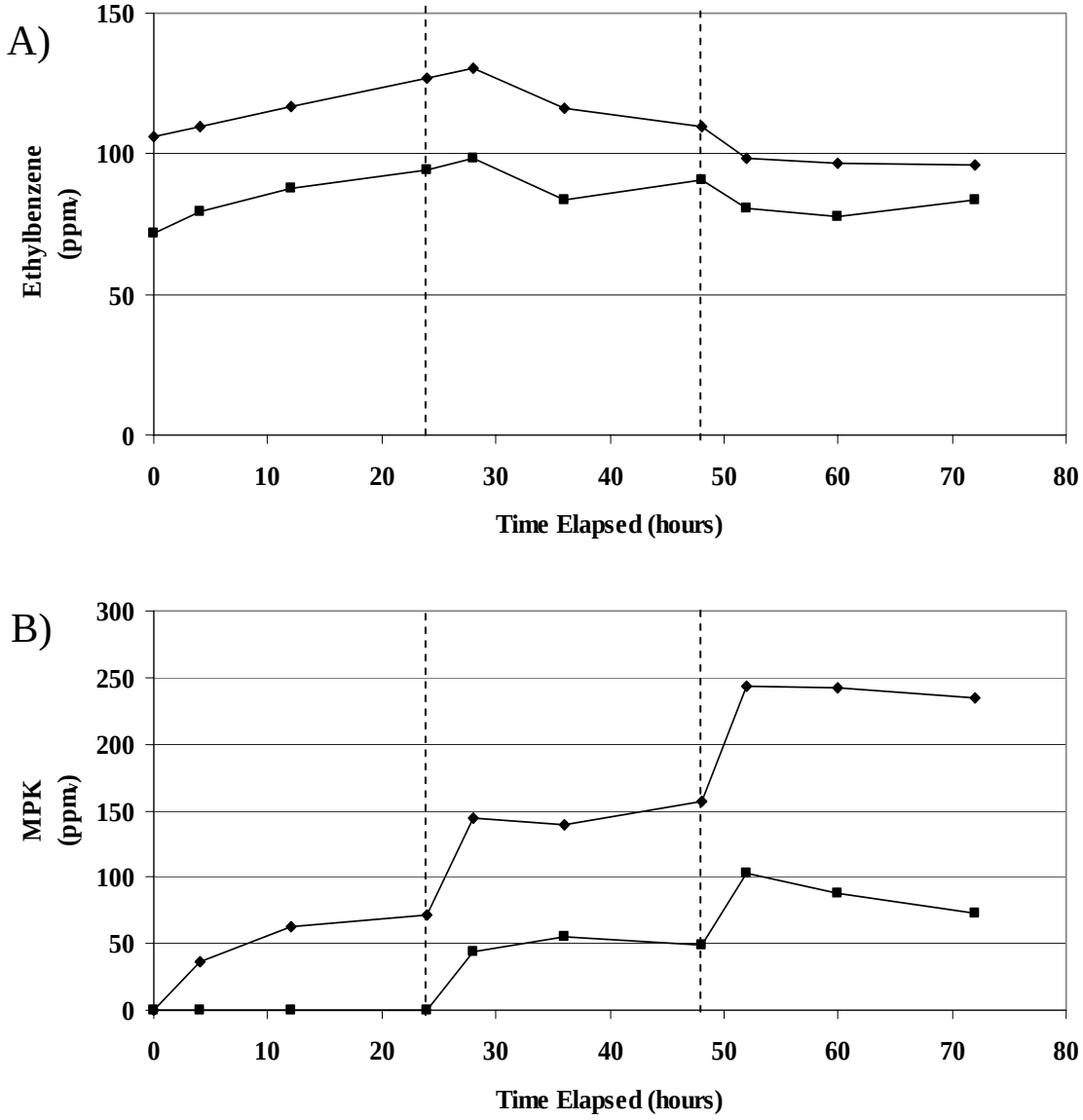

Figure 6-5: Ethylbenzene and Methyl Propyl Ketone Concentrations in the Inlet Biofilter Section – A) Inlet (◆) and Outlet (■) Ethylbenzene Concentrations; B) Inlet (◆) and Outlet (■) Methyl Propyl Ketone Concentrations

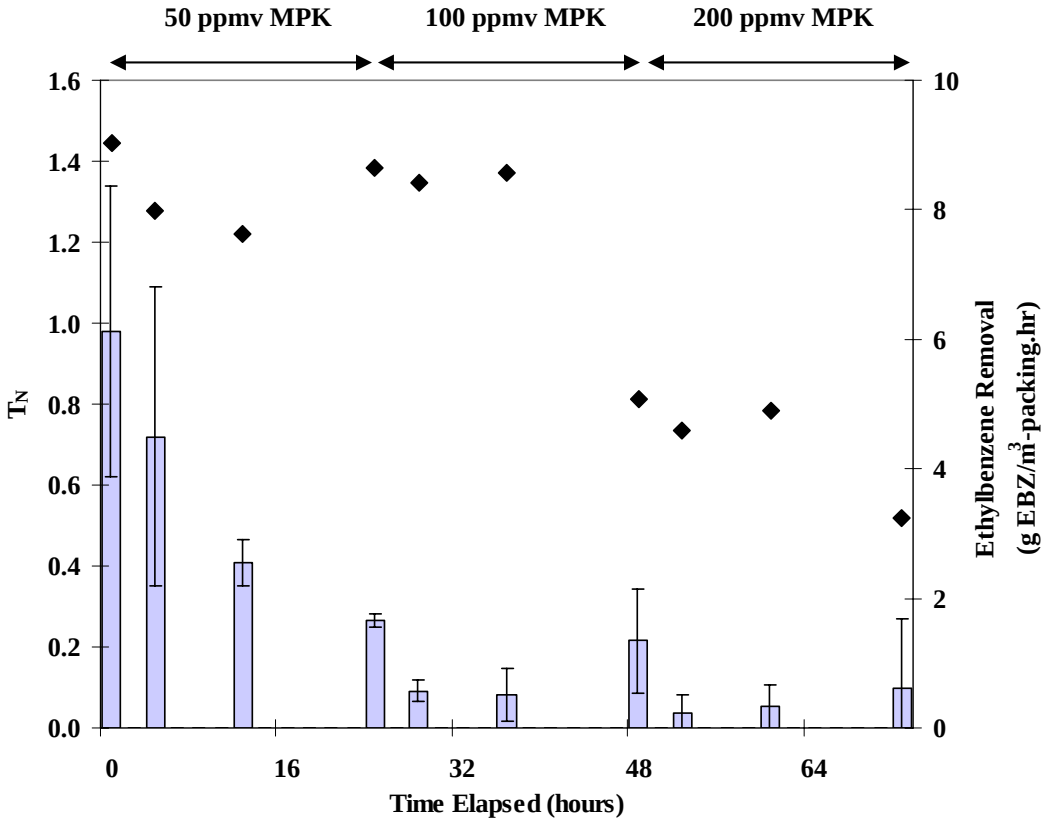

Figure 6-6: Effect of Methyl Propyl Ketone (MPK) on Ethylbenzene (EBZ) Degradation in the Inlet Biofilter Section: Ethylbenzene Removal (◆) and Relative Target Expression Number (bars). Error bars represent one standard deviation.

6.2.3. Ethylbenzene and o-Xylene

As expected, the introduction of o-xylene into the biofilter feed had little to no effect on T_N values (Table 6-4) and ethylbenzene removal (Figure 6-7). Average T_N values oscillated between 0.89 and 1.03 (Figure 6-8). This result is similar to that obtained in the batch reactor study described in Chapter 5.

Table 6-4: Comparative Threshold Data for Biofilter Treating Ethylbenzene and o-Xylene

Treatment Condition	Time Elapsed (hours)	C_T *ElHDO*	C_T 18S rDNA	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[13] Time zero / Previous time point
100 ppm_v EBZ + 50 ppm_v o-X	0	***26.03***[14]	***15.4***	***-10.63***	***1.00***	0.91	0.13	ns / ns
		27.02	16.34	-10.68	0.97			
		26.35	15.33	-11.02	0.76			
	12	26.4	16.14	-10.26	1.29	1.05	0.21	ns / ns
		26.78	16.02	-10.76	0.91			
		26.76	16.05	-10.71	0.95			
100 ppm_v EBZ + 100 ppm_v o-X	24	27.23	16.62	-10.61	1.01	0.89	0.20	ns / ns
		27.42	16.78	-10.64	0.99			
		27.14	15.91	-11.23	0.66			
	36	26.57	16.21	-10.36	1.21	1.02	0.16	ns / ns
		26.73	16.03	-10.7	0.95			
		27.21	16.44	-10.77	0.91			
100 ppm_v EBZ + 200 ppm_v o-X	48	25.89	15.36	-10.53	1.07	0.98	0.08	ns / ns
		26.11	15.36	-10.75	0.92			
		26.52	15.82	-10.7	0.95			
	60	27.78	17.32	-10.46	1.13	0.96	0.14	ns / ns
		27.74	16.96	-10.78	0.90			
		27.94	17.1	-10.84	0.86			
	72	26.67	16.23	-10.44	1.14	1.03	0.22	ns / ns
		26.94	16.55	-10.39	1.18			
		27.05	16.06	-10.99	0.78			

[13] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 48 hours was collected to data collected at 36 hours). sig: significant, ns: non-significant, α=0.15

[14] This sample was used as the calibrator.

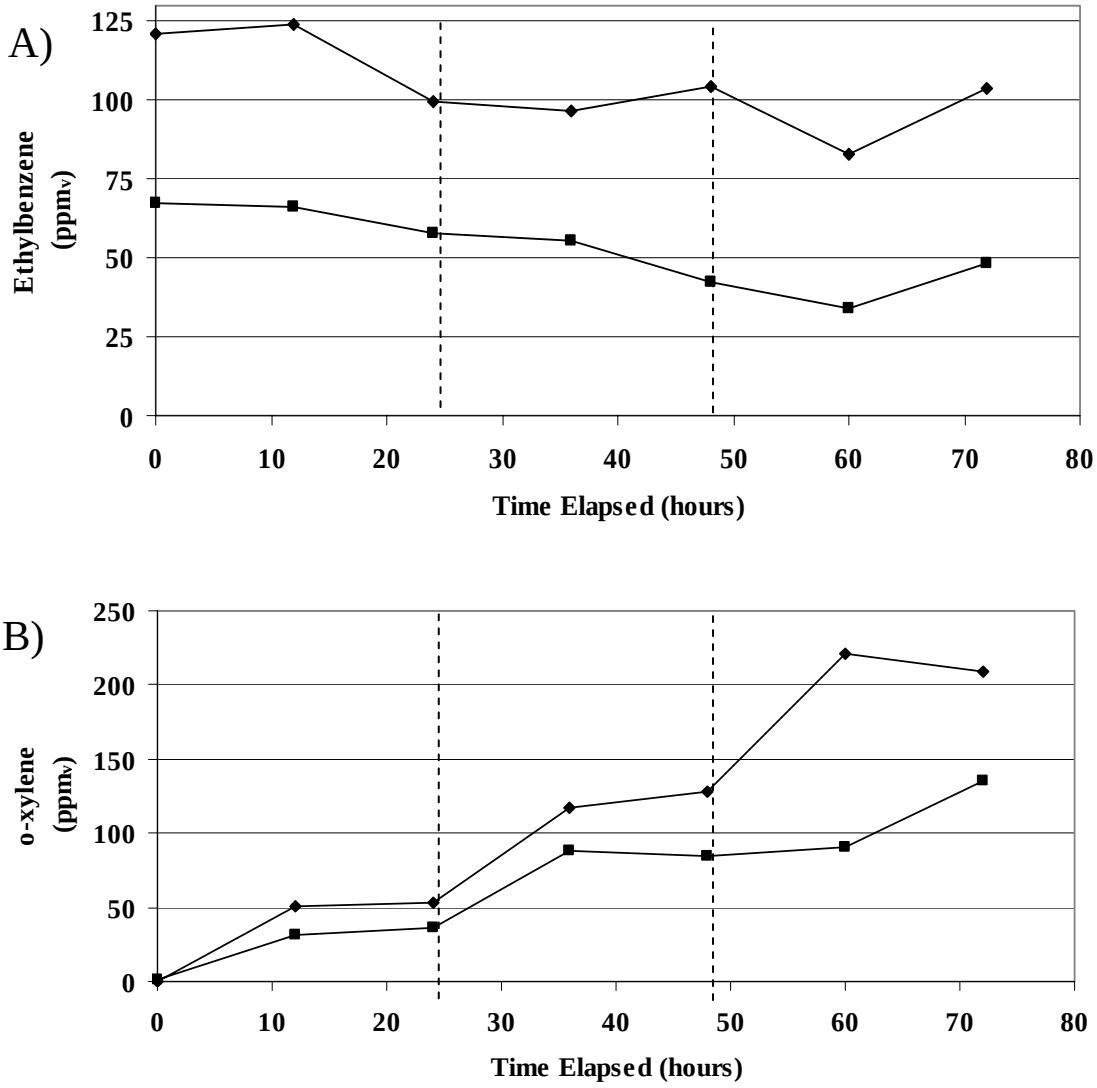

Figure 6-7: Ethylbenzene and o-Xylene Concentrations in the Inlet Biofilter Section – A) Inlet (◆) and Outlet (■) Ethylbenzene Concentrations; B) Inlet (◆) and Outlet (■) o-Xylene Concentrations

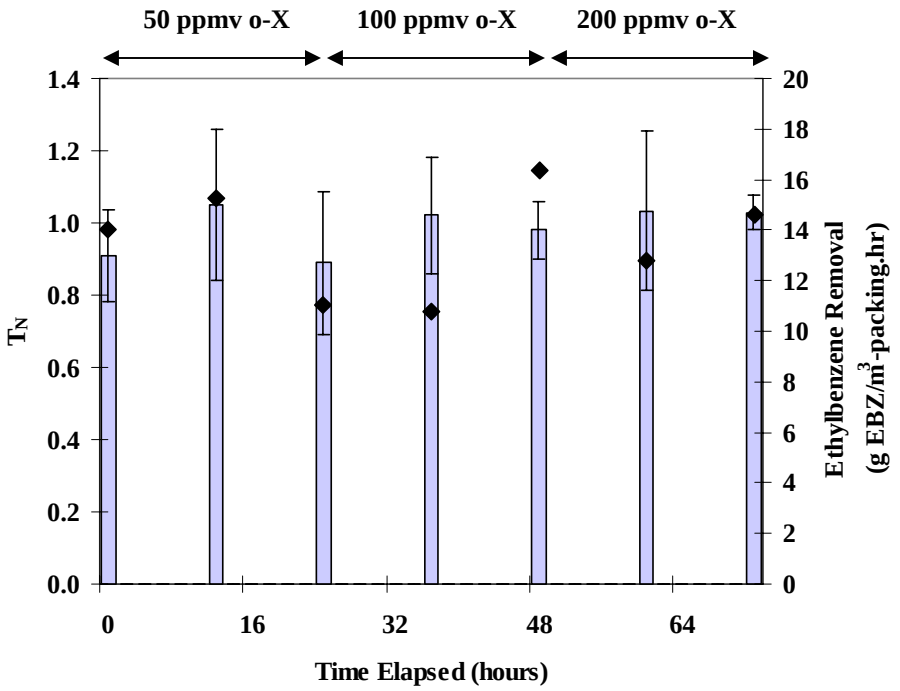

Figure 6-8: Effect of o-Xylene (o-X) on Ethylbenzene (EBZ) Degradation in the Inlet Biofilter Section: Ethylbenzene Removal (◆) and Relative Target Expression Number (bars). Error bars represent one standard deviation.

6.2.4. Ethylbenzene and Phenyl acetate

T_N values, as outlined in Table 6-5, did not dramatically increase until 48 hours after the start of the experiment and 24 hours after 100 ppm_v phenyl acetate was provided to the biofilter. Ethylbenzene removal rates also remained relatively constant (Figure 6-9). It appears that the phenyl acetate loading has to reach some threshold level before the *ElHDO*'s expression level increases, and, even then, the response is not immediate. Indeed, the *ElHDO* expression level did not change during the first 12 hours after 100 ppmv phenyl acetate was introduced to the column. During the last phase, with the

highest phenyl acetate loading, the expression increased almost 10-fold from that at the beginning of the experiment (Figure 6-10). Simultaneously, the ethylbenzene removal decreased sharply.

Table 6-5: Comparative Threshold Data for the VPB Treating a Mixture of Ethylbenzene and Phenyl Acetate

Treatment Condition	Time Elapsed (hours)	C_T *ElHDO*	C_T 18S rDNA	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[15] Time zero / Previous time point
100 ppm_v EBZ + 50 ppm_v PhAc	0	***27.77***[16]	***16.2***	***-11.57***	***1.00***	0.82	0.26	N/A / N/A
		27.87	16.21	-11.66	0.94			
		27.57	15.07	-12.5	0.52			
	4	28.06	16.01	-12.05	0.72	1.31	0.52	ns / ns
		27.02	16.06	-10.96	1.53			
		27.48	16.66	-10.82	1.68			
	12	26.9	15.82	-11.08	1.40	1.40	0.05	sig / ns
		26.74	15.59	-11.15	1.34			
		26.52	15.48	-11.04	1.44			
	24	27.37	15.73	-11.64	0.95	0.93	0.27	ns / sig
		27.26	15.93	-11.33	1.18			
		28.05	15.86	-12.19	0.65			
100 ppm_v EBZ + 100 ppm_v PhAc	28	27.52	15.11	-12.41	0.56	0.61	0.14	ns / ns
		27.64	15.69	-11.95	0.77			
		27.77	15.21	-12.56	0.50			
	36	27.45	15.02	-12.43	0.55	0.60	0.05	ns / ns
		27.41	15.21	-12.2	0.65			
		27.96	15.67	-12.29	0.61			
	48	24.35	14.535	-9.815	3.38	2.88	0.52	sig / sig
		25.46	15.12	-10.34	2.35			
		25.68	15.65	-10.03	2.91			
100 ppm_v EBZ + 200 ppm_v PhAc	52	25.45	15.97	-9.48	4.26	4.44	0.42	sig / sig
		25.49	16.22	-9.27	4.92			
		25.61	16.09	-9.52	4.14			
	60	23.95	15.75	-8.2	10.34	10.27	0.46	sig / sig
		24.41	16.13	-8.28	9.78			
		24.12	15.97	-8.15	10.70			
	72	23.78	15.47	-8.31	9.58	9.36	0.70	sig / sig
		23.99	15.73	-8.26	9.92			
		23.4	14.93	-8.47	8.57			

[15] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 48 hours was collected to data collected at 36 hours). sig: significant, ns: non-significant, α=0.15

[16] This sample was used as the calibrator.

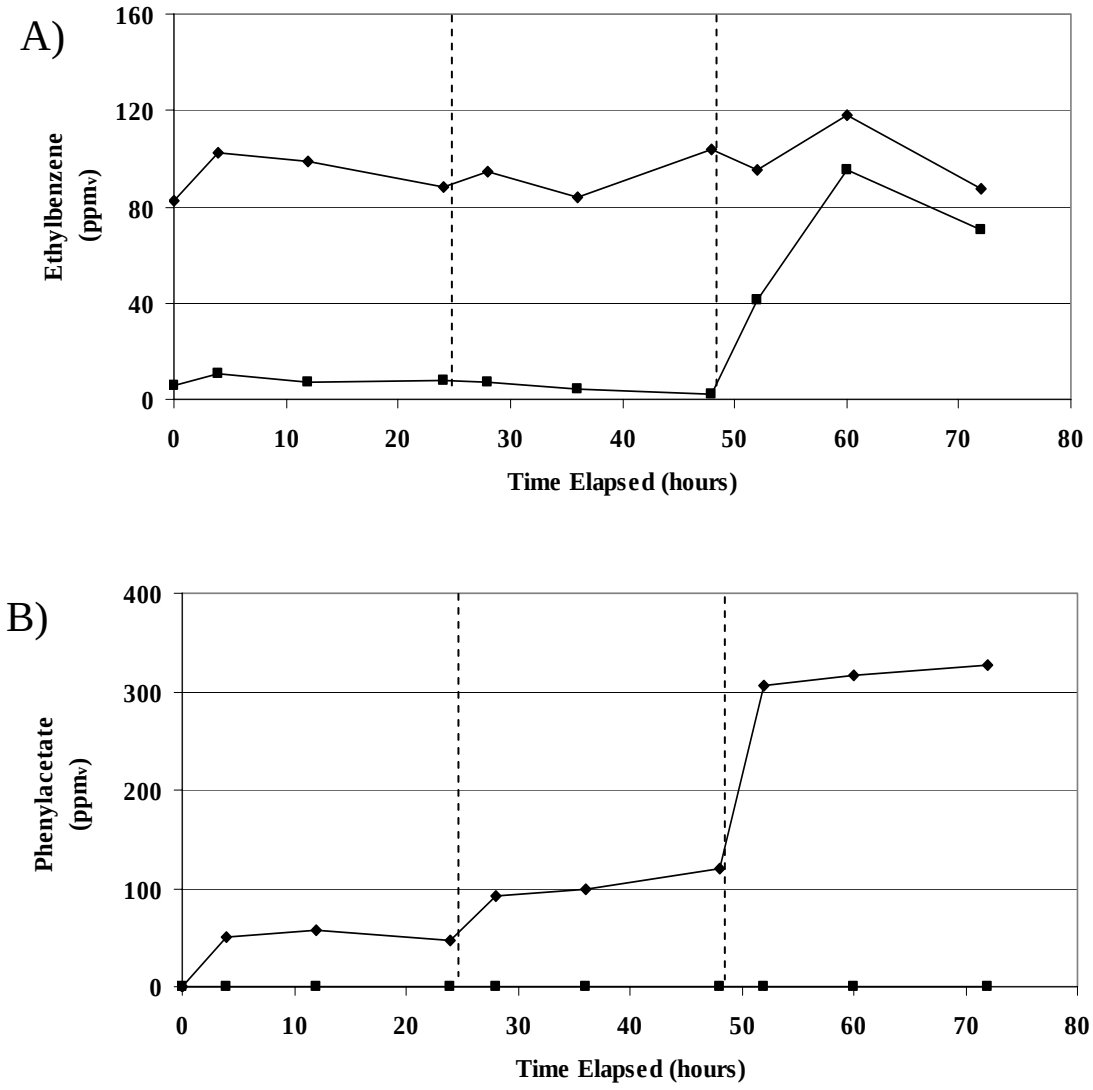

Figure 6-9: Ethylbenzene and Phenyl Acetate Concentrations in the Inlet Biofilter Section – A) Inlet (◆) and Outlet (■) Ethylbenzene Concentration; B) Inlet (◆) and Outlet (■) Phenyl Acetate Concentration

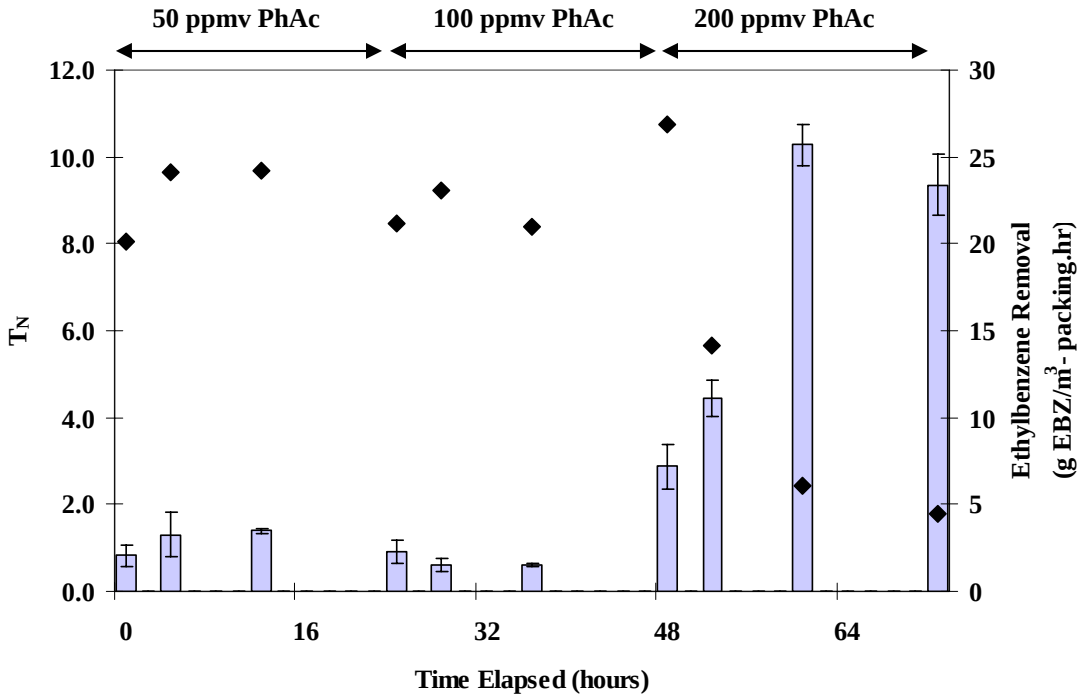

Figure 6-10: Effect of Phenyl Acetate (PhAc) on Ethylbenzene (EBZ) Degradation in the Inlet Biofilter Section: Ethylbenzene Removal (◆) and Relative Target Expression Number (bars). Error bars represent one standard deviation.

6.2.5. Ethylbenzene, Methyl Propyl Ketone and Phenyl Acetate

The simultaneous introduction of both repressor and inducer compounds caused each regulatory substrate to negate each other's effect on gene expression. T_N values remained relatively constant (Table 6-6) and ethylbenzene removal rates also remained steady (Figure 6-11 and 6-12).

Table 6-6: Comparative Threshold Data for the VPB Treating a Mixture of Ethylbenzene, Methyl Propyl Ketone and Phenyl Acetate

Treatment Condition	Time Elapsed (hours)	C_T *ElHDO*	C_T 18S rRNA	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[17] Time zero / Previous time point
100 ppm_v EBZ + 50 ppm_v PhAc + 50 ppm_v MPK	0	***26.96***[18]	***15.99***	***-10.97***	***1.00***	1.03	0.14	N/A / N/A
		26.78	15.67	-11.11	0.91			
		26.8	16.08	-10.72	1.19			
	4	26.82	16.05	-10.77	1.15	1.22	0.23	ns / ns
		26.52	15.6	-10.92	1.04			
		26.63	16.23	-10.4	1.48			
	12	27.39	16.44	-10.95	1.01	1.00	0.17	ns / ns
		27.42	16.66	-10.76	1.16			
		27.46	16.21	-11.25	0.82			
	24	27.22	16.04	-11.18	0.86	0.64	0.25	ns / ns
		27.95	15.57	-12.38	0.38			
		27.89	16.34	-11.55	0.67			
100 ppm_v EBZ + 100 ppm_v PhAc + 100 ppm_v MPK	28	25.77	14.52	-11.25	0.82	0.97	0.29	ns / ns
		25.71	15.12	-10.59	1.30			
		25.83	14.49	-11.34	0.77			
	36	26.76	14.55	-12.21	0.42	0.59	0.31	ns / ns
		25.9	14.84	-11.06	0.94			
		26.52	14.22	-12.3	0.40			
	48	26.24	14.91	-11.33	0.78	0.73	0.04	ns / ns
		27.2	15.73	-11.47	0.71			
		26.13	14.64	-11.49	0.70			
100 ppm_v EBZ + 200 ppm_v PhAc + 200 ppm_v MPK	52	23.66	11.8	-11.86	0.54	0.77	0.20	ns / ns
		24.3	13.16	-11.14	0.89			
		24.34	13.17	-11.17	0.87			
	60	24.99	13.83	-11.16	0.88	0.57	0.27	ns / ns
		24.58	12.26	-12.32	0.39			
		24.57	12.41	-12.16	0.44			
	72	24.8	13.81	-10.99	0.99	0.61	0.33	ns / ns
		25.05	13.89	-11.16	0.88			
		25	13.5	-11.5	0.69			

[17] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 48 hours was collected to data collected at 36 hours). sig: significant, ns: non-significant, α=0.05

[18] This sample was used as the calibrator.

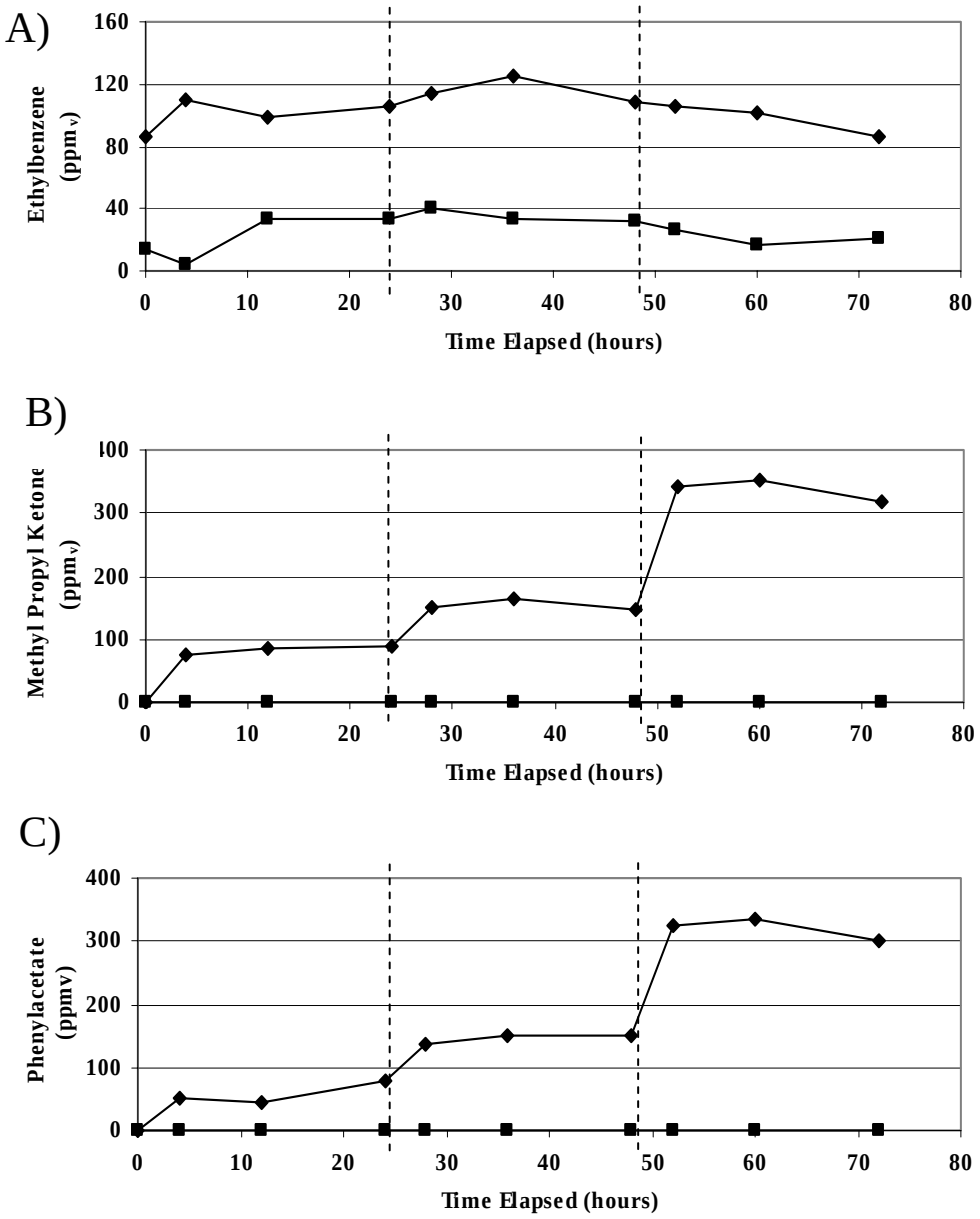

Figure 6-11: Ethylbenzene, Methyl Propyl Ketone and Phenyl Acetate Concentrations in the Inlet Biofilter Section – A) Inlet (◆) and Outlet (■) Ethylbenzene Concentration; B) Inlet (◆) and Outlet (■) Methyl Propyl Ketone Concentration; C) Inlet (◆) and Outlet (■) Phenyl Acetate Concentration

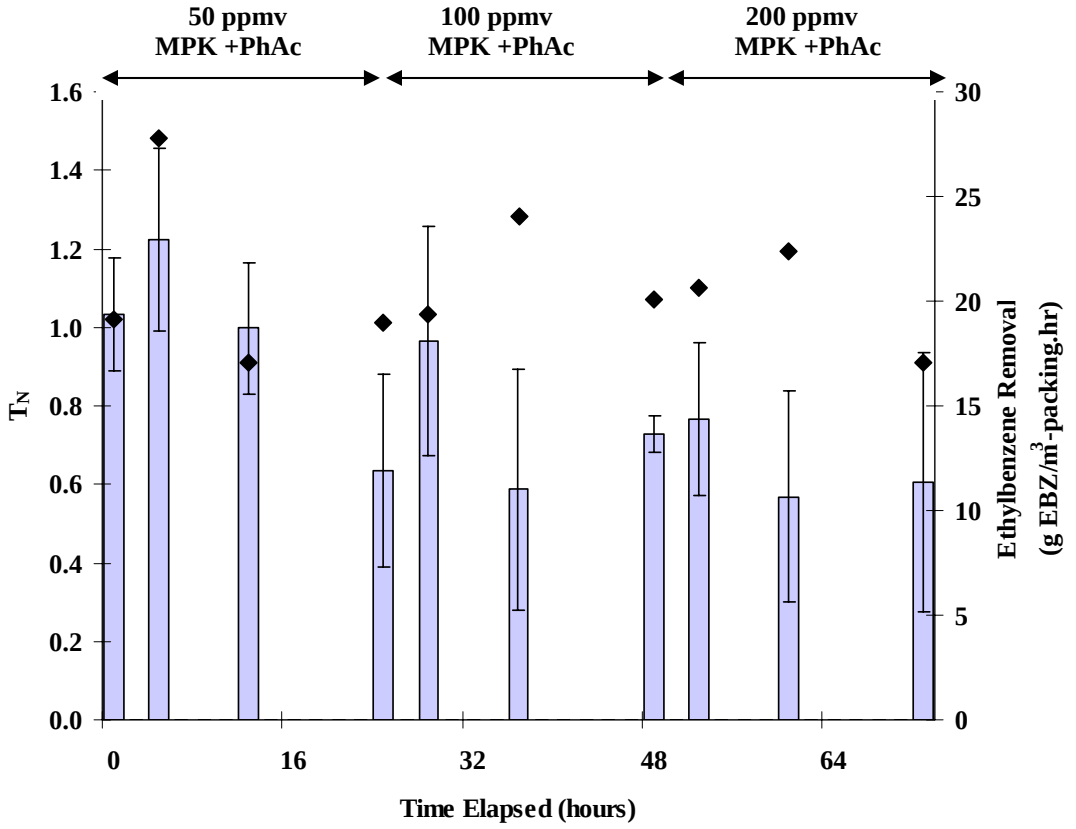

Figure 6-12: Effect of Methyl Propyl Ketone (MPK) and Phenyl Acetate (PhAc) on Ethylbenzene (EBZ) Degradation in the Inlet Biofilter Section: Ethylbenzene Removal (◆) and Relative Target Expression Number (bars). Error bars represent one standard deviation.

6.3. DISCUSSION

To determine if qRT-PCR can be used to determine how the presence of regulatory substrates affects *ElHDO* expression in a VPB, mixtures of 100 ppm_v ethylbenzene and 50-200 ppm_v of a regulatory substrate were fed to the VPB. Differences in ethylbenzene degradation and *ElHDO* gene expression were monitored by comparing the ethylbenzene removal rate and T_N values in the inlet section of the biofilter over time.

After the startup phase was completed, operational conditions (ethylbenzene loading, nitrogen availability, moisture content, pH and pressure drop) were relatively stable in the VPB. Most of the nitrogen available was in the form of ammonium. This result was expected since the nitrogen was supplied to the reactor in this form and the cells then convert the available nitrogen for assimilation. The most important parameter affecting ethylbenzene removal was found to be moisture content. Previous research showed that moisture contents greater than 15% were necessary for optimal operation of a fungal VPB inoculated with *E. lecanii-corni* (Woertz et al., 2001). Once the moisture content was reestablished at levels exceeding 15%, ethylbenzene removal returned to the levels prior to the drying phase.

The introduction of regulatory substrates into the VPB yielded similar effects on gene expression to those observed in the batch reactor study. An increase in *ElHDO* expression was observed with phenyl acetate, while no effect on T_N was observed with o-xylene and a decrease in *ElHDO* expression was observed when methyl propyl ketone was present. Microscale level data (i.e., T_N values) remained approximately constant (close to 1.0) at the low and medium phenyl acetate loading (during the first 36 hours of operation). At the lowest phenyl acetate loading, no increase in gene expression was observed. At the intermediate phenyl acetate loading level, an increase in T_N was observed but took place 12-24 hours after the introduction of the regulatory substrate. An increase in T_N at the highest phenyl acetate loading was observed. This increase, however, was not immediate; it appears that a threshold phenyl acetate concentration needs to be reached before the expression level increases. The response occurred somewhat faster at the highest loading (i.e., within 12 hours of introduction). The

macroscale results in the VPB, however, were quite different from those observed in the batch studies in the case of the highest phenyl acetate loading. Indeed, while an increase in specific substrate utilization rate was observed at the highest phenyl acetate loading in batch studies, a decrease in removal rate was seen in the VPB at the highest phenyl acetate loading.

Several factors could have contributed to the phenyl acetate results. It is clear from the phenyl acetate T_N values that a certain threshold level exists before the high expression levels are activated and the cells preferentially degrade phenyl acetate over ethylbenzene. It is possible that in the batch reactor study, this threshold had not in fact been reached since the phenyl acetate concentrations were approximately 30 μM and the ethylbenzene concentrations were 20 μM. As a result the ratio of ethylbenzene to phenyl acetate is initially 3:2 in the batch experiments versus 2:1 in the last phase of the VPB experiment. The most likely explanation for the decrease in ethylbenzene consumption is based on cellular energetic considerations. Because phenyl acetate is an intermediate in the ethylbenzene degradation pathway (see Figure 2-4 and 2-5), *E. lecanii-corni* cells would have to expend less energy to degrade phenyl acetate as compared to ethylbenzene. Therefore it is likely that if cells were already degrading the substrates as efficiently as possible and two substrates available, they would degrade phenyl acetate, thereby leaving the ethylbenzene untouched. It is also possible that *E. lecanii-corni* cells would have different thresholds in a batch reactor system as compared to a VPB because of differences inherent to each setup. For instance, limiting conditions are more likely to be present in a VPB as compared to a batch reactor. While all nutrients are equally distributed in a batch reactor, that is not the case for VPB. Even though excess nutrients

were provided in both cases, it is quite likely that some uneven nutrient distribution may have occurred in the VPB.

In the presence of methyl propyl ketone, no effect on ethylbenzene removal was observed at low methyl propyl ketone concentrations (50 ppm_v) and a delayed response was observed at the medium and high methyl propyl ketone concentrations (100 and 200 ppm_v). T_N decreased by 73% in the first 24 hours and then another 65% in the 4 hours after 100 ppm_v methyl propyl ketone was introduced to the feed. The T_N values in the biofilter remained suppressed (and relatively constant if one considers the error bars) throughout the rest of the MPK experiment. Because responses were much more rapid at the microscale, this also indicates that the T_N values act as a leading indicator and could be used to predict imminent failure of a VPB.

The reason T_N acts as a leading indicator is in large part coupled to the fact that T_N values are directly related to the regulation of mRNA rather than the regulation of existing protein. Gene expression in eukaryotic systems involves a number of steps including transcription, RNA processing, transport, translation and mRNA turnover (Cao and Parker, 2001; van Hoof and Parker, 2002; Brown and Johnson, 2001). The protein that is ultimately the product of the translation step is the molecule that carries out the degradation reaction. Therefore, it can be expected that ethylbenzene degradation and mRNA levels will not be instantaneously correlated but rather that these two parameters will be correlated over a period of time. While the repression effect caused by the introduction of methyl propyl ketone into the VPB has a rapid effect on mRNA production and, by extension, on T_N values, the effect on ethylbenzene degradation was delayed. A possible explanation for this is that a sufficient level of enzymes may have

still been available for degradation because the level of enzyme was not corrected immediately but rather over a period of time following mRNA level control.

Based on these results, it appears that the regulation occurs mostly at the transcriptional level. The main mechanism of transcriptional regulation in eukaryotic cells is directly linked to the structure of the mRNA molecules. Using deadenylation, eukaryotic cells can exercise fine control over the structure and stability of mRNA. Nascent transcripts originating from the transcription step in the nucleus by RNA polymerase II undergo coordinated endonucleotic cleavage and polyadenylation (Colgan and Manley, 1997; Barabino and Keller, 1999). These steps dictate in large part how the mRNA molecules will be regulated. The proper mRNA 3'-end formation is important both for the release of the transcript from the site of transcription and for its subsequent export to the cytoplasm. Once in the cytoplasm, the polyA tail enhances translation initiation and mRNA stability (Eckner et al., 1991; Long et al., 1995; Huang and Carmichael, 1996; Custodio et al., 1999; Brodsky and Silver, 2000). Because the mRNA adenylation is responsible for how well the mRNA will be able to "survive" and make a protein, it is consistent with the idea that deadenylation is responsible for mRNA decay. It has been shown that the polyA tail is critical for mRNA stability and that deadenylation is the first and rate-limiting step in general mRNA decay in yeast and mammalian cells (Decker and Parker, 1993; Hsu and Stevens, 1993; Mulhrad et al., 1994; Couttet et al., 1997). Using these mechanisms, cells have the ability to control mRNA and protein production in response to changing environmental conditions.

Many gene expression studies relate to up-regulated genes. In this case, the expression level changes generally correspond to several orders of magnitude (e.g., T_N

values varying from 1 to 10). Because this change is large, a standard deviation of 0.5 becomes small relative to the overall change. However, when studying down-regulation using the comparative method, one is considering changes from 1 to 0. On this scale, this same standard deviation (i.e., 0.2 to 0.5) becomes very large in a statistical sense. Therefore, while clear down-regulation can be detected (e.g., when T_N values change from 1.0 to 0.3), small changes (e.g., when T_N values change from 0.1 to 0.05) cannot be accurately quantified. In the latter case, the inherent variability in the qRT-PCR makes gene expression changes difficult to quantify. The standard deviations obtained in this study are consistent with other published results which were obtained using the comparative method (Semighini et al., 2002; Schmittgen and Zakrajesh, 2000). Because this method involves a large degree of manual labor, a certain level of error can be expected, which is most likely the cause for the relatively high standard deviations.

When analyzing differences at low T_N values, the conventional methods such as degradation profiles would provide a much better understanding of what is occurring in the biofilter than the gene expression observations. For instance, it is intuitive that because the ethylbenzene removal decreases at the medium and high methyl propyl ketone loadings that T_N would also most likely continue to drop. However, this conclusion cannot be drawn based on the qRT-PCR data obtained during the two last phases of the experiment (medium and high methyl propyl ketone loadings). This is because qRT-PCR is not sensitive enough to detect changes in expression when T_N levels are low. Under these conditions, the macroscale level measurement (although delayed) is a better indicator of change.

The final important result from this substrate mixture study is that the simultaneous introduction of two regulatory compounds (phenyl acetate and methyl propyl ketone) negated each others effect. Indeed, T_N values remained close to 1 throughout the 3-day experiment indicating that *ElHDO* expression did not vary regardless of the phenyl acetate and methyl propyl ketone loadings (50, 100 and 200 ppm_v). Ethylbenzene removal was also found to remain relatively constant independently of each compound's loading, that is also consistent with the gene expression results. These data suggest that the introduction of phenyl acetate (inducer) negated the repression previously caused by methyl propyl ketone when it alone was introduced into the air stream with ethylbenzene. qRT-PCR gene expression data could therefore be used to quantify at which loading a specific compound could increase the removal efficiency of another compound which is more difficult to treat in a specific waste stream.

Many VPB research studies have looked at substrate interactions and analyzed the impact of one compound's presence on the other's degradation potential (Madigan et al., 2000; Zheng et al., 2001; Aizpuru et al., 2001; Battacharya et al, 2001; Lovanh et al., 2002). None of these analyses, however, have looked at how the expression of a specific gene is modified in response to the introduction of another compound in the mixture. Because similar trends were observed in the batch reactor studies (Chapter 5) and in the VPB studies (this chapter), it is beneficial to first quantify gene expression changes as a result of the introduction of another substrate in a batch reactor and then extend those results to experimental systems such as a VPB. In addition, because running a qRT-PCR analysis is quite rapid (less than 6 hours from biomass extraction to qRT-PCR amplification), qRT-PCR has the potential to become a very efficient tool to study substrate mixture interactions in environmental engineering systems.

While the qRT-PCR method appears to have utility in quantifying gene expression changes associated with substrate mixture interactions, there are a number of other questions relating to VPB research that have the potential to be answered using this technique. To this end, the remaining two chapters of this dissertation present how the qRT-PCR comparative threshold method can be used to study the effect of varying nutrient concentrations and shutdown/restart conditions on gene expression.

Chapter 7: Effect of Nitrogen on Gene Expression

In Chapters 5 and 6, the effect of substrate mixtures on gene expression was studied. In this chapter, the effect of nitrogen availability on gene expression is considered. Because nutrients must be provided to maintain VPB performance, delineating the minimum nitrogen requirements could potentially reduce costs. The effect of nitrogen concentration was first observed in batch reactor systems and then in a biofilter.

7.1. BATCH REACTOR STUDY

The following section describes the series of experiments performed to study the effect of varying nitrogen concentration on *ElHDO* expression. This phase of the study was performed in batch reactors.

7.1.1. Methods

Batch reactors were set up as previously described using 250-mL bottles containing 20 mL of basal medium with varying concentrations of $(NH_4)_2 \cdot SO_4$. The specific $(NH_4)_2 \cdot SO_4$ concentrations used were 0, 1, 2, 3, 4 and 5 g/L. Each bottle was inoculated with *E. lecanii-corni* spores at a concentration of $5x10^4$ cells/mL. Three bottles were set up for each nitrogen concentration tested. Ethylbenzene was provided as the sole carbon source at a concentration of 20 μM. The ethylbenzene was replenished at

the same concentration (20 μM) after each feeding was consumed. Ethylbenzene levels were monitored by gas chromatography as previously described in Chapter 3.

Cells were incubated in the bottles at room temperature on a shaker and harvested 12 days after inoculation. Suspended cells were transferred to 15-mL plastic tubes and centrifuged for 5 minutes at 4000 rpm. The supernatant was removed and the remaining cells were added to the tube and centrifuged again. The cell pellet was then transferred to a microfuge tube, centrifuged for 1 minute and the supernatant removed. The pellet was frozen in liquid nitrogen and cells were disrupted by grinding with a mortar and pestle. Total RNA isolation and cDNA synthesis were performed as previously described in Chapter 4.

7.1.2. Results

The nitrogen concentration had a significant impact on *ElHDO* expression (Table 7-1 and Figure 7-1). T_N values were the lowest when no nitrogen was present in the basal medium. At this concentration, expression was shown to be approximately 7% of that observed at the highest nitrogen concentration. The expression levels slightly increased when 1 g/L of $(NH_4)_2.SO_4$ was introduced into the medium. Under these nutrient conditions, *ElHDO* expression was approximately 31% of the expression levels observed at the highest nitrogen concentration. At ammonia sulfate concentrations above 2 g/L of $(NH_4)_2.SO_4$, however, the expression levels leveled off. Increases in $(NH_4)_2.SO_4$ beyond 2 g/L had little effect on *ElHDO* expression in batch reactors.

Table 7-1: Gene Expression as a Function of Nitrogen Concentration in Batch Reactors.

Ammonium Sulfate Concentration (g $(NH_4)_2.SO_4$ /L)	**C_T** ***ElHDO***	**C_T** ***18S rRNA***	**ΔC_T**	**$2^{-\Delta\Delta C_T}$**	**Average T_N**	**Standard Deviation T_N**	**t-test[19]** 5 g$(NH_4)_2.SO_4$ /L / Previous data point
0	26.70	10.95	-15.75	0.06	0.07	0.01	sig / N/A
	25.96	10.63	-15.33	0.08			
	27.11	11.39	-15.72	0.06			
1	24.29	10.01	-14.28	0.16	0.30	0.14	sig / sig
	23.74	10.88	-12.86	0.44			
	23.77	10.43	-13.34	0.31			
2	23.46	11.03	-12.43	0.59	0.93	0.30	ns / sig
	22.88	11.33	-11.55	1.08			
	23.11	11.62	-11.49	1.13			
3	23.12	11.34	-11.78	0.92	0.87	0.05	ns / ns
	23.01	11.05	-11.96	0.81			
	23.06	11.22	-11.84	0.88			
4	23.05	11.47	-11.58	1.06	0.87	0.19	ns / ns
	23.23	11.02	-12.21	0.68			
	23.65	11.78	-11.87	0.87			
5	***22.38[20]***	***10.72***	***-11.66***	***1.00***	0.97	0.15	N/A / ns
	22.85	10.88	-11.97	0.81			
	22.67	11.16	-11.51	1.11			

[19] t-test performed by comparing data to 5 g/L data point and previous data point (e.g., data collected with 5 g/L was compared to data collected with 4 g/L). sig: significant, ns: non-significant, α=0.10

[20] This sample was used as the calibrator.

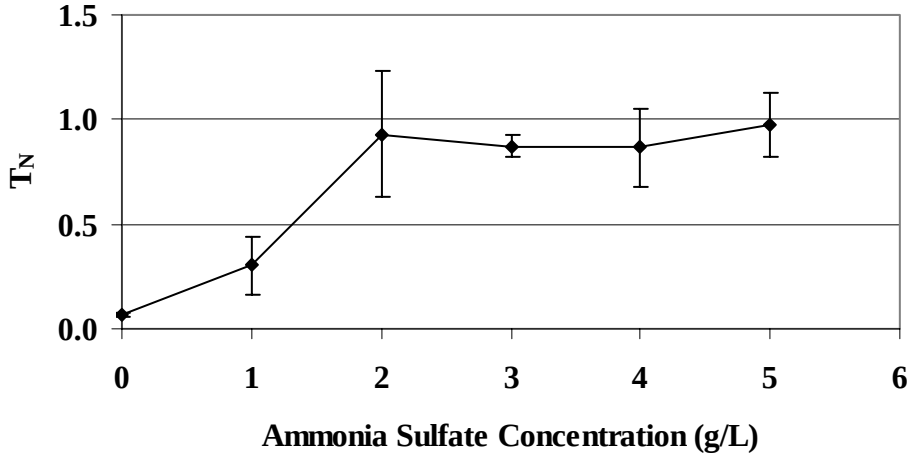

Figure 7-1: T_N as a Function of Ammonia Concentration in Batch Reactors

7.2. BIOFILTER STUDY

A similar study to that performed in the previously described batch reactor study was then run in a biofilter. The following section outlines the methods and results obtained for this study.

7.2.1. Methods

A VPB similar to that described in Chapter 6 was operated for a short-term experiment at a low nitrogen loading. Ethylbenzene was supplied at 100 ppm_v. The column was operated at a residence time of 1 minute. Basal medium supplemented with 1 g/L $(NH_4)_2 \cdot SO_4$ was supplied for 25 days to the bioreactor using a nebulizer as previously described. The concentration of $(NH_4)_2 \cdot SO_4$ was then increased to 4 g/L for 15

days. Three individual packing samples were taken from the inlet section. Packing samples for gene expression analysis were collected on Day 20 (1 g/L $(NH_4)_2 \cdot SO_4$ basal medium supplied) and Day 38 (4 g/L $(NH_4)_2 \cdot SO_4$ basal medium supplied) of VPB operation. RNA isolation and cDNA synthesis were performed individually on each of the triplicate samples. Moisture content, pH, biomass levels, pressure drop and nutrient concentration were also measured periodically throughout this experiment using the methods described previously.

7.2.2. Results

As evident in Figure 7-2, *ElHDO* expression levels in the VPB were quite similar at both nitrogen loadings (1 and 4 g/L $(NH_4)_2 \cdot SO_4$). *ElHDO* expression appears to be a little lower in both VPB measurements as compared to the 5 g/L $(NH_4)_2 \cdot SO_4$ calibrator sample in the batch system (Table 7-2 and Figure 7-2).

Table 7-2: Effect of Nitrogen on Target Expression Numbers

Growth Condition	**C_T** ***ElHDO***	**C_T** ***18S rRNA***	**ΔC_T**	**$2^{-\Delta\Delta C_T}$**	**Average T_N**	**Standard Deviation T_N**	**t-test[21]**
Batch ($5\ g\ (NH4)_2 \cdot SO_4/L$)	***22.35[22]***	***14.08***	***-8.27***	***1.00***	1.15	0.17	N/A
	21.95	14.05	-7.90	1.29			
	22.21	13.93	-8.28	1.00			
	21.96	14.07	-7.89	1.30			
VPB (Day 20)	23.02	14.15	-8.87	0.66	0.79	0.13	N/A
	22.88	14.27	-8.61	0.79			
	23.01	14.69	-8.32	0.97			
VPB (Day 38)	22.83	14.11	-8.72	0.73	0.60	0.26	ns
	22.83	13.20	-9.63	0.39			
	22.10	13.67	-8.43	0.90			
	22.62	13.39	-9.23	0.52			

[21] t-test performed by comparing data from Day 38 to Day 20. sig: significant, ns: non-significant, α=0.10

[22] This sample was used as the calibrator.

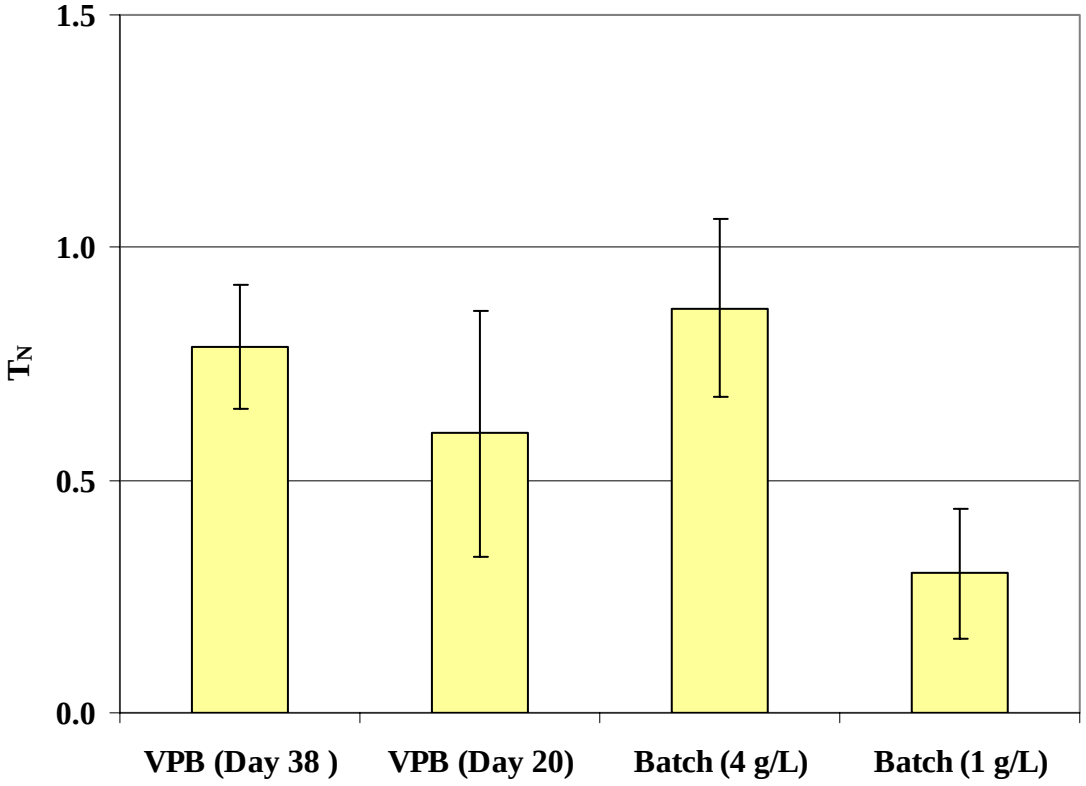

Figure 7-2: Target Expression Number in the VPB and Batch Reactor as a Function of Nitrogen Supply

After the initial start-up phase (Days 0-10), ethylbenzene removal in the inlet biofilter section fluctuated but remained high (upwards of 75%) from days 10 to 26, at which point the removal efficiency started to decline (Figure 7-3). This drop coincided with a decrease in moisture content (Figure 7-4). The pressure drop in the top section started to increase shortly after this drop in moisture content (Day 28 of operation). Leachate pH remained stable between 4.8 and 5.2 throughout the experiment. Nitrogen levels in the biofilm started at high levels and rapidly stabilized at a low level of approximately 0.05 g NH_4-N/g COD (see Figure 7-5). The increase in nitrogen supplied

to the biofilter on Day 25 did not lead to an increase in available nitrogen in the biofilm. No measurable NO_3^- or NO_2^- was detected. All available nitrogen was in the form of NH_4^+.

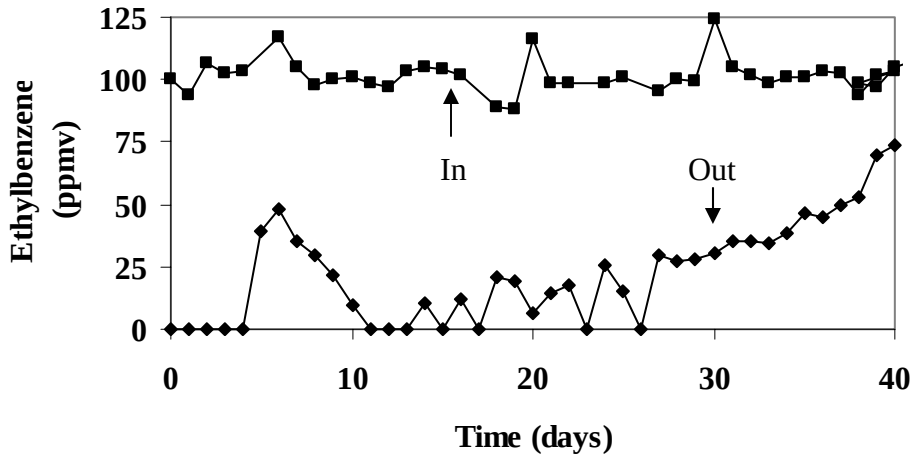

Figure 7-3: Ethylbenzene Concentrations across the Inlet VPB Section

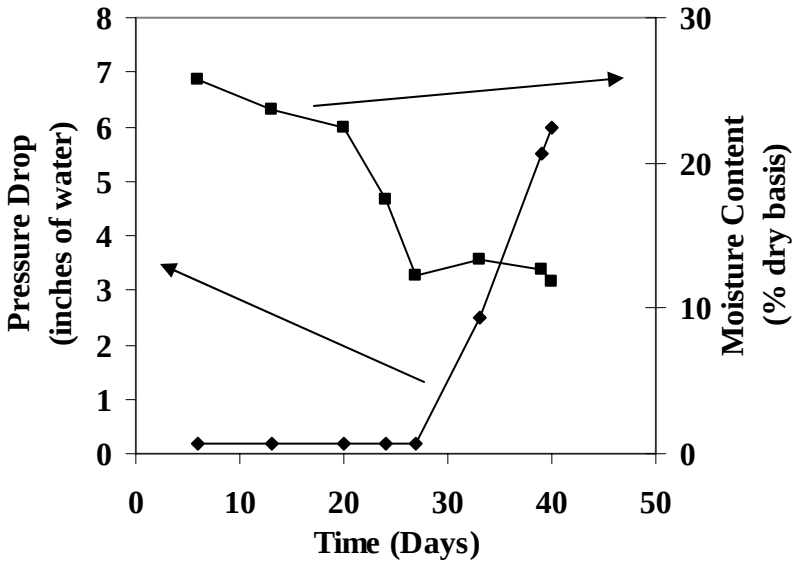

Figure 7-4: Pressure Drop and Moisture Content across the Inlet Section of the VPB

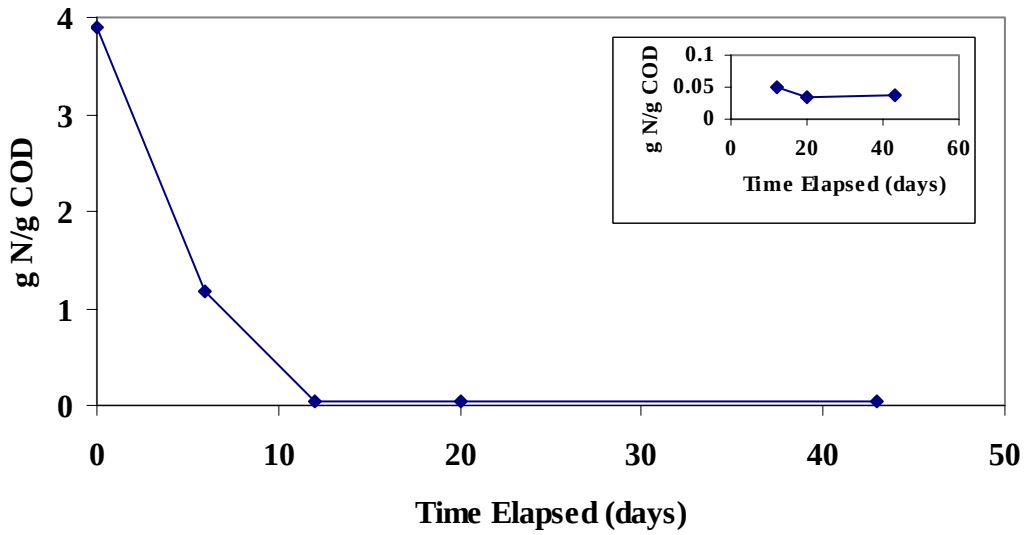

Figure 7-5: Nitrogen Level as a Function of COD in the Inlet VPB Section

7.3. DISCUSSION

Optimal microbial degradation of organic carbon sources in biofilters relies on several parameters including pH, moisture content, temperature, contact time, packing media and nutrient availability (such as nitrogen, phosphorous, sulfur and trace elements). The combination of these factors dictate how well the microbial population is able to degrade the supplied carbon sources. Because nitrogen makes up approximately 15% of the dry cell weight, it is one of the most crucial components associated with the successful operation of VPBs and therefore is one of the most important parameters to monitor during VPB operation (Yang et al., 2002).

A variety of packing media are used in biofiltration. The media can be made of inert material (foam, ceramic or plastic) or can be composed of organic materials such as compost (Swanson and Loehr, 1997; Song and Kinney, 2000; Moe and Irvine, 2000; Woertz et al., 1997; Gibbins and Loehr, 1997). One of the advantages of using compost is that some nitrogen is already available for microbial growth. However, Gibbins and Loehr (1998) showed that toluene degradation decreased by 69% when using aged compost bed media with depleted nitrogen and therefore even when using this type of material, either external nitrogen must be provided or new packing media must be added. Inert media, including the Celite ceramic pellets used in this study, do not provide any nitrogen for microbial growth and therefore all of the nitrogen must be provided externally to the bioreactor.

A handful of studies have looked at the effect on treatment efficiency of varying the form of nitrogen supplied (i.e., nitrate vs. ammonia) and mass of nitrogen supplied (Gibbons and Loehr, 1992; Morgenroth et al., 1996; Dirk-Faitakis and Allen, 2000; Weckhuysen et al., 1993; Barnes et al., 1995; Yang et al., 2002). In all cases, an increase in nitrogen supply was linked with an increase in removal efficiency. Yang et al. (2002) found that in the case of NH_3, increasing nitrogen concentrations in the biofilm increased pollutant removals up to a certain level. Once a certain nitrogen threshold was exceeded, higher nitrogen concentrations in fact reduced the overall performance of a VPB treating methanol. All of the VPB research studying nitrogen linked performance (degradation of a specific compound) to a measurement of available nitrogen which was performed using physical/chemical methods. No studies have attempted to link nitrogen availability to biological activity in the biofilm.

In this research, experiments were devised to determine if a correlation between nutrient level and *ElHDO* expression could be found. Because nitrogen is an important nutrient in cellular growth, it was expected that a similar result would be obtained to that observed for the substrate mixture study described in Chapter 6 where the introduction of a repressor or inducer compound caused expression to decrease or increase. In this case, expectations were that at low nutrient levels, where nutrient availability is a limiting factor, expression levels would be low whereas at higher nutrient levels, expression levels would be high. This is in fact what was observed in the batch reactor study (Figure 7-1). However, once this experiment was performed in a VPB, very different results were obtained.

In the VPB, expression levels were found to remain approximately constant when comparing *ElHDO* expression levels between biomass samples collected on Day 20 and Day 38. Prior to Day 20, the VPB had been supplied basal medium containing only 1 g/L $(NH_4)_2.SO_4$. Although this level was subsequently increased to 4 g/L, the *E1HDO* expression levels remained steady. The experimental conditions in the VPB were significantly different than those in the batch reactor and thus it is not surprising that E1HDO expression levels were different. Indeed, conditions in a well-mixed batch reactor are homogenous. Samples are shaken and therefore all cells are exposed to similar levels of carbon and nitrogen at all times. In addition, because cells are suspended in the basal medium, they have access to similar quantities of nutrients on a continuous basis. This situation is quite different in a flow-through system such as a VPB. In this case, as the biomass level increases, the potential for channeling also increases (i.e. gas follows a preferential path rather than being equally distributed through the pores). Therefore, ethylbenzene may not be distributed equally along the bioreactor.

This also holds true for nutrient distribution. As more pore space is filled in the bioreactor, the nutrient solution may not be able to access all parts of the bioreactor and nitrogen may become unevenly distributed. As a result, carbon and nitrogen loadings may not be equal for all cells in a VPB.

Columns under stressed conditions have a higher potential of developing channeling. As shown in Figure 7-4, the moisture content dropped from 23% to 12% between Day 20 and 25. While fungi can maintain higher activity levels at lower moisture contents than bacteria (Cox et al., 1993), previous research with *E. lecanii-corni* has shown that moisture contents above 15% are necessary for optimal activity (Woertz, 2002). These dry conditions result in sporulation causing the production of conidiophore and conidia on the surface of the biofilm. The process of sporulation has been well studied in *Saccharomyces cerevisiae* as a result of stress conditions such as nutrient deprivation (Klein et al., 1994).

In this case, not only was the nitrogen loading low, the overall condition in the biofilter was also dry, which would cause the filamentation process to be even more amplified. To circumvent the dry conditions, manual wetting of the column was attempted by pouring basal medium over the inlet section on Day 23. However, the fungal cells were already in their filamentous morphology and the wetting caused the bioreactor to clog even more rapidly. This is possibly demonstrated by the pressure drop which rapidly increased from 0.2 inches of water on Day 27 to 6.0 inches of water on Day 40. Under these conditions, severe channeling is very likely and therefore uneven distribution of nitrogen should be expected.

Because the removal ethylbenzene removal efficiency decreased significantly in the first section between when the samples were collected (inlet concentrations of 98 and 97 ppm_v and outlet concentrations of 6 and 56 ppm_v for Days 20 and 38 respectively), the T_N values should have been significantly different from each other. On Day 20, 94% of the inlet ethylbenzene was consumed in the inlet section as compared to 42% on Day 38. Because three individual samples were collected at different locations in the inlet section at each sampling time, it is quite possible that cells from each packing sample were receiving different nutrient and carbon loadings. One sample could possibly skew the average in one direction or the other. The VPB T_N values range from 0.39 to 0.90 on Day 20 (1 g/L $(NH_4)_2 \cdot SO_4$) and from 0.66 to 0.97 on Day 38 (4 g/L $(NH_4)_2 \cdot SO_4$). Samples with high T_N values may have been obtained from zones within the biofilter that were receiving more nitrogen. Based on these qRT-PCR results, it is not possible to draw any conclusions about optimal nitrogen requirements in biofilters.

Strictly considering physico-chemical measurements of nitrogen levels, the nitrogen available per unit biomass was similar on the two days that are being compared in this study (Figure 7-5). In both cases, this measurement shows that approximately 0.04 g N/g COD is available. In the case of *E. lecanii-corni*, a direct link between expression levels and nitrogen concentration was observed in batch reactors (Figure 7-1), and therefore because nitrogen levels are similar, by extension, expression levels should also be similar. However, since VPB performance has also been linked to nitrogen availability in past biofilter research (Gribbins and Loehr, 1998), one would expect removal efficiency to also be similar. However, this is not the case. The removal efficiency was much lower on Day 38 indicating that in this case, these two parameters cannot be directly correlated. As previously mentioned, on Day 38, the moisture content

was much lower and the pressure drop much higher as compared to Day 20 and therefore channeling was quite likely. One of the effects of channeling is to essentially reduce the residence time. As the residence time decreases, a subsequent decrease in degradation efficiency is expected.

This experiment was valuable in determining some of the shortcomings associated with qRT-PCR when applied to VPBs. While in homogenous samples, it provides very specific information with regards to optimal nutrient requirements, when applied to more heterogeneous situations, the results are much less clear. One of the problems becomes deciding where samples should be collected. As was demonstrated, T_N values varied over a wide range for packing collected at the same time point but at different locations from one packed bed section of the VPB. If severe channeling and uneven distribution of the nutrients were in fact occurring, it would be difficult to collect a representative biomass sample from such a heterogeneous system.

Nonetheless, on average, similar *ElHDO* expression levels were observed at both time points, which corroborate the nitrogen availability results. Therefore, in this case, while the qRT-PCR results do provide information about molecular level expression, this information is not as useful as in the case of substrate mixtures presented in Chapter 6. Expression levels were shown to be a leading indicator of the ultimate degradation of ethylbenzene when a repressor was introduced into the column feed and therefore could be used to predict column failure. In the case of nitrogen availability, the expression values alone could not predict column failure. The T_N values provide only information about how much enzyme the organisms are producing without taking into consideration other parameters that could be more important such as those describing all of the effects

of channeling. No matter how much enzyme the microbes are producing, as long as the actual substrates do not have enough time to contact the enzymes (reduced residence time) the efficiency will drop. In this case, pressure drop is a much more important parameter.

In conclusion, nutrient levels were shown, using qRT-PCR, to affect *ElHDO* expression levels in homogenous samples such as batch reactors. This method can therefore be used to determine optimal nutrient concentrations for expression of a gene of interest in simpler systems. When applied to a stressed VPB, qRT-PCR results as related to nutrient concentration were much less definitive especially when several other parameters are affecting column operation, such as low moisture content and high pressure drop. In this case the expression data were not useful as a stand alone piece of information. However, in combination with other macroscale level measurements such as pressure drop, a better understanding of reactions occurring in the biofilter can be obtained. In the next chapter, the utility of qRT-PCR is analyzed as it relates to VPBs operated under transient conditions.

Chapter 8: Effect of VPB Shutdown and Restart on Gene Expression

In this phase of the research, the utility of qRT-PCR was assessed during a series of shutdown and restart experiments. Specifically, the goal of these experiments was to determine if qRT-PCR could provide a direct measure of how quickly biofilm gene expression shuts down when the carbon source supply is removed and then how quickly microbial gene expression recovers upon substrate re-introduction.

8.1. METHODS

A VPB was operated as previously described (Section 6.1.1) at a constant ethylbenzene loading of 26 $g/m^3/hr$. Basal medium containing a high nitrogen concentration (5 g/L $(NH_4)_2SO_4$) was supplied continuously to the column using a nebulizer. The biofilter was operated for a period of approximately 40 days prior to beginning the shutdown tests. Three shutdown periods were evaluated in this experiment. During each shutdown test, the ethylbenzene supply to the column was discontinued for a period of 1, 3 or 7 days and then turned back on for period of 3 days. The air and nutrient solution were still provided during the shutdown periods. Composite packing samples consisting of 3 pellets were collected from the inlet section of the biofilter at time 0, 4, 12 and 24 hours after the ethylbenzene supply was discontinued and at time 0, 4, 12, 24, 48 and 72 hours after the ethylbenzene feed was resumed. Biomass extraction, RNA isolation, cDNA synthesis and qRT-PCR were performed as described previously.

8.2. RESULTS

T_N values obtained for the 1-day shutdown experiment are summarized in Table 8-1 and Figure 8-1. As shown in Figure 8-1, *ElHDO* expression gradually decreased over the 24-hour period following the removal of ethylbenzene from the inlet air stream. The expression decreased by 32, 72 and 93 % in the first 4, 12 and 24 hours, respectively. Upon re-introduction of ethylbenzene, the gene expression levels recovered to pre-shutdown levels within 4 hours and remained at approximately the same level over the subsequent 3-day monitoring period (Figure 8-1).

Table 8-1: 1-Day Shutdown

Time	C_T *ElHDO*	C_T *18S rRNA*	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[23] Time zero / Previous data point
Shutdown Time: 0 hour	***27.81***[24]	***19.34***	***-8.47***	***1.00***	1.24	0.37	N/A / N/A
	27.69	19.30	-8.39	1.06			
	27.43	19.70	-7.73	1.67			
Shutdown Time: 4 hour	28.03	19.60	-8.43	1.03	0.85	0.37	ns / ns
	29.47	19.76	-9.71	0.42			
	27.99	19.66	-8.33	1.10			
Shutdown Time: 12 hour	28.72	17.68	-11.04	0.17	0.27	0.09	sig / ns
	28.62	18.63	-9.99	0.35			
	28.59	18.31	-10.28	0.29			
Restart Time: 0 hour	28.21	16.65	-11.56	0.12	0.09	0.03	sig / sig
	28.32	15.66	-12.66	0.05			
	28.88	16.86	-12.02	0.09			
Restart Time: 4 hour	28.47	20.06	-8.41	1.04	0.88	0.17	ns / sig
	28.36	19.74	-8.62	0.90			
	28.40	19.42	-8.98	0.70			
Restart Time: 12 hour	28.76	19.70	-9.06	0.66	0.67	0.07	ns / ns
	28.56	19.66	-8.90	0.74			
	28.78	19.58	-9.20	0.60			
Restart Time: 24 hour	28.41	19.79	-8.62	0.90	0.62	0.27	ns / ns
	28.71	19.44	-9.27	0.57			
	28.83	18.93	-9.90	0.37			
Restart Time: 48 hour	28.51	19.55	-8.96	0.71	0.66	0.05	ns / ns
	29.06	19.94	-9.12	0.64			
	28.67	19.53	-9.14	0.63			
Restart Time: 72 hour	27.54	19.03	-8.51	0.97	0.94	0.33	ns / ns
	27.08	18.93	-8.15	1.25			
	27.89	18.68	-9.21	0.60			

[23] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 4 hours was compared to data collected at 0 hours). sig: significant, ns: non-significant, α=0.05

[24] This sample was used as the calibrator.

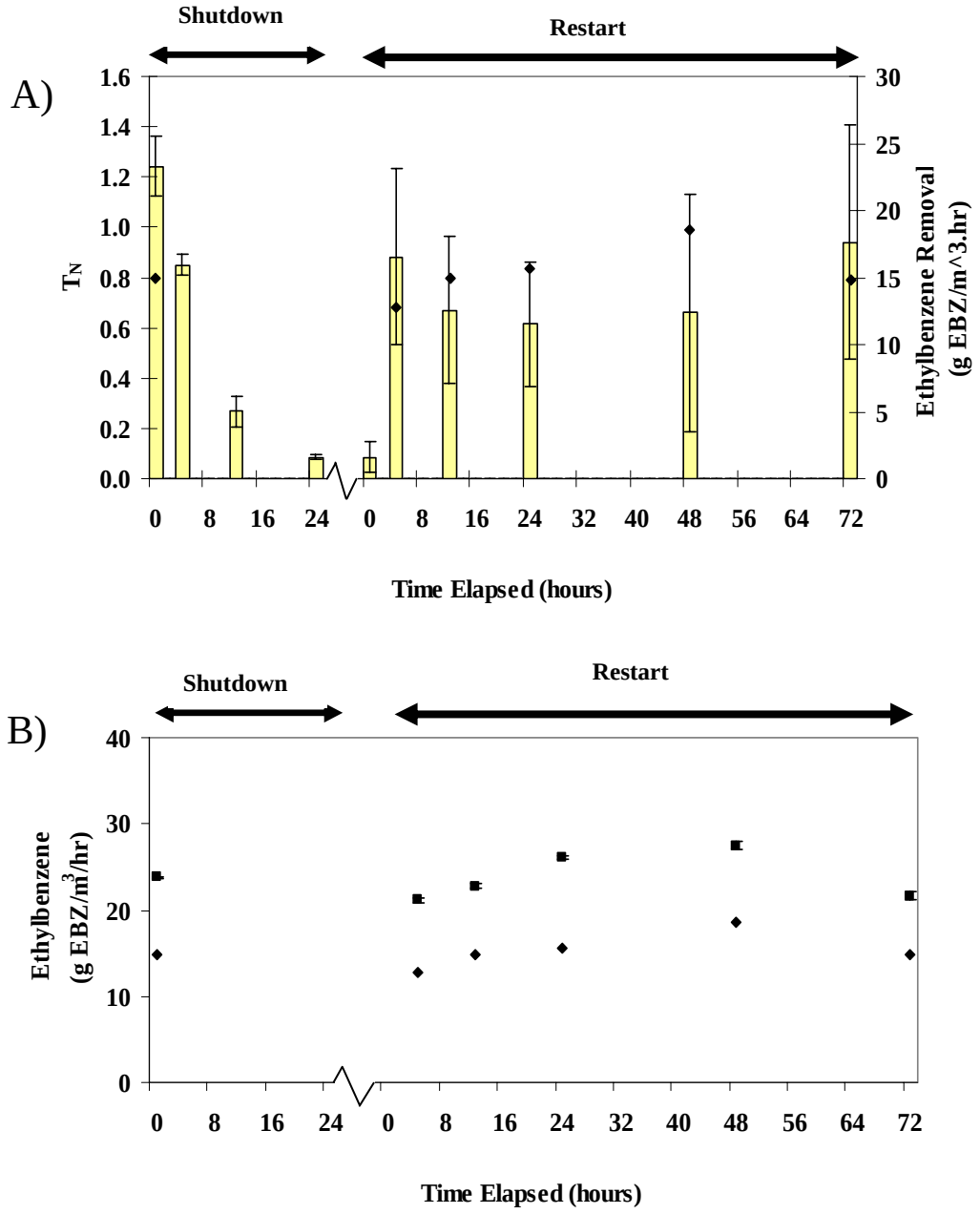

Figure 8-1: 1-Day Shutdown – A) Target Expression Numbers; B) Ethylbenzene Loading (■) and Removal (◆) in the Inlet Biofilter Section

Results for the 3-day shutdown experiment are presented in Table 8-2 and Figure 8-2. *ElHDO* expression did not decrease during the first 12 hour period following shutdown. However, within 24 hours, a 94% decrease in expression was observed. Upon re-introduction of the carbon source, the expression level rapidly increased to levels almost 3-fold higher than the levels observed prior to shutdown. The expression then decreased and stabilized at levels close to 1.0.

Table 8-2: 3-Day Shutdown

Time	C_T *ElHDO*	C_T *18S rRNA*	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[25] Time zero / Previous time point
Shutdown Time: 0 hours	***27.43***[26]	***15.91***	***-11.52***	***1.00***	1.04	0.12	N/A / N/A
	27.29	15.70	-11.59	0.95			
	27.03	15.75	-11.28	1.18			
Shutdown Time: 4 hours	26.42	15.35	-11.07	1.37	1.22	0.29	ns / ns
	26.66	15.64	-11.02	1.41			
	26.66	14.96	-11.70	0.88			
Shutdown Time: 12 hours	26.11	14.28	-11.83	0.81	0.99	0.21	ns / ns
	25.90	14.66	-11.24	1.21			
	25.98	14.39	-11.59	0.95			
Shutdown Time: 24 hours	24.12	11.43	-12.69	0.05	0.04	0.02	sig / sig
	24.49	11.65	-12.84	0.05			
	24.73	11.74	-12.99	0.02			
Restart Time: 0 hours	37.79	25.16	-12.63	0.46	0.17	0.26	sig / ns
	40.00	22.92	-17.08	0.02			
	38.49	20.57	-17.92	0.01			
Restart Time: 4 hours	26.35	16.74	-9.61	3.76	3.58	0.45	sig / sig
	26.71	16.81	-9.90	3.07			
	26.29	16.74	-9.55	3.92			
Restart Time: 12 hours	26.49	13.76	-12.73	0.43	0.61	0.38	ns / sig
	26.27	13.28	-12.99	0.36			
	26.76	15.31	-11.45	1.05			
Restart Time: 24 hours	27.29	13.23	-14.06	0.17	0.77	0.58	ns / ns
	26.96	15.17	-11.79	0.83			
	27.13	16.01	-11.12	1.32			
Restart Time: 48 hours	27.38	14.75	-12.63	0.46	0.40	0.07	sig / ns
	27.16	14.39	-12.77	0.42			
	27.23	14.11	-13.12	0.33			
Restart Time: 72 hours	25.61	14.14	-11.47	1.04	0.65	0.36	ns / ns
	26.37	14.07	-12.30	0.58			
	26.89	13.74	-13.15	0.32			

[25] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 4 hours was compared to data collected at 0 hours). sig: significant, ns: non-significant, α=0.05

[26] This sample was used as the calibrator.

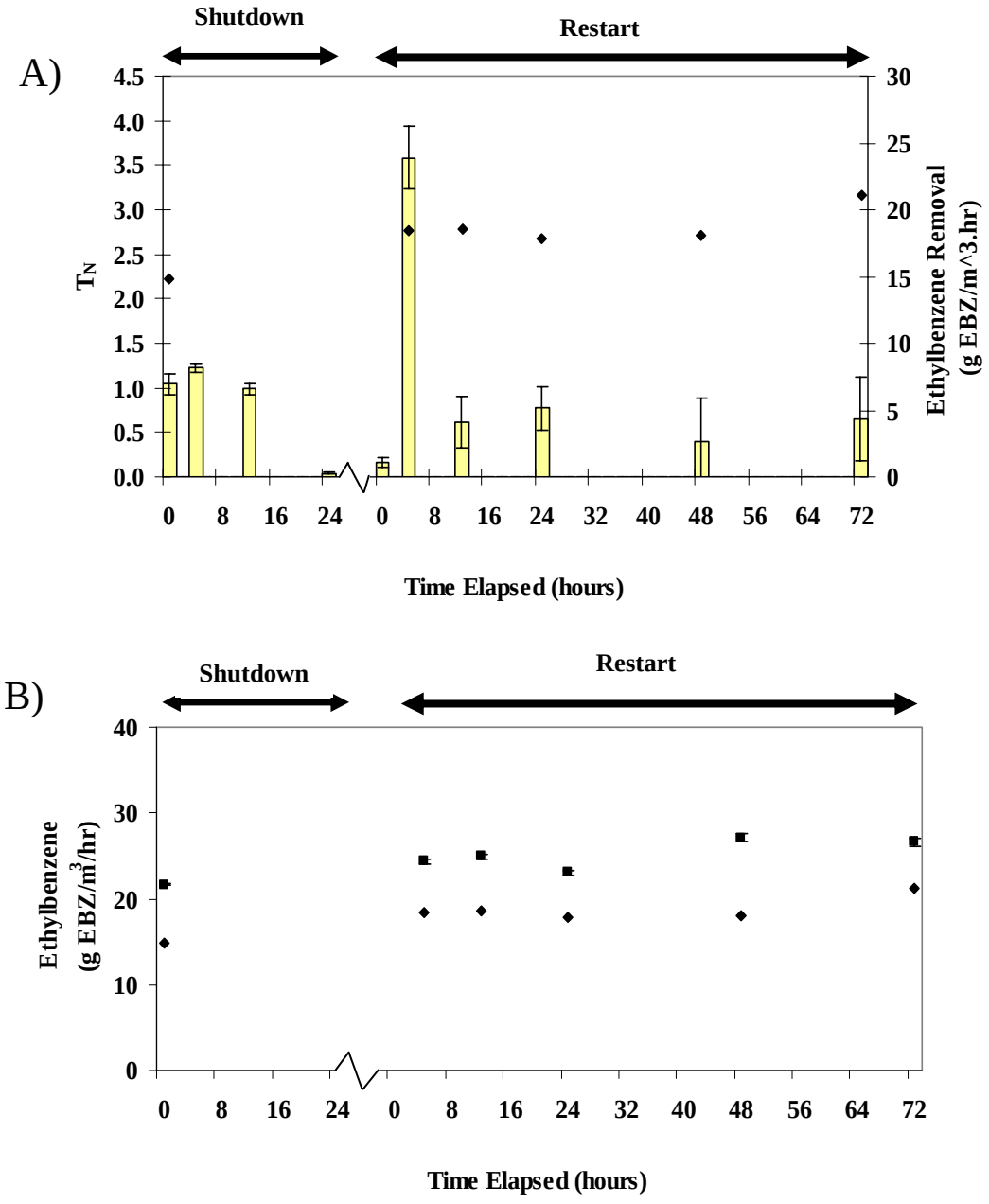

Figure 8-2: 3-Day Shutdown – A) Target Expression Numbers; B) Ethylbenzene Loading (■) and Removal (◆) in the Inlet Biofilter Section

Results obtained during the 7-day shutdown experiment can be found in Table 8-3 and Figure 8-3. Similar to the 1-day shutdown results, *ElHDO* expression gradually

decreased once the ethylbenzene supply was removed. Expression levels were 54, 70 and 80 % lower after 4, 12 and 24 hours, respectively. Upon re-introduction of the carbon source, the expression level returned to pre-shutdown values within 4 hours and remained at those levels.

Table 8-3: 7-Day Shutdown

Time	C_T *ElHDO*	C_T *18S rRNA*	ΔC_T	$2^{-\Delta\Delta C_T}$	Average T_N	Standard Deviation T_N	t-test[27] Time zero / Previous data point
Shutdown Time: 0 hours	***22.73***[28]	***11.48***	***-11.25***	***1.00***	1.12	0.12	N/A / N/A
	22.7	11.76	-10.94	1.24			
	22.44	11.34	-11.1	1.11			
Shutdown Time: 4 hours	24.54	12.48	-12.06	0.57	0.52	0.04	sig / sig
	24.98	12.7	-12.28	0.49			
	24.63	12.41	-12.22	0.51			
Shutdown Time: 12 hours	24.35	11.38	-12.97	0.30	0.34	0.06	sig / sig
	24.31	11.79	-12.52	0.41			
	24.03	11.1	-12.93	0.31			
Shutdown Time: 24 hours	24.35	10.99	-13.36	0.23	0.22	0.01	sig / ns
	24.51	11.03	-13.48	0.21			
	24.65	11.26	-13.39	0.23			
Restart Time: 0 hours	24.74	10.52	-14.22	0.13	0.17	0.06	sig / ns
	25.15	11.08	-14.07	0.14			
	24.42	11.09	-13.33	0.24			
Restart Time: 4 hours	23.71	12.81	-10.9	1.27	0.87	0.35	ns / ns
	23.72	12.01	-11.71	0.73			
	23.62	11.68	-11.94	0.62			
Restart Time: 12 hours	24.14	13.56	-10.58	1.59	1.39	0.29	ns / ns
	24.36	13.19	-11.17	1.06			
	24.16	13.52	-10.64	1.53			
Restart Time: 24 hours	23.96	13.09	-10.87	1.30	1.04	0.25	ns / ns
	24.25	13.01	-11.24	1.01			
	24.32	12.77	-11.55	0.81			
Restart Time: 48 hours	23.93	13.54	-10.39	1.82	1.33	0.47	ns / ns
	24.21	12.76	-11.45	0.87			
	24.16	13.29	-10.87	1.30			
Restart Time: 72 hours	23.95	13.11	-10.84	1.33	1.42	0.47	ns / ns
	24.43	13.2	-11.23	1.01			
	23.82	13.52	-10.3	1.93			

[27] t-test performed by comparing data to time zero and previous data point (e.g., data collected at time 4 hours was compared to data collected at 0 hours). sig: significant, ns: non-significant, α=0.05

[28] This sample was used as the calibrator.

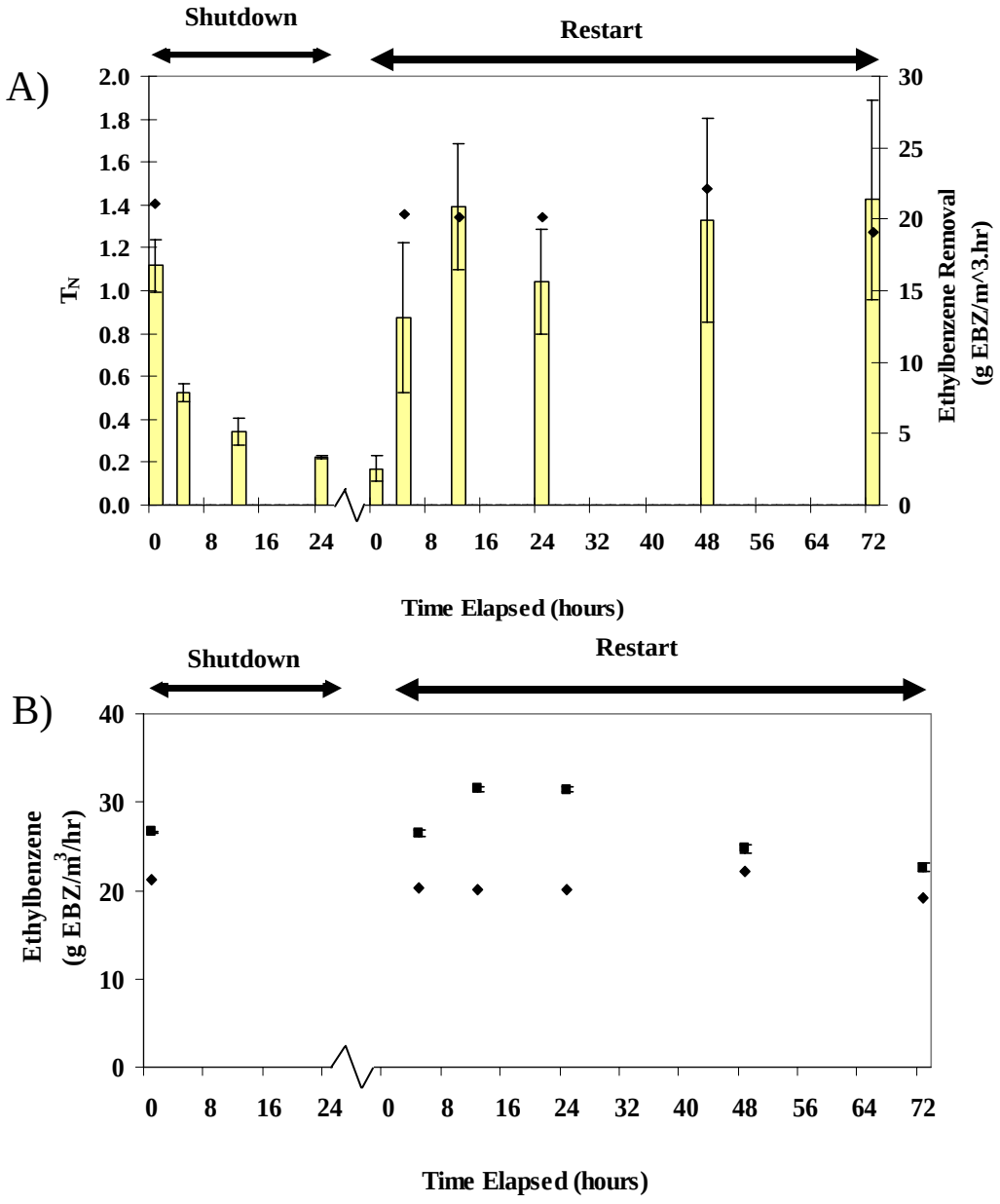

Figure 8-3: 7-Day Shutdown – A) Target Expression Numbers; B) Ethylbenzene Loading (■) and Removal (◆) in the Inlet Biofilter Section

8.3. DISCUSSION

While VPBs have been shown to be reliable for removing a variety of organic compounds, their long-term high performance can still be troublesome especially when they are subjected to dynamic loading conditions. Transient feed conditions (i.e., shutdown for a length of time and then restart) are common in industrial applications such as paint booths or factory applications. During the shutdown period, loss of biomass activity or the formation of inactive zones within the biofilter can result in poor performance upon when the feed is resumed. Reliable operation must be achieved for biofilters to be a useful treatment technology.

Several studies have found a substantial reduction in biomass activity when the feed carbon source is discontinued or when the load conditions drastically increase (i.e., spike in contaminant concentration) in mainly bacterial systems. The amount of time required for the biomass to regain their full degradation capacity in the case of a shutdown or the ability for the microbial population to degrade the additional substrate in a column feed spike varies with each study. van Groenestijin and Hesselink (1994) showed that a 2-day shutdown period resulted in a 30 to 60% decrease in biomass activity. 48 hours after the feed was resumed, contaminant degradation had returned to 100% of the pre-shutdown level. In another study, Martin et al. (1996) showed that degradation was restored within 9 to 24 hours after a 4-day shutdown period. Wani et al. (1998) found that increasing the length of the starvation period caused an increase in the length of the re-acclimation period in a biofilter treating reduced sulfur and VOC emissions. After a 7-day shutdown, 2-2.5 days of re-acclimation were needed to return to

full hydrogen sulfide degradation capacity. Mohseni and Allen (1997) found that 48 hours after a spike of α-pinene, the microbial population had acclimated to the higher load conditions. Deshusses (1997) found that 2-5 hours were needed to reach steady removal when hexane, acetone, 1-propanol and/or methyl isobutyl ketone were added to a steady methyl ethyl ketone feed. While some of these studies did look at general biomass activity, they did not look specifically at molecular level activity in the biofilm. This level of investigation could help in developing a better understanding of molecular level responses to transient loads. The specific goal of this phase of the research was to assess and determine if qRT-PCR could be used to accurately quantify changes in *ElHDO* gene expression as a result of discontinuing the ethylbenzene feed to the VPB.

Expectations were that when the organic contaminant was no longer present in the inlet stream, *ElHDO* gene expression would decline and would then recover upon the reintroduction of ethylbenzene in the VPB feed. Results obtained for the 1-day and 7-day shutdown experiments follow this expected trend. Gene expression did slowly shutdown over a 24 hour period after the VPB ethylbenzene supply was shut off. Upon the re-introduction of ethylbenzene, *ElHDO* expression levels in the biofilm returned to the original levels within 4 hours. In both cases (1- and 7-day shutdown tests), the results at both macro- and micro-scale indicated similar trends. *ElHDO* expression gradually decreased after ethylbenzene was shutoff and ethylbenzene removal in the recovered within four hours after column restart. These data indicate that qRT-PCR can be sensitive enough to analyze biofilm level gene expression patterns.

Fungal recovery times after a carbon starvation period are significantly shorter than those observed in bacterial systems (Cox et al., 1997; Woertz, 2002). In this

research, a 4-hour time window was sufficient to regain full degradation capacity regardless of the length of starvation. Other biofiltration research in bacterial systems has shown that 9 to 48 hours have been required to allow the microbial population to recover (van Groenestijin and Hesselink, 1994; Martin et al., 1996; Wani et al., 1998). One reason for the slow recovery of bacterial-based VPBs may be that a portion of the biomass actually dies during the starvation period. Therefore, upon startup, new cells must be created that can express the genes necessary to make the protein required for degradation. The faster recovery of gene expression in the case of *E. lecanii-corni* may be due to a lower death rate for fungal cells as compared to bacteria. Because this research used a comparative quantitative method to analyze gene expression, and since qRT-PCR is sensitive enough to determine gene expression in a biofilm, this same method could be used to determine if the bacterial and fungal relative expression rates vary significantly. Using the comparative qRT-PCR method, one could determine the gene expression level of a specific enzyme as compared to the ribosomal RNA level (*16S rRNA* or *18S rRNA* for bacteria and fungi, respectively). If the lower degradation capacity in bacterial systems is in fact linked to cell death, the ratio should remain similar. However, if the degradation efficiency is linked to a slower production of the enzyme and therefore is linked to gene regulation associated with carbon starvation, this ratio would be much lower. Nonetheless, the faster recovery for both gene expression and bioreactor performance in fungal bioreactors during transient operation show why they are an attractive alternative over bacterial systems.

Because transient loading is a common occurrence and because full degradation recovery does vary based on the microbial population and the chemicals being treated, several research studies have looked at the development of mathematical models to

predict the effect of these transient loads on treatment efficiency (Zarook et al., 1997a; Zarook et al., 1997b; Amanullah et al., 1999; Jorio et al., 2003; Deshusses et al., 1995; Den and Pirbazari, 2002; Rittmann et al., 2002). In general, these models incorporate mixing phenomena, convection and dispersion in the gas phase, oxygen limitation effects, adsorption phenomena, interphase mass transfer between the gas and the aqueous biofilm and general biodegradation kinetics. None of these models take into consideration molecular level microbial gene expression for a specific chemical and microorganism. Because the degradation relies on the production of proteins that carry out each specific reaction, these models could provide more accurate predictions by combining these parameters (such as T_N as a function of time and substrate concentration) with general physical/chemical properties. Because no tools have been available in the past to quantify the gene expression levels as a function of environmental conditions, such data was not available for incorporation into biofiltration models. This is an area where future research could be performed.

The results obtained during the 3-day shutdown phase were significantly different than the results obtained during the 1- and 7-day shutdown experiments. The expected gradual decrease in gene expression was not observed as in the other two experiments. In addition, the expression level upon reintroduction of the carbon source jumped up to levels much higher than the original levels and then stabilized.

The results obtained for the shutdown and restart experiments show that the application of qRT-PCR for following gene expression patterns in VPBs can, at times, be rather challenging. As previously mentioned in Chapter 7 for the nitrogen experiments, while biomass extraction, RNA isolation, cDNA synthesis and qRT-PCR amplification

are quite reproducible, the results of this analysis rely to a great extent on the quality of the initial biomass sample. Because VPBs tend to be heterogeneous by nature, obtaining a representative composite sample can be rather difficult. The nitrogen VPB experiment presented in Chapter 7 was performed using a lower nitrogen basal medium (1 g/L $(NH_4)_2.SO_4$) to provide nutrients in the column as compared to the high nitrogen basal medium (5 g/L $(NH_4)_2.SO_4$) used in the transient loading experiments. Because a low nitrogen level is associated with a higher degree of filament formation in *E. lecanii-corni*, less filamentation should have occurred in the shutdown and restart experiments where excess nitrogen was provided as compared to the nitrogen study (Woertz, 2002). Therefore, cell morphology should have remained more homogenous and therefore less channeling should have been present in the column. Under the high nitrogen loading conditions, it was expected that taking a representative composite sample would not be a difficult task. However, based on these results, it appears that the samples collected 4 and 12 hours after shutdown and then 4 hours after startup, may not have been entirely representative. In this specific case, the collection of multiple composite samples would have helped in determining if the *ElHDO* gene expression levels for the 3-day shutdown experiment were in fact different from the 1- and 7-day shutdown experiments.

Since it can be anticipated that a small degree of channeling and uneven nutrient distribution will be present in all VPBs, especially with increased length of operation, it can be expected that the biofilm on some of the packing material will be heterogeneous. Therefore, the potential for grabbing non-representative samples exists. This problem, as previously mentioned, could be circumvented by increasing the number of samples taken. However, taking additional samples would bring up other issues namely biofilm stability. Because laboratory-scale columns are quite small, each time packing samples are

removed, new media is placed in the reactor to replenish that lost so as to maintain the same packing volume in the column at all times. Because past VPB research has shown that the operational history of a VPB affects biofilm consistency, it is reasonable to assume that the biofilm on new packing would be substantially different from old packing. For this reason, increasing the number of samples in short-term experiments would impact biofilm stability and therefore is not a good option. A nice follow-up experiment to this research would be to repeat these studies in a larger scale bioreactor to determine if there is a large variability in gene expression levels in each sample.

In conclusion, when a representative sample is obtained, qRT-PCR is sensitive enough to assess microscale level changes in a VPB biofilm. Using this tool, this research showed that gene expression slowly declined over a 24-hour period following the removal of carbon from the reactor. Upon re-introduction of the carbon substrate, gene expression recovered within 4 hours. The recovery time was short and is one of the reasons why fungal VPBs can be more attractive than their bacterial counterparts when operated under adverse conditions such as transient operation. This research did, however, show that one of the main challenges associated with qRT-PCR when applied to VPBs, is in obtaining representative samples during short-term experiments. Therefore, while this method definitely shows potential for determining gene expression changes, it may be better suited for larger bioreactors where a larger number of samples can be removed from the reactor without affecting the microbial biofilm and for longer term laboratory-scale experiments where a substantial number of samples can be collected over a longer period of time.

Chapter 9: Conclusions

The main objective of this dissertation was to determine if gene expression studies could be used to delineate how substrate interactions and bioreactor operation affect pollutant degradation in a VPB. To this end, this research focused on a fungal biofiltration system degrading ethylbenzene. Specifically, the effect of single substrate (ethylbenzene) and multiple substrates on ethylbenzene gene expression, as well as that of two key operating parameters (nitrogen availability and shutdown/restart conditions) were determined. The specific objectives and a summary of findings for this dissertation project are listed below.

1. **Identify a gene in the ethylbenzene degradation pathway in *E. lecanii-corni* that can be used in gene expression studies.**

   Ethylbenzene degradation by *E. lecanii-corni* was shown to occur via a pathway similar to one used by bacterial species. Styrene, phenyl acetate and homogentisate are intermediates involved in ethylbenzene metabolism. The degradation process involves a homogentisate dioxygenase at the ring-cleavage step. A 360-bp fragment of the *ElHDO* gene was isolated and used in a standard Northern blot gene expression study. Results indicate that *ElHDO* is induced by ethylbenzene, confirming its role in ethylbenzene degradation by *E. lecanii-corni*.

2. **Optimize the qRT-PCR technology to allow quantification of gene expression in a fungal biofilm degrading ethylbenzene only.**

   qRT-PCR was optimized using the comparative threshold method to describe gene expression associated with ethylbenzene degradation. This method compares the relative expression of a gene of interest (*ElHDO*) to a housekeeping gene (*18S rRNA*). Total RNA quantities ranging from 25 pg to 1 μg were used to develop standard curves for each target. Results show that the threshold cycle number and log RNA are linearly correlated over this entire range. In addition, the qRT-PCR efficiency is similar for both target genes implying that the comparative threshold method can be used to successfully quantify relative gene expression using these two targets.

3. **Determine whether the qRT-PCR tool can be used to understand how substrate mixtures, nitrogen availability and shutdown/restart conditions affect ethylbenzene degradation in a VPB.**

   - In homogeneous systems, such as a well-mixed batch reactor, the qRT-PCR tool was shown to accurately depict changes in gene expression levels when regulatory substrates such as methyl propyl ketone or phenylacetate were introduced or when nitrogen concentrations were modified. The specific regulatory effect that each parameter had on the degradation of the primary substrate (in this case ethylbenzene) was identified and quantified. For instance, nitrogen levels below 2 g/L $(NH_4)_2.SO_4$ repressed *ElHDO* expression. Similarly, methyl propyl ketone repressed *ElHDO* expression and ethylbenzene degradation, while phenylacetate induced both. However, although ethylbenzene degradation rates and gene expression levels were

correlated in all cases, the variations for these two parameters were not numerically proportional.

- In the more heterogeneous VPB system, the qRT-PCR tool was also used successfully to quantify gene expression level changes resulting from the introduction of substrate mixtures and transient feed conditions. As in the batch studies, methyl propyl ketone suppressed up to 95% of *ElHDO* gene expression, although there was a lag between the down regulation of the gene and the subsequent drop in the ethylbenzene degradation. Phenyl acetate induced gene expression, but, at high concentrations, actually suppressed ethylbenzene degradation in the VPB. When the column was subjected to both methyl propyl ketone and phenylacetate as well as ethylbenzene, the individual regulatory effects of each compound were negated and no effect on *ElHDO* expression was observed. In the transient feed studies, *ElHDO* expression was shown to decrease gradually over a period of 24 hours following the removal of ethylbenzene from the column feed. Gene expression recovery, however, was very rapid (within 4 hours) upon the reintroduction of ethylbenzene. This rapid recovery rate for both gene expression and bioreactor performance, as compared to bacterial VPBs, is one of the reasons fungal VPBs may be better suited when transient operation and other adverse operating conditions are encountered.
- In addition to the specific findings noted above, several broader conclusions can be drawn from this phase of the research. First, the qRT-PCR technology is a powerful tool that provides a better understanding of regulatory control in the biofilm. Using this tool, the direct impact of regulatory substrates on gene expression can be quantified in substrate mixture studies. For instance, in the

case of *ElHDO* down-regulation, fine control at the transcriptional level was shown to occur. Gene expression acted as a leading indicator and therefore gene expression data could be used to predict future ethylbenzene degradation. In the case of up-regulation of ethylbenzene degradation, the tool showed how gene expression data could be used to understand preferential microbial degradation patterns (i.e., *E. lecanii-corni* degraded phenyl acetate preferentially over ethylbenzene). Second, nitrogen availability as a function of biomass concentration was found to dictate gene expression levels. As long as the nitrogen level remains above a threshold, expression remained relatively constant. Third, during the transient loading experiment, gene expression data were able to accurately describe how quickly *ElHDO* expression levels responded to shutdown and restart conditions. Because specific gene expression quantification can be obtained as a function of time, qRT-PCR data could be used in predictive numerical models.

4. Assess whether qRT-PCR accurately reflects macroscale pollutant degradation observed in a fungal VPB.

Overall, qRT-PCR was found to accurately represent *ElHDO* gene expression in the VPB biofilm and provide information relating to macroscale level pollutant degradation. In the case of the substrate mixture study with methyl propyl ketone (repressor), T_N was in fact found to be a leading indicator. Because T_N values are a measure of mRNA levels, they are in turn ultimately correlated by a series of regulatory steps to enzyme production. Since the protein (i.e., enzyme) is the molecule which carries out the actual degradation, it is logical that T_N should act as a

leading indicator of degradation. This result implies that qRT-PCR could be used in an automated system to predict VPB failure as a result of changing environmental conditions. The method does, however, have several potential shortcomings that should be considered when interpreting the data. First, because VPB biofilms can be heterogeneous by nature, collecting representative samples can be challenging in certain instances. In particular, when working with a fungal VPB, obtaining a representative sample can be difficult if filamentation has occurred. In some cases, more samples may be required for a truly representative analysis. Second, while the qRT-PCR method was found to be sensitive enough for quantifying large gene expression changes (e.g. T_N values changing from 1.1 to 0.3), it was not always sensitive enough to compare changes between conditions which have small T_N (e.g. T_N values changing from 0.1 to 0.05). In the latter case, variations cannot be quantified due to the magnitude of the standard deviation relative to the T_N values.

The work presented in this dissertation represents the first application of qRT-PCR to an environmental system. Because the goal of this project was to assess the utility of this tool, a rather "simple" model system was chosen for this research. In this system, qRT-PCR was found to be a powerful tool. Although some limitations were encountered, overall this technology was used successfully to accurately quantify molecular level changes. Additional research will be required to assess the utility of this method to describe more complex environmental systems (i.e., multiple microbial species and substrate mixtures). In addition, while this body of research was applied to VPBs, the results obtained in this dissertation are quite general in nature and can be expanded to other areas of environmental engineering such as monitoring the evolution of key enzyme expression levels at natural attenuation sites and wastewater treatment plants.

In conclusion, the data that can be obtained using qRT-PCR provide insight into molecular level phenomena occurring in the biofilm. Because gene expression levels are quantifiable, these data have the potential to be incorporated into numerical models that study the relationship between VPB performance and parameters important to VPB operation, including microbial response to feed composition changes, nutrient requirements and transient operation. qRT-PCR results provide a new type of data, which in combination with the more conventional data, can be used to better understand the intricacies associated with VPBs and other biological systems relevant to environmental engineering.

Appendix A

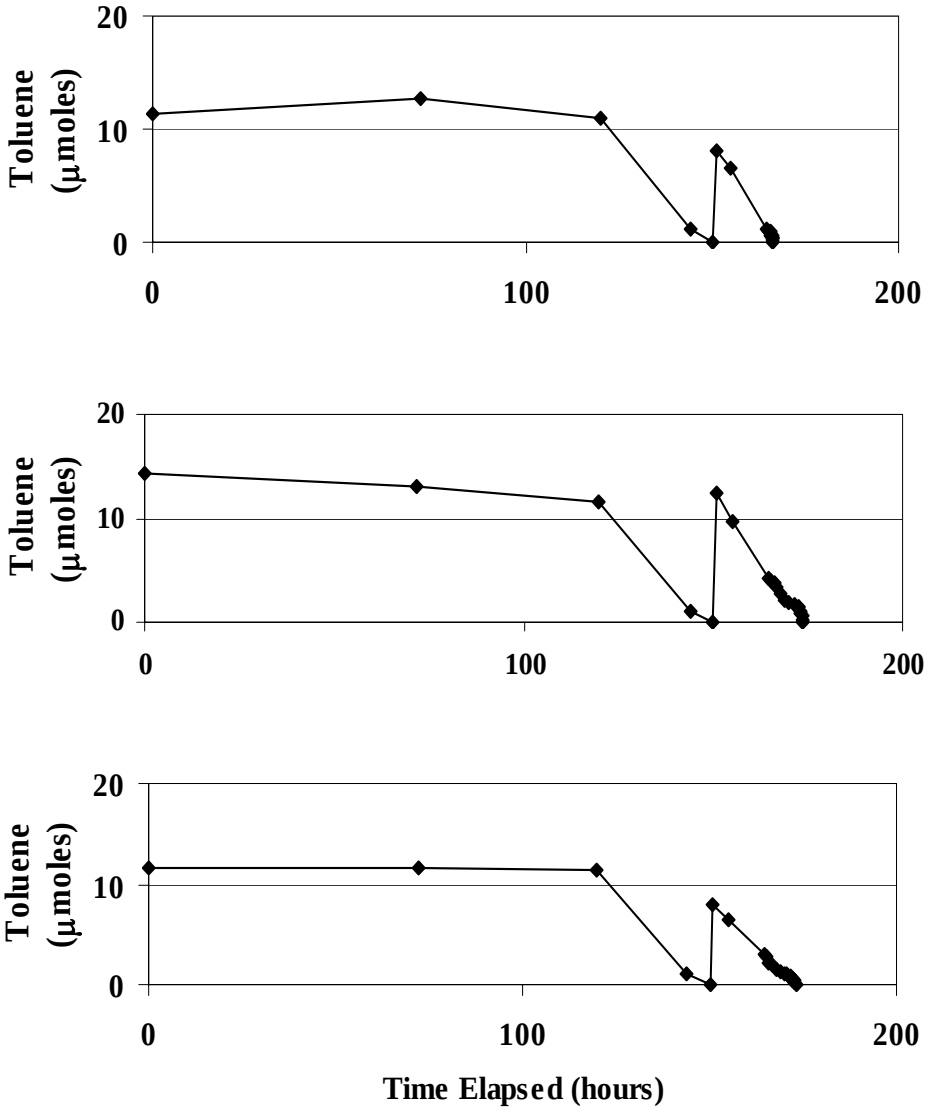

Figure A1: Toluene Degradation by *E. lecanii-corni* (1 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

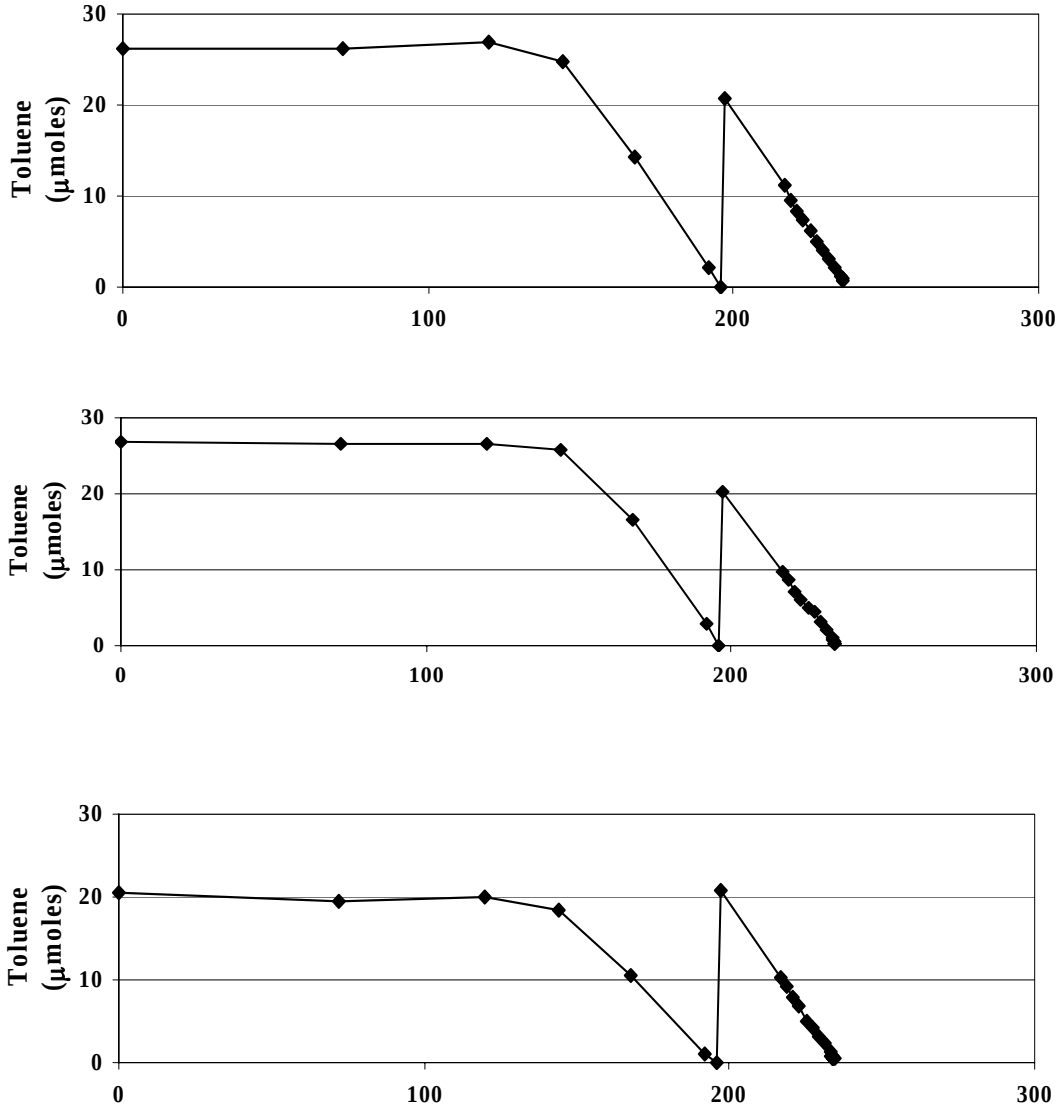

Figure A2: Toluene Degradation by *E. lecanii-corni* (2 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

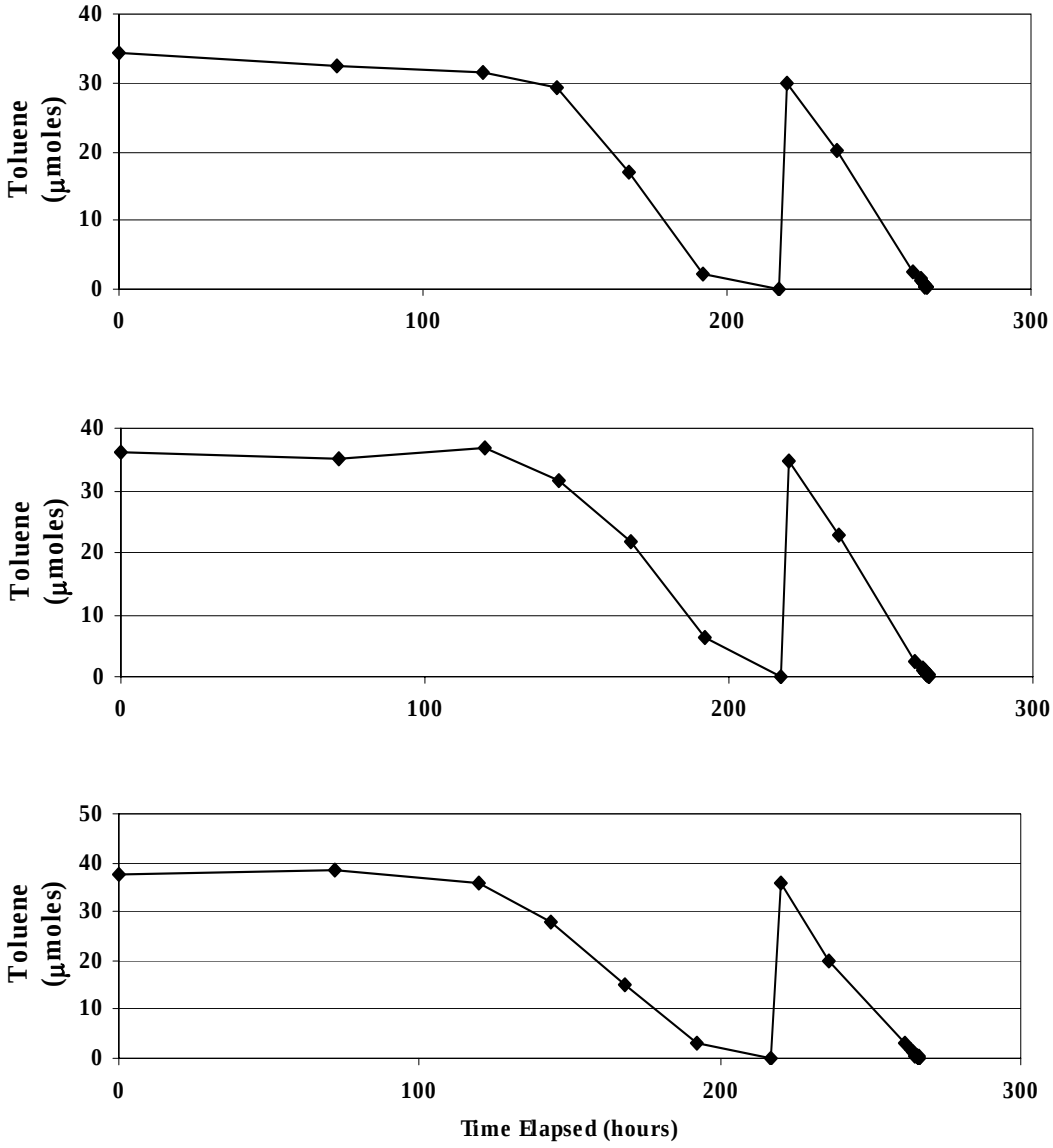

Figure A3: Toluene Degradation by *E. lecanii-corni* (4 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

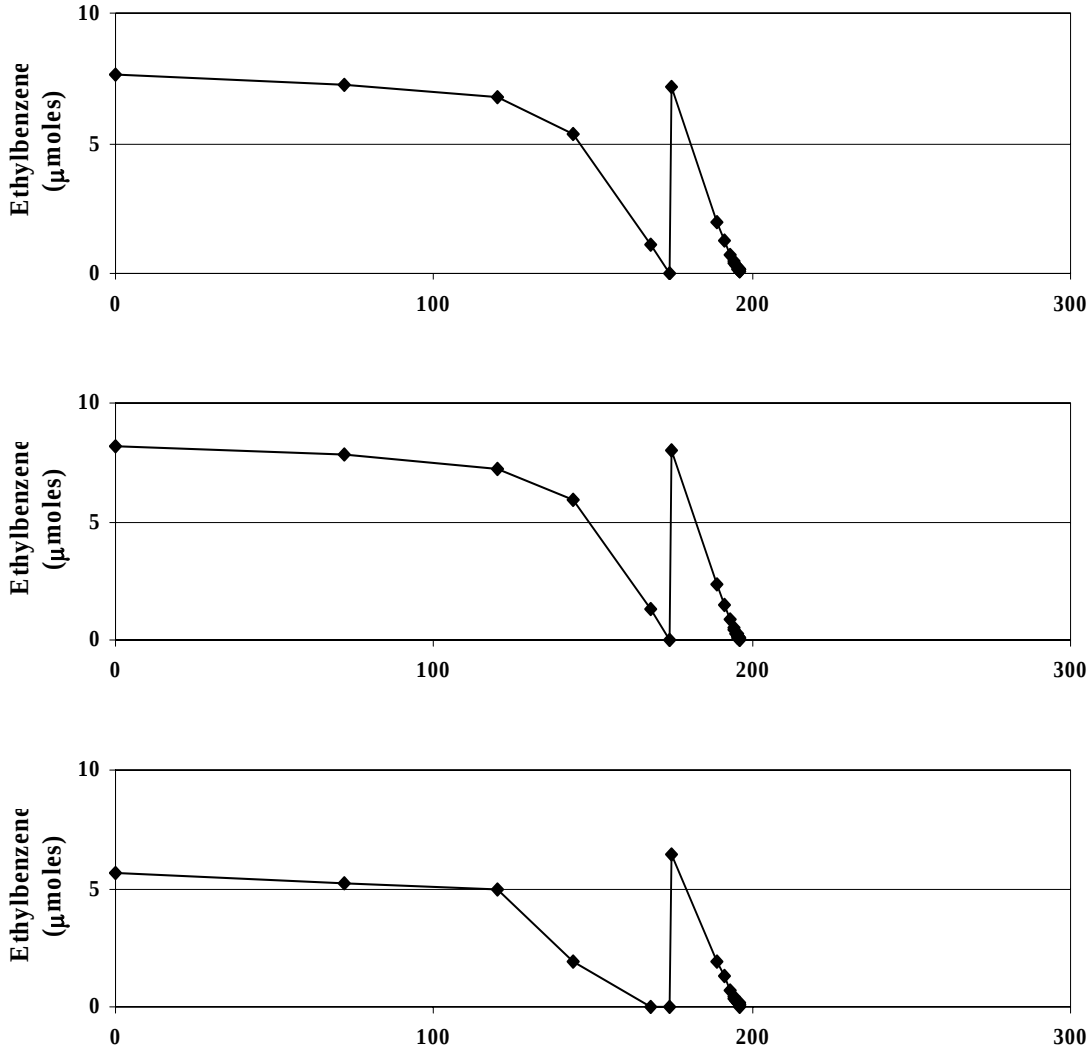

Figure A4: Ethylbenzene Degradation by *E. lecanii-corni* (1 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

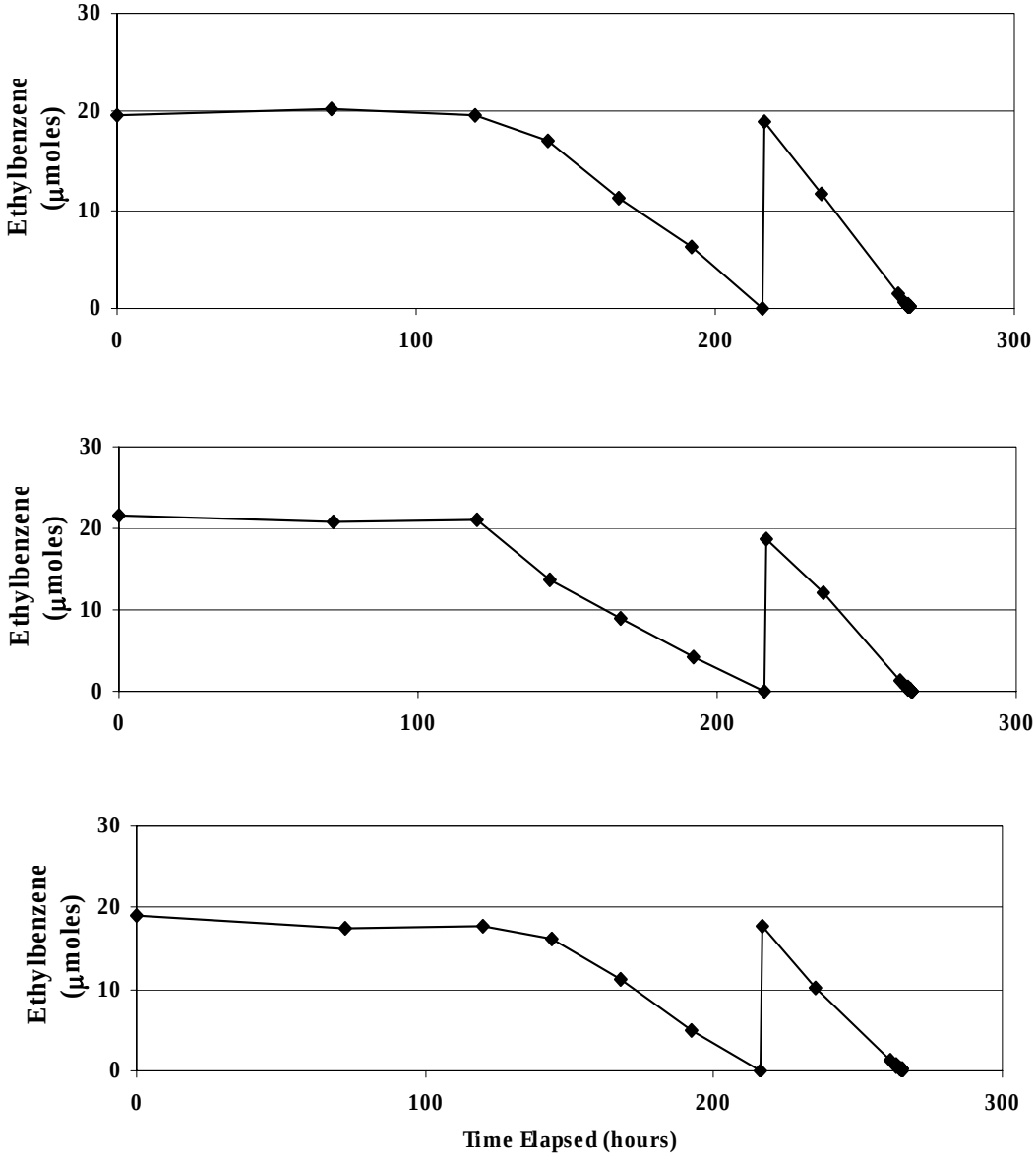

Figure A5: Ethylbenzene Degradation by *E. lecanii-corni* (2 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

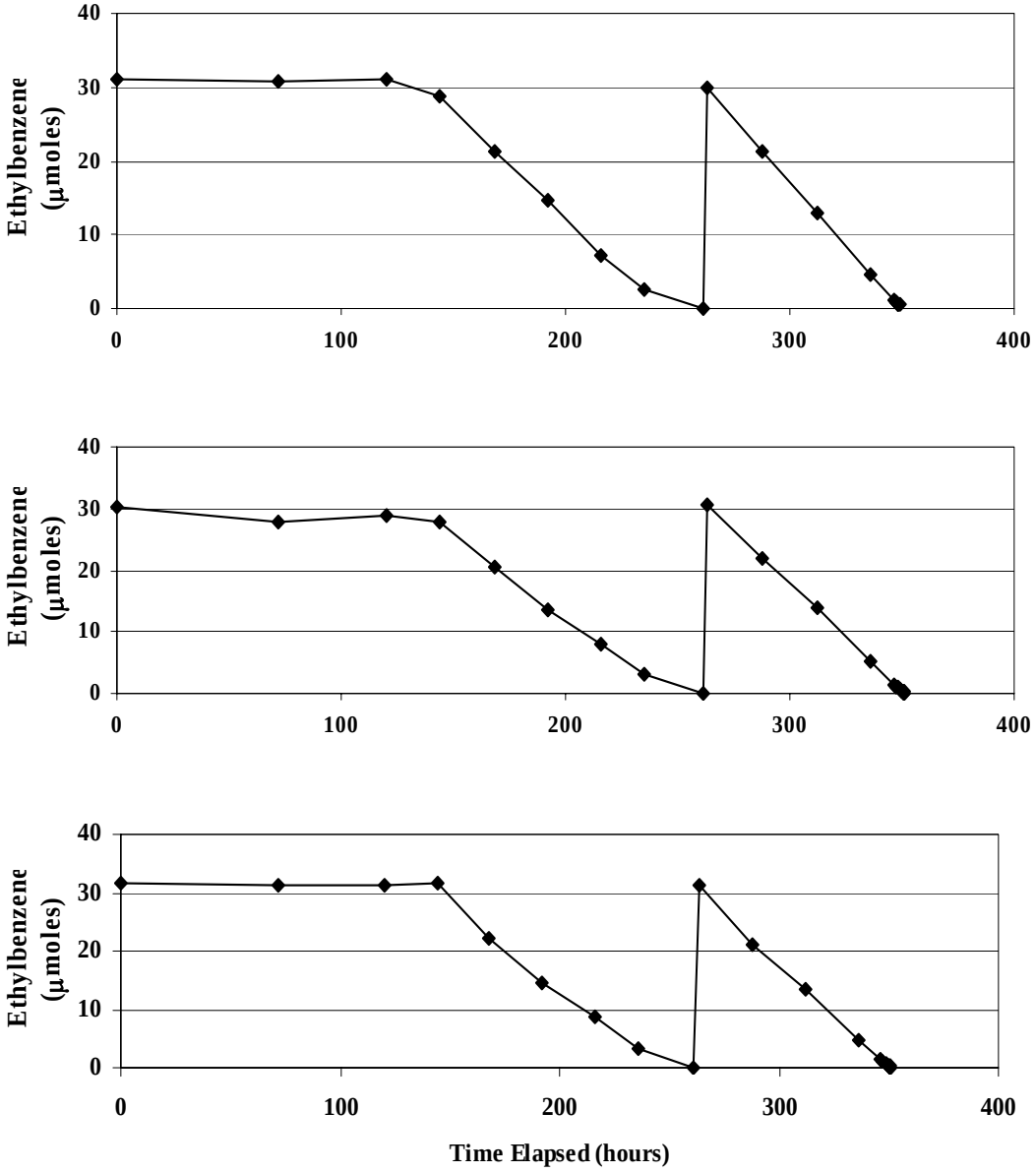

Figure A6: Ethylbenzene Degradation by *E. lecanii-corni* (4 µL injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

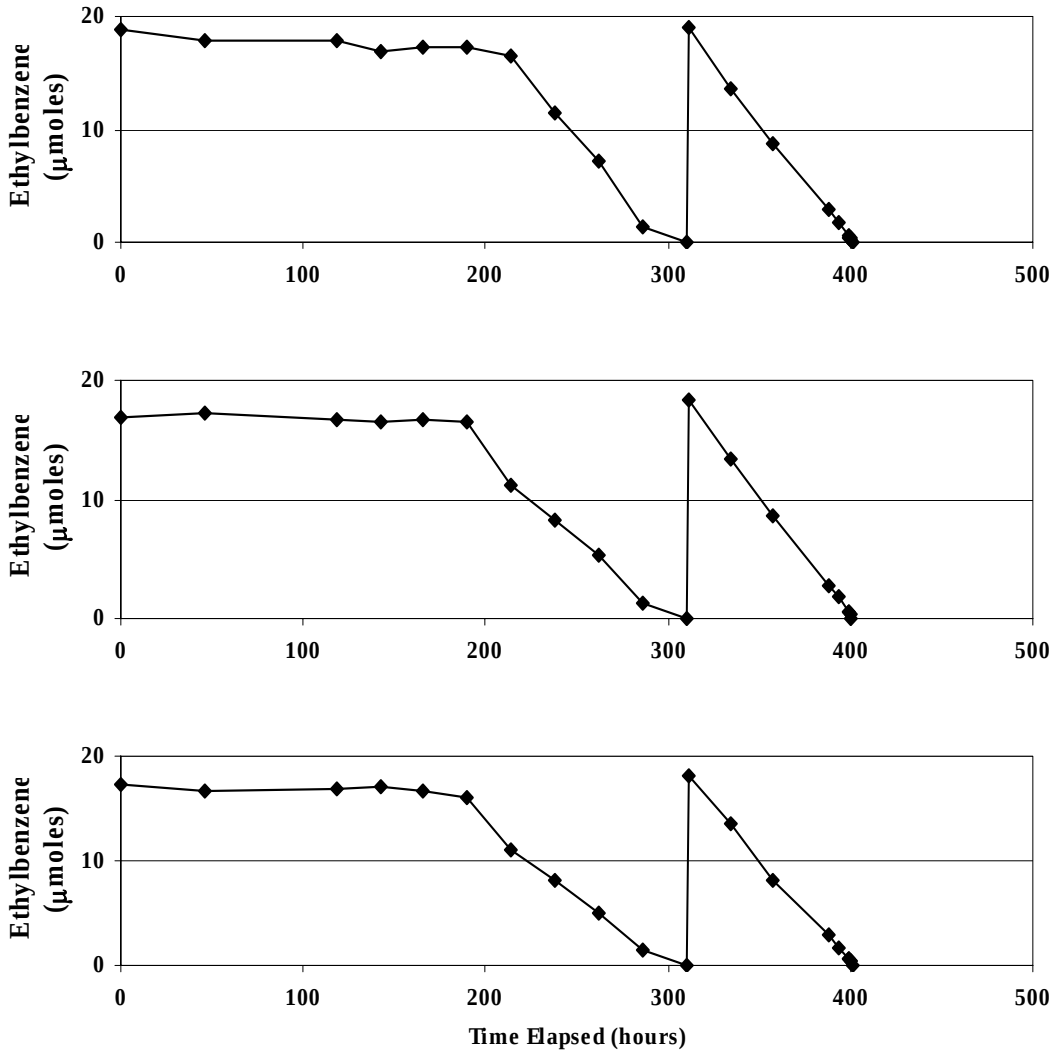

Figure A7: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the absence of methyl propyl ketone (2 µL Ethylbenzene injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

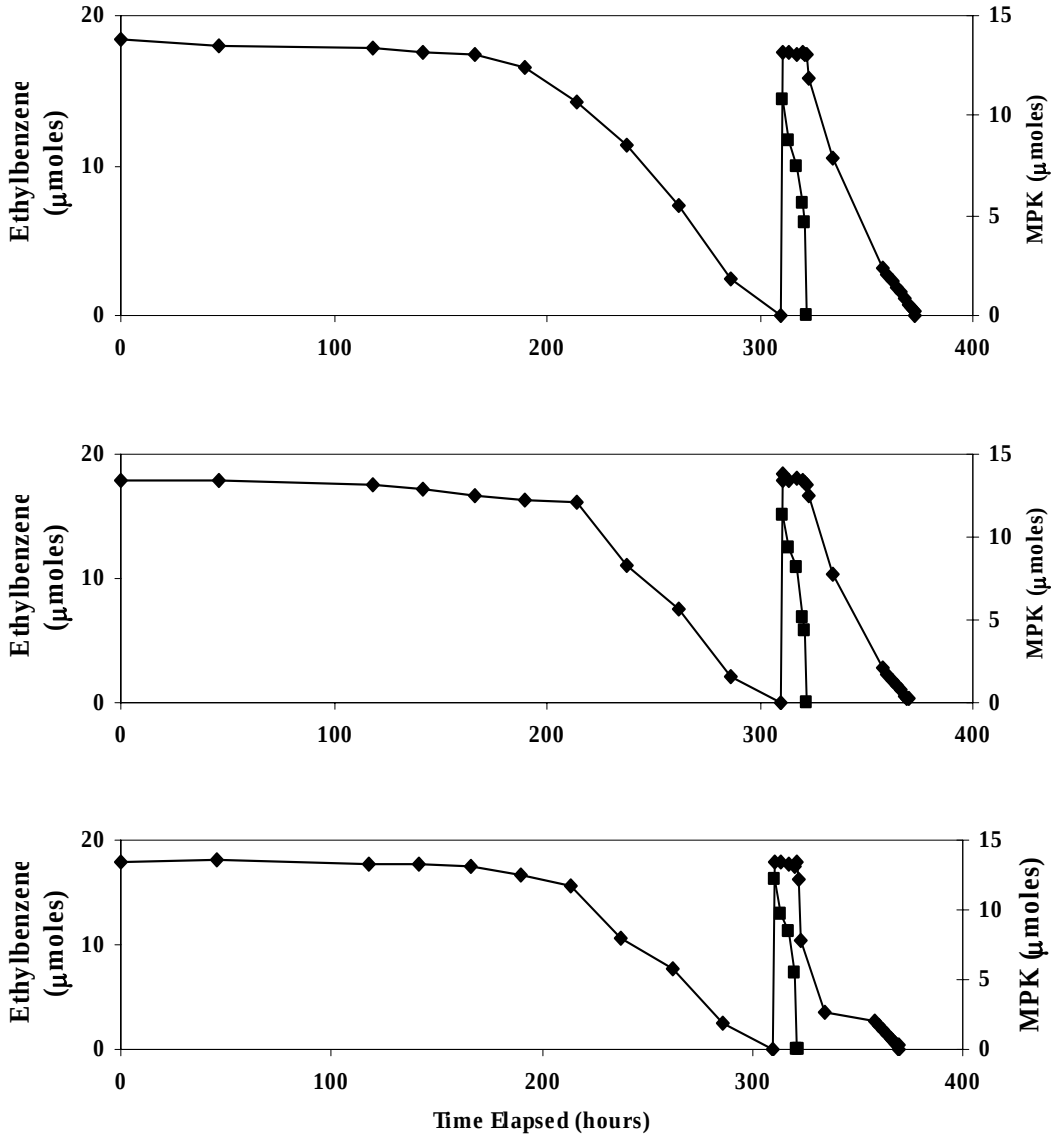

Figure A8: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of methyl propyl ketone (■) (2 µL Ethylbenzene and 1 µL methyl propyl ketone injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

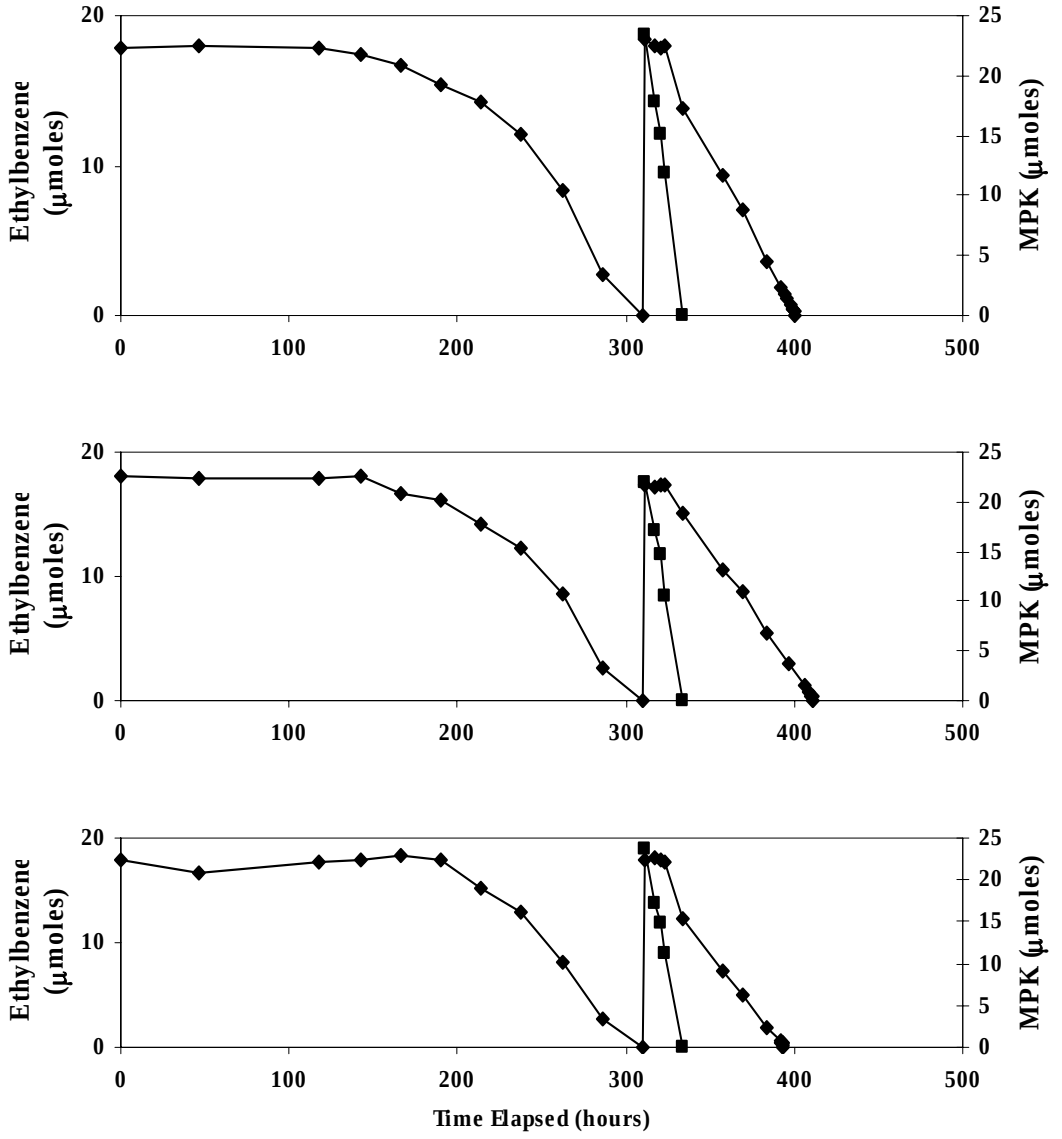

Figure A9: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of methyl propyl ketone (■) (2 µL Ethylbenzene and 2 µL methyl propyl ketone injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

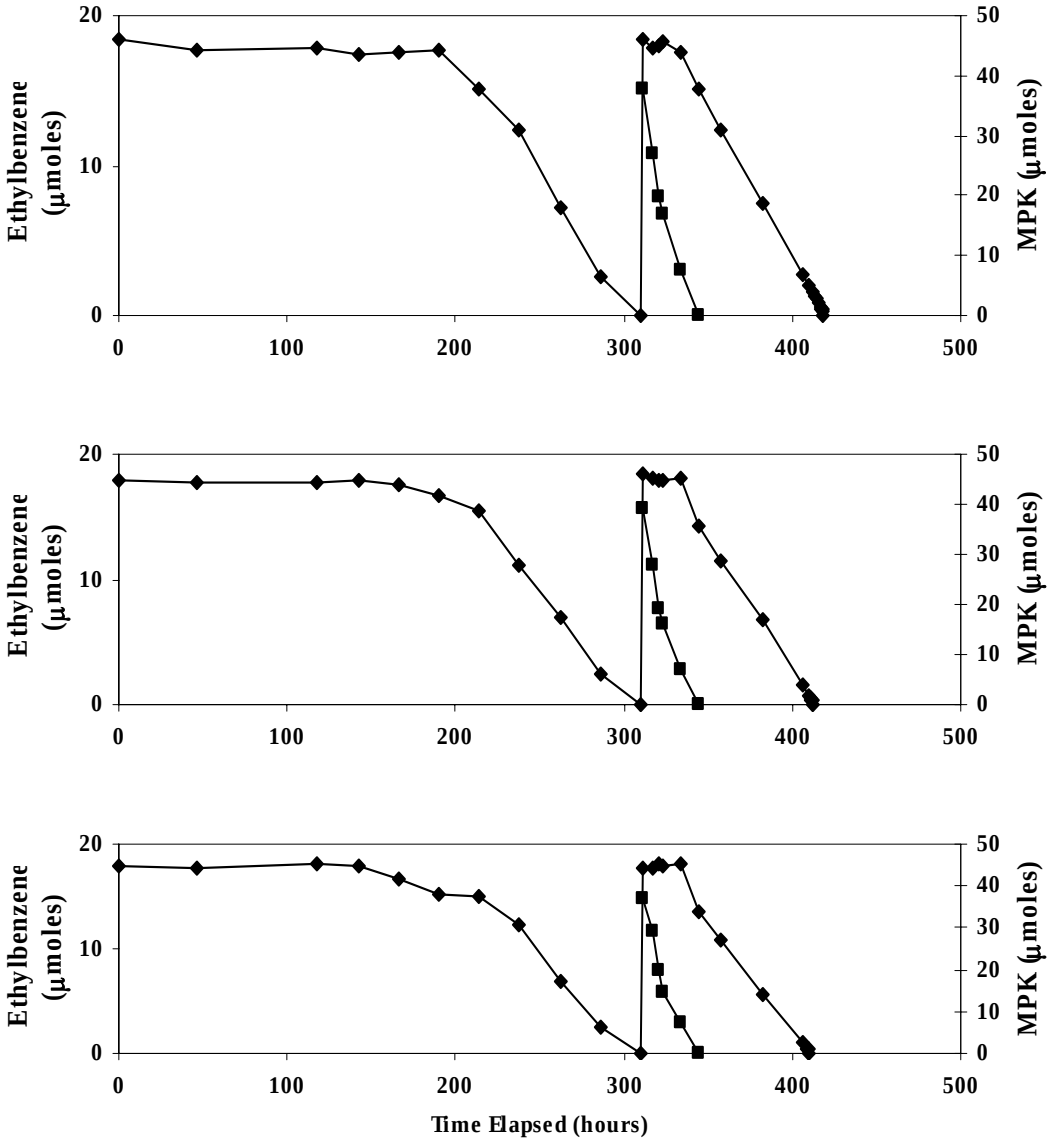

Figure A11: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the absence of phenyl acetate (2 µL Ethylbenzene and 4 µL methyl propyl ketone injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

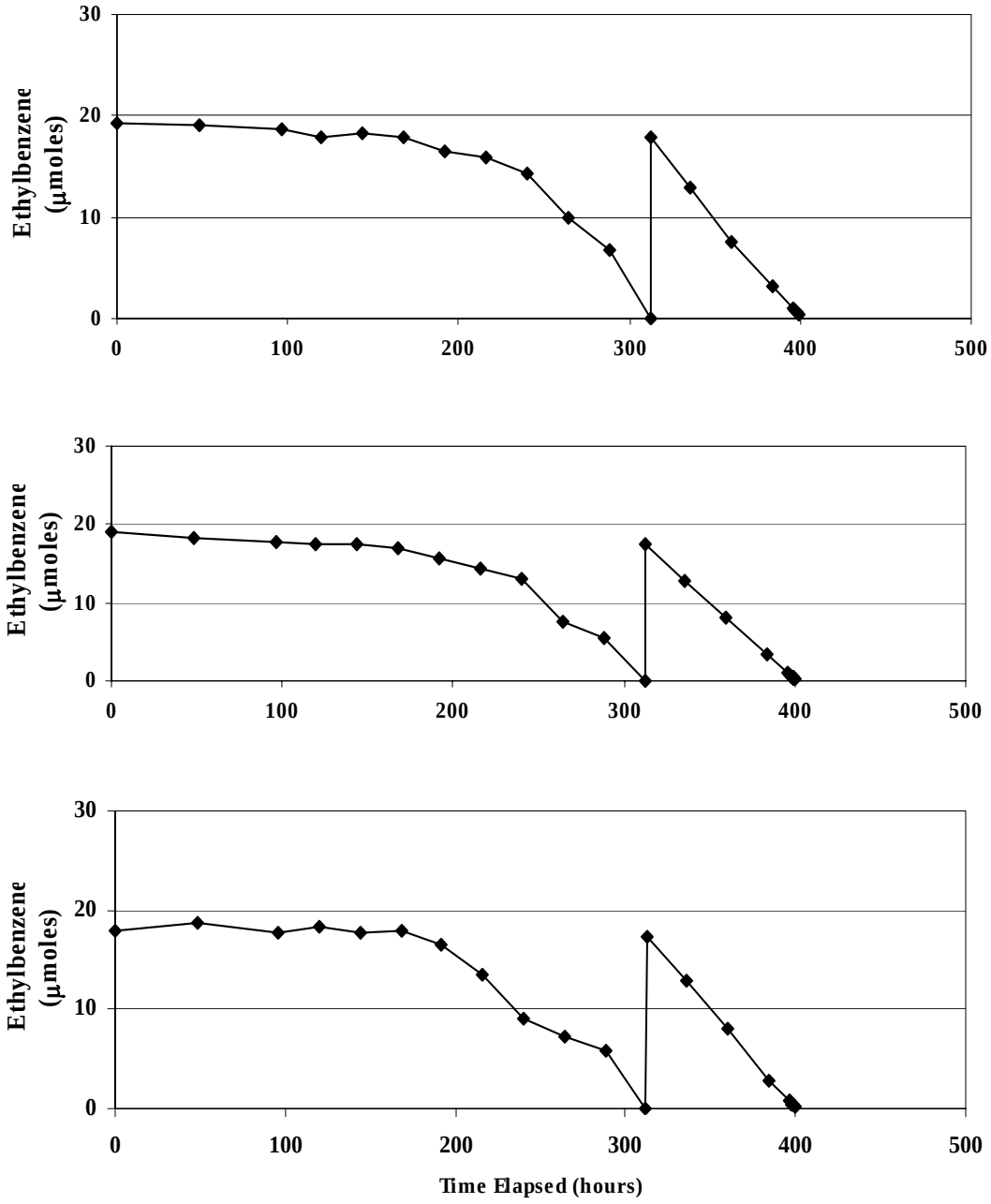

Figure A11: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the absence of phenyl acetate (2 µL Ethylbenzene injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

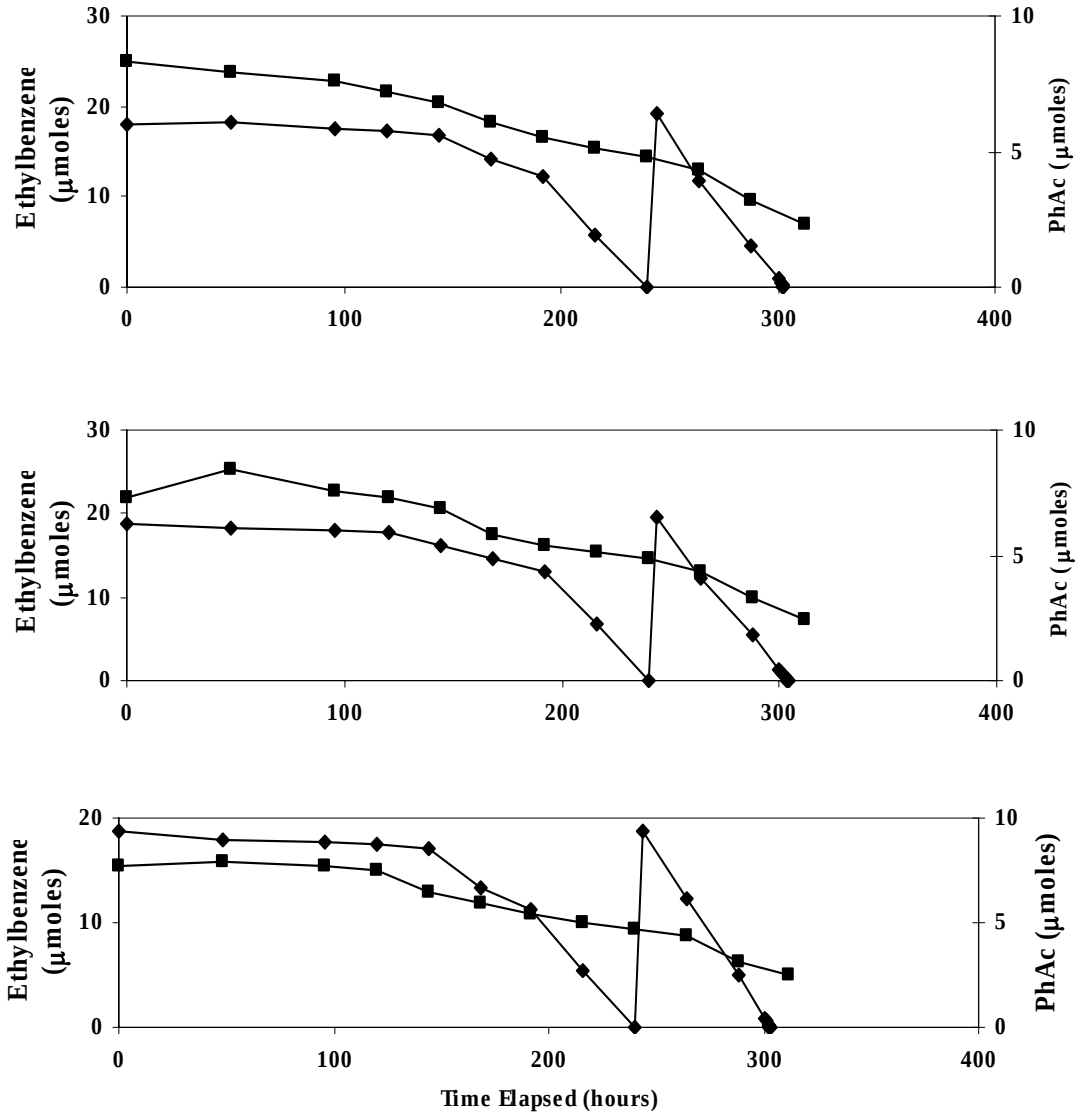

Figure A12: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of phenyl acetate (■) (2 µL Ethylbenzene and 1 µL phenyl acetate injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

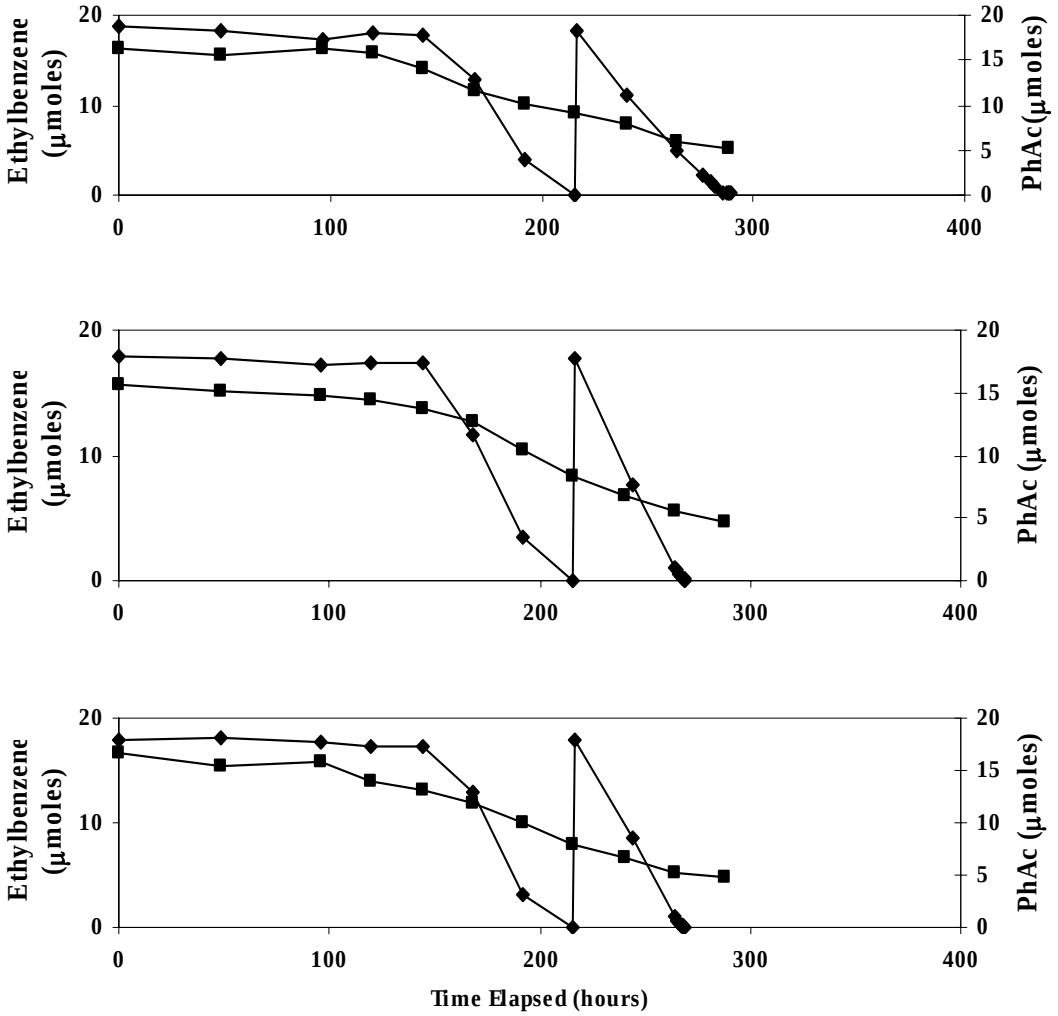

Figure A13: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of phenyl acetate (■) (2 µL Ethylbenzene and 2 µL phenyl acetate injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

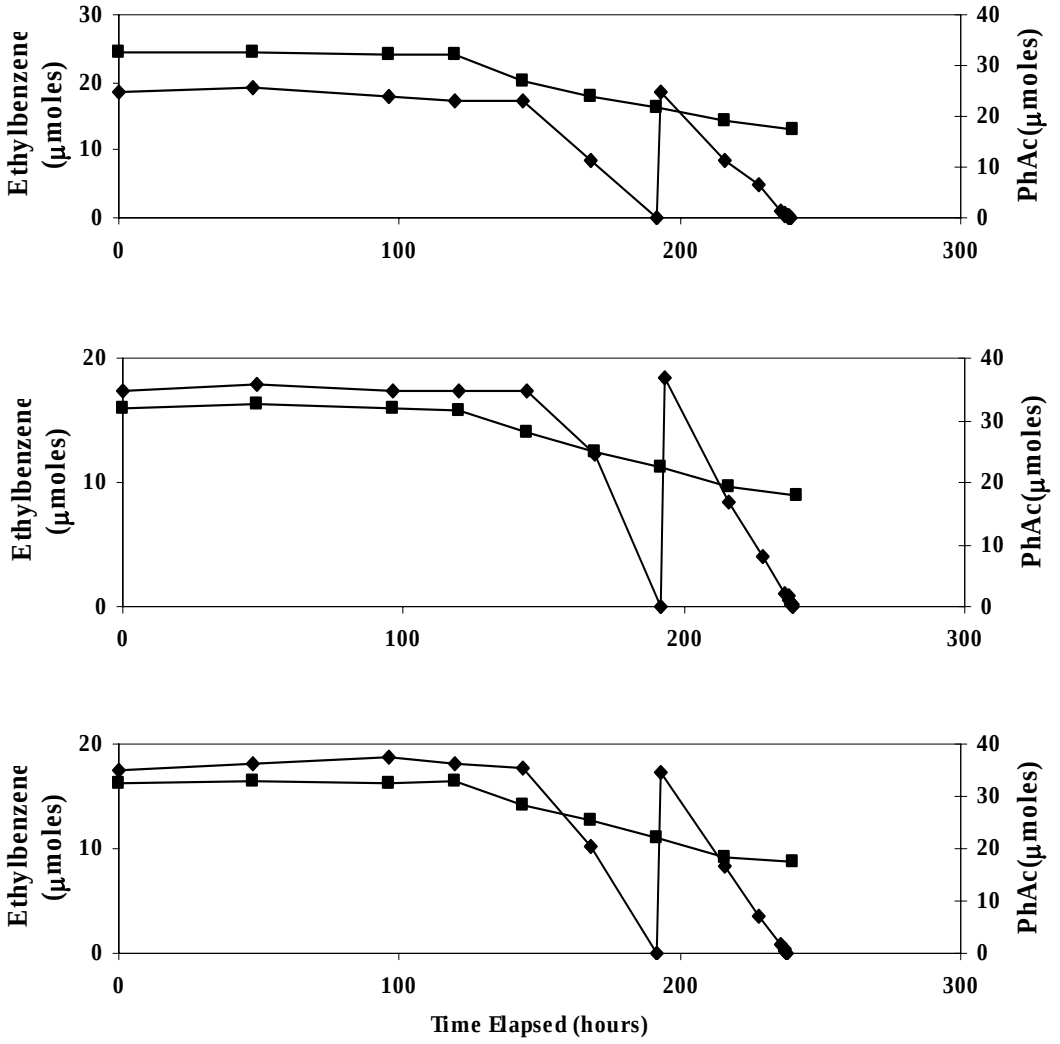

Figure A14: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of phenyl acetate (■) (2 µL Ethylbenzene and 4 µL phenyl acetate injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

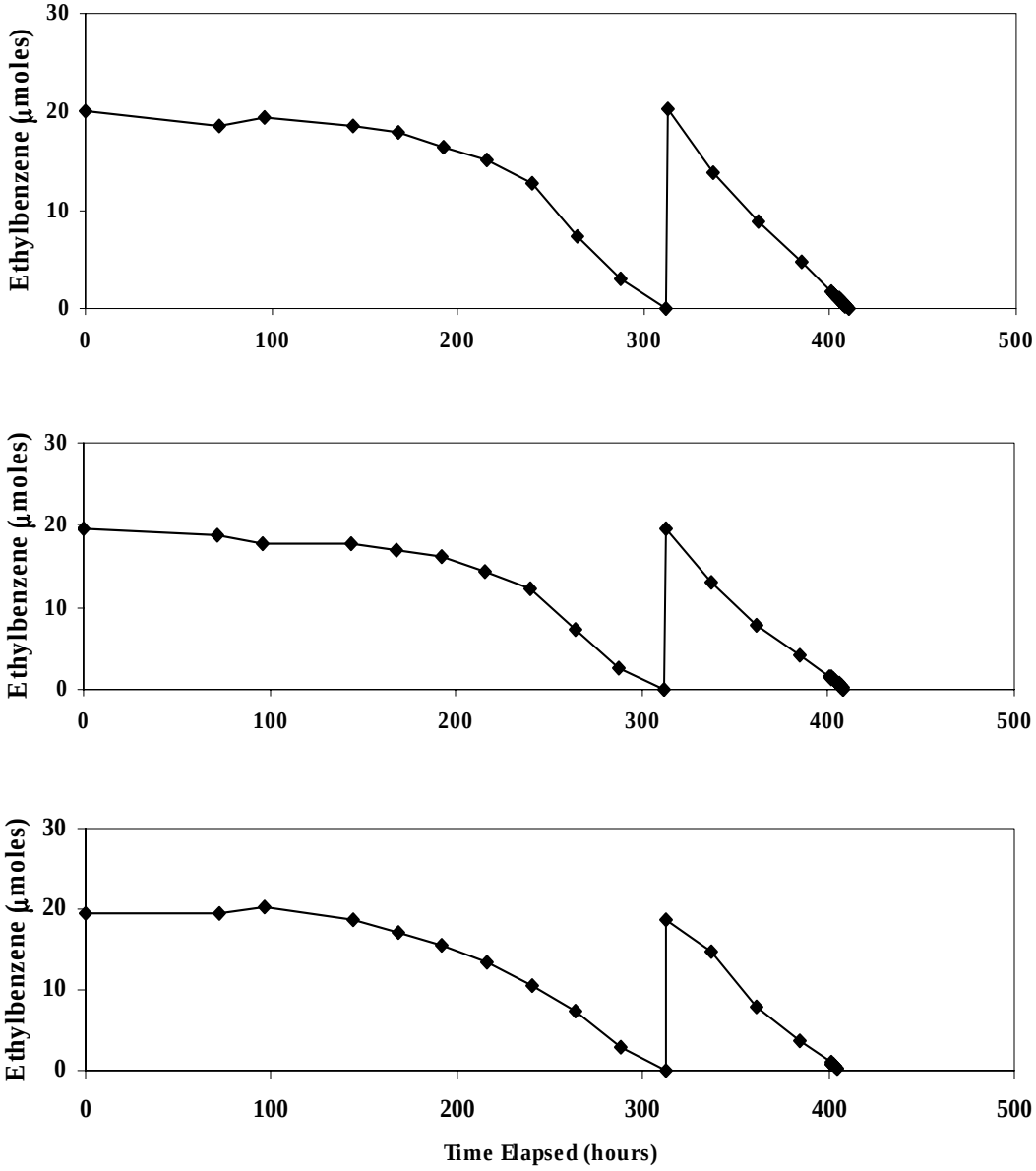

Figure A15: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the absence of o-xylene (2 µL Ethylbenzene injection). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

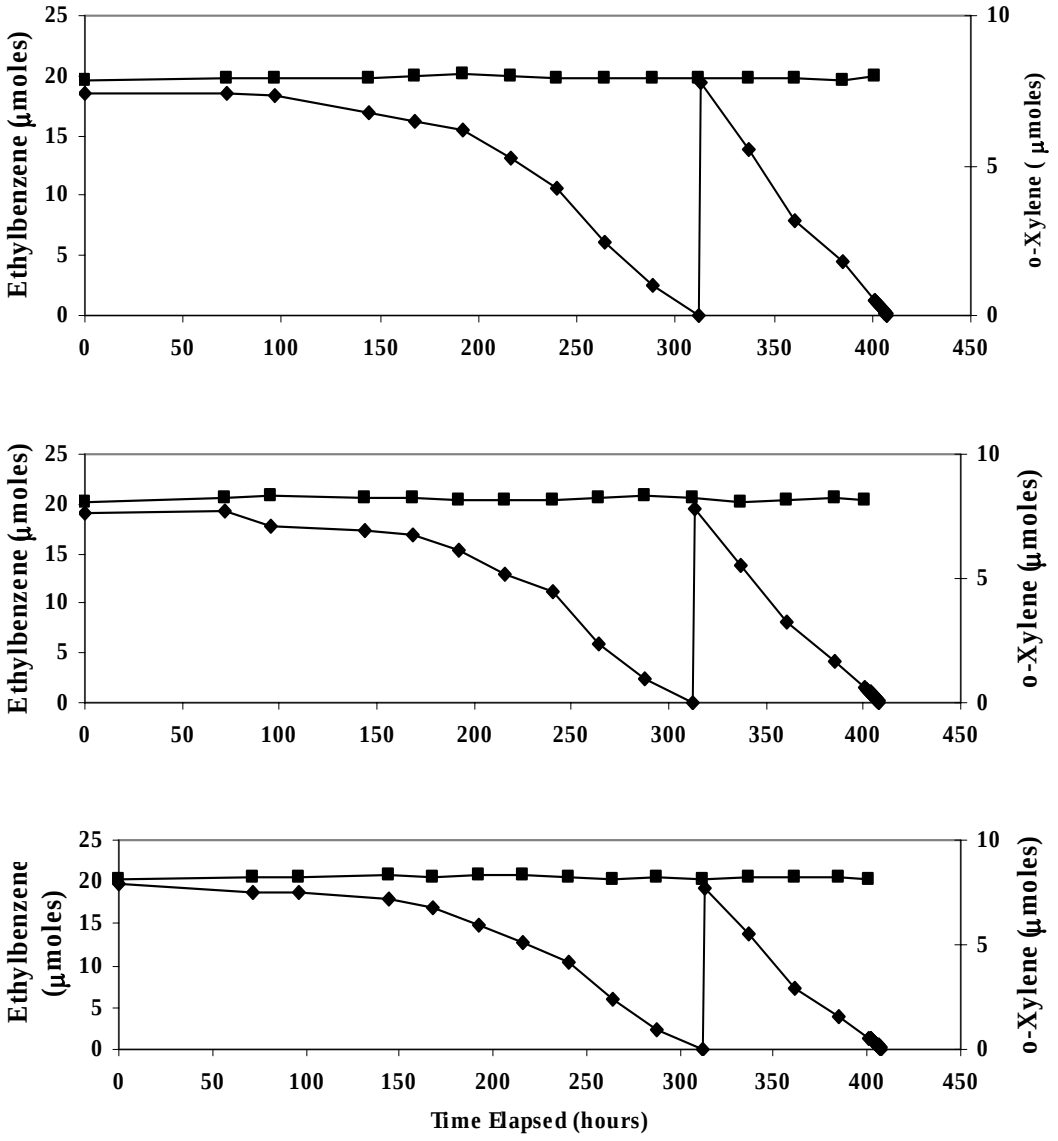

Figure A16: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of o-xylene (■) (2 µL Ethylbenzene and 1 µL o-xylene injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

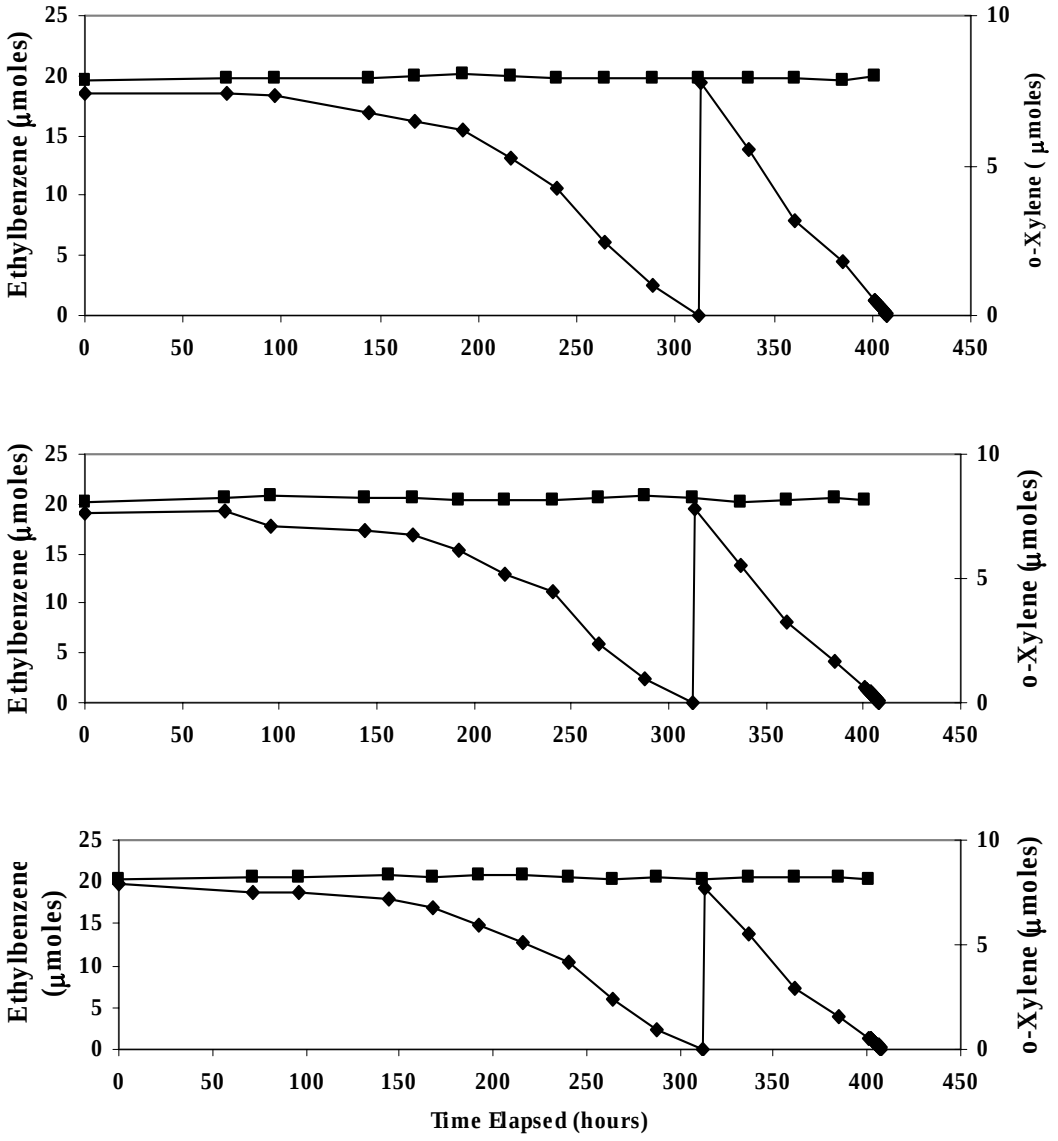

Figure A17: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of o-xylene (■) (2 µL Ethylbenzene and 2 µL o-xylene injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

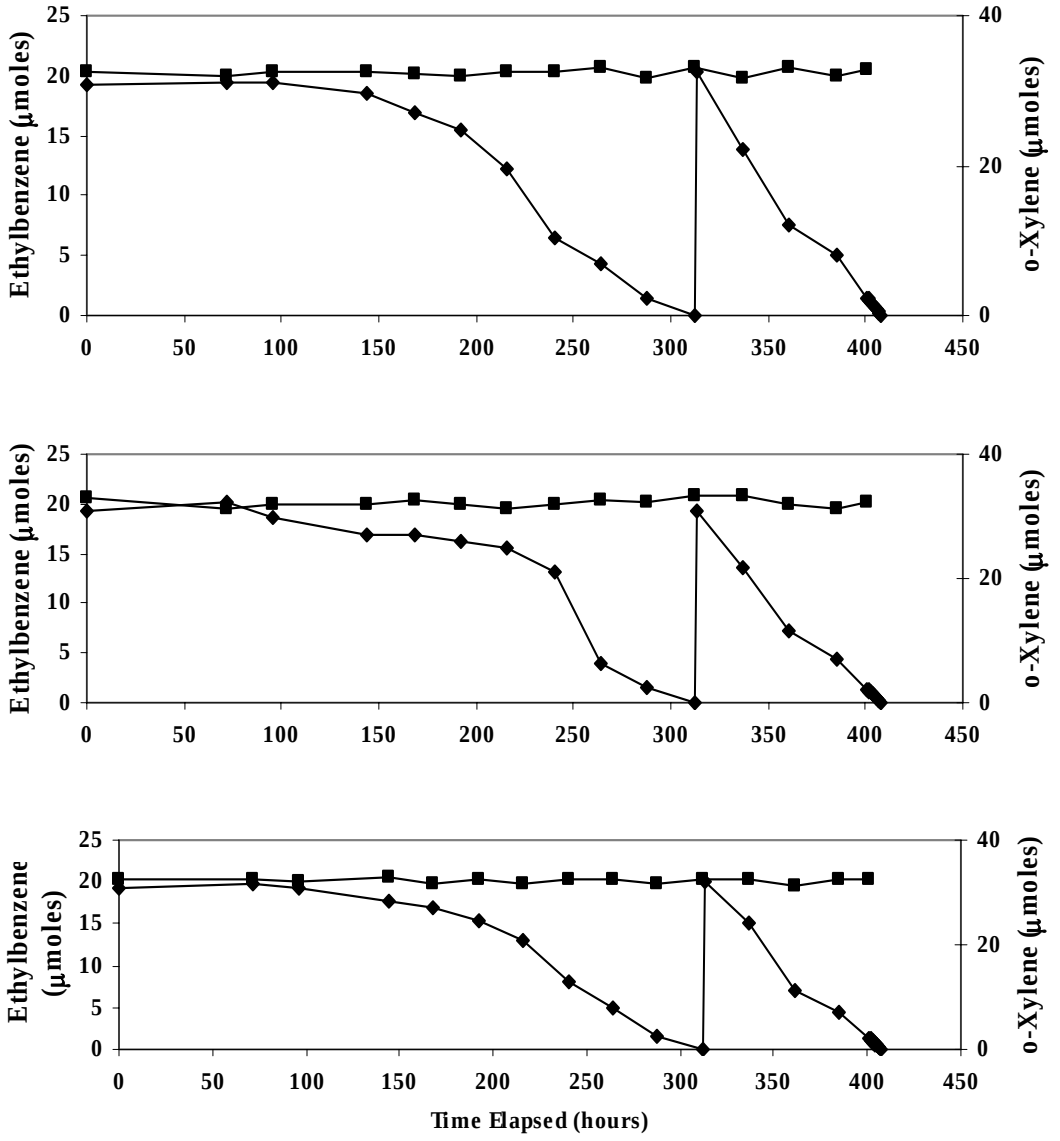

Figure A18: Ethylbenzene (◆) Degradation by *E. lecanii-corni* in the presence of o-xylene (■) (2 µL Ethylbenzene and 4 µL o-xylene injections). Experiment was performed in triplicate. Each graph represents depletion in one bottle.

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Vita

Claudia Kneller Gunsch was born in Sherbrooke, Canada on May 17, 1977, the daughter of Marilyn Rose Kneller and Samuel Kneller. She graduated in July 1994 from Lycée Saint Pierre in Brunoy, France before heading to Purdue University to pursue a Bachelor of Science in Civil Engineering. Upon completion of her degree in May 1998, she moved to South Carolina to work on a Master's of Science in Environmental Engineering and Science at Clemson University. In August 2000, she relocated to Texas to pursue a Doctorate of Philosophy in Civil Engineering at the University of Texas.

Permanent address: 8820 Tiombe Bend, Austin, TX 78749

This dissertation was typed by the author.