Biotechnology and Bioengineering

# Relative Gene Expression Quantification in a Fungal Gas-Phase Biofilter

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Received 15 November 2006; accepted 12 February 2007

Published online 22 February 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21393

**ABSTRACT:** Monitoring of gas-phase biofilter performance generally relies on macroscale measurements that neglect the molecular level phenomena that can control the biodegradation process. The present study was undertaken to determine whether or not quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) could detect changes in relative gene expression resulting from feed variations typically encountered in the field. Specifically, homogentisate-1,2-dioxygenase, *ElHDO*, expression was quantified as a function of short-term chemical feed variations and shutdown period in a biofilter seeded with a pure culture of the fungus *Exophiala lecanii-corni*. *ElHDO* was previously shown to be involved in ethylbenzene degradation in *E. lecanii-corni*. Overall, relative gene target expression numbers ($T_N$) were consistent with gas-phase biofilter performance during each short-term experiment although no direct mathematical correlation was found between $T_N$ and ethylbenzene removal rate. During the chemical feed experiments, no effect on $T_N$ was measured in the presence of *o*-xylene which does not affect *ElHDO* expression. In the presence of phenylacetate, an inducer of *ElHDO*, $T_N$ increased once a threshold substrate concentration was exceeded. When methyl propyl ketone, a repressor of *ElHDO*, was introduced, $T_N$ decreased rapidly and acted as a leading indicator of bioreactor failure. In the transient loading experiments, *ElHDO* expression slowly decreased over a 24-h time period when the ethylbenzene feed was discontinued, but rapidly recovered upon its re-introduction. These results indicate that qRT-PCR reflects microbial activity changes that occur in gas-phase biofilters in response to short-term changes in feed conditions and provides a useful complement to the macroscale measurements typically collected.

Biotechnol. Bioeng. 2007;98: 101–111.

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**KEYWORDS:** biofiltration; qRT-PCR; gene expression quantification; relative quantification; fungi; ethylbenzene

## Introduction

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 for VOC-contaminated waste gas streams (Groenestijn and Hesselink, 1993; Melse and Van der Werf, 2005). In gas-phase 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. Gas-phase biofilters have been used successfully to treat a wide range of contaminated streams (Aizpuru et al., 2001; Arnold et al., 1997; Christen et al., 2002; Li and Moe, 2005; Ortiz et al., 2003; Pol et al., 1994; Woertz et al., 2001).

Because chemical feed conditions in biofilters are generally dynamic, an important area of research is to investigate microbial changes resulting from these dynamic conditions (Jang et al., 2006; Yoon and Park, 2002). In general, contaminant mixtures are degraded in gas-phase biofilters in an order corresponding to the relative ease of biodegradation of each individual compound. For instance, Aizpuru et al. (2001) report that oxygenated compounds are degraded prior to aromatic and halogenated compounds. Similarly, Prado et al. (2004) found that formaldehyde

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Contract grant sponsor: National Science Foundation
Contract grant number: BES #9875881
Contract grant sponsor: Texas Advanced Technology Program
Contract grant number: ATP-314

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degradation is affected by the inlet methanol concentration. This effect is amplified when low moisture content, nutrient levels or pH conditions are also present (Prado et al., 2004). When the feed carbon source is either discontinued or when the carbon load is rapidly increased (i.e., spike in contaminant concentration), a substantial reduction in bacterial biomass activity has been shown to occur. The time required for the biomass to regain full degradation capacity in the case of a shutdown or the ability for the microbial population to degrade the additional substrate, when a feed concentration change occurs, varies for different studies (Deshusses et al., 1995; Groenestijn and Hesselink, 1993; Martin and Loehr, 1996). In general, full degradation capacity can be restored within 2–48 h for shutdown periods less than 2 days (Deshusses et al., 1995; Groenestijn and Hesselink, 1993; Martin and Loehr, 1996). For longer shutdown periods, up to 3 days may be needed to return to full treatment capacity. However, it is unclear if the duration of the re-acclimation period is linked to microbial death or simply to a shift in cellular metabolic activity.

Most biofiltration 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 treatment performance to these bulk parameters (Atoche and Moe, 2004; Auria et al., 1998; Martin and Loehr, 1996; Prado et al., 2004). However, one limitation of bulk measurements is that they do not provide a direct link between treatment performance and parameters describing the actual in situ microbial biodegradation. A recent study conducted by Garcia-Peña et al. (2005) demonstrated that there was good agreement between toluene oxygenase activity and toluene degradation capacity with the fungus *Paecilomyces variotii*. Nonetheless, while a number of methods are under development for use in biofiltration and more broadly in the biological treatment field, relatively few published studies have focused on understanding the relationship between the performance of these engineered biological treatment systems and microbial transcriptional activity (Corredor et al., 2004; Devers et al., 2004; Han and Semrau, 2004; Johnson et al., 2005). Furthermore, most of these studies have not focused on measuring gene expression in gas-phase biofilters under realistic operating conditions (e.g., non-sterile feed with low contaminant concentrations typically found in gas-phase biofilter applications). Rather, most studies have dealt with pure cultures under very controlled conditions which are not representative of actual industrial operation. Thus, it is difficult to determine the utility of such methods for field applications. The main objective of this research was to determine if quantitative gene expression measurements could detect changes in gene expression resulting from feed variations that may be encountered in the field. Specifically, relative gene expression quantification was used to measure how short-term chemical feed fluctuations and shutdowns impact pollutant degradation in a fungal gas-phase biofilter degrading ethylbenzene.

## Materials and Methods

### Biofilter Operation

A biofilter was constructed as shown in Figure 1A and packed with Celite R-635 (Celite Corp., Lompoc, CA) [0.6-cm *D*, 0.8-cm *H* and 20-μm pore size]. The bioreactor consisted of three stainless-steel sections each 15.5 cm I.D. and 34 cm in height. Each section was packed to a depth of 25 cm, leaving a 9-cm plenum between each section. The packing medium was presoaked for 24 h in nutrient basal medium previously described (Gunsch et al., 2005). *Exophiala lecanii-corni* was inoculated into the biofilter 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 as described previously until the cells reached a concentration of $10^9$ as determined by hemacytometer count (Gunsch et al., 2005). The biofilter

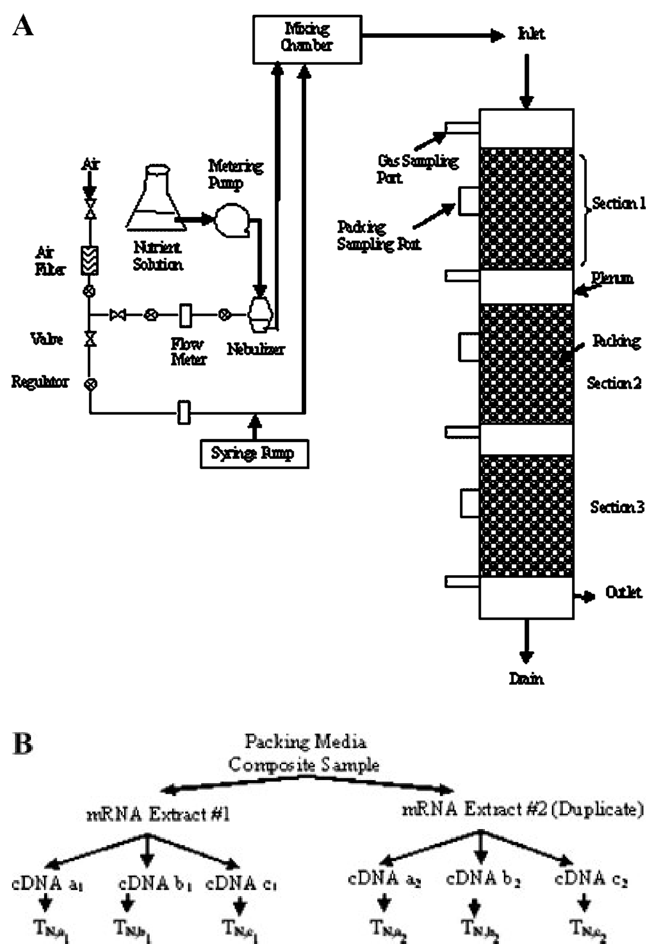


**Figure 1.** **A**: Vapor phase bioreactor system. **B**: Biomass sampling and analysis. Each $T_N$ value represents the ratio of $X_{n,q}$ to $X_{n,cb}$. $X_{n,q}$ represents the *ElHDO* expression number normalized to the *18S rRNA* expression number under a given experimental condition; $X_{n,cb}$ represents the *ElHDO* expression number normalized to the *18S rRNA* expression number at the time zero baseline condition. Details of the mathematical analysis are described in Gunsch et al. (2006).



DOI 10.1002/bit

column retained approximately 90% of the inoculum solution that was poured over the reactor packing media. Samples of the biofilm were collected weekly and transferred to R2A plates (Difco Laboratories, Detroit, MI). The testing confirmed that *E. lecanii-corni* was the dominant species in the gas-phase biofilter throughout this study. No other fungi or bacteria were observed on the plates.

The column was operated at room temperature (20–23°C) in a down-flow fashion. The humidified, synthetic waste gas stream provided to the top of the bioreactor was generated by mixing two separate air streams. The first air stream (building air with 10% relative humidity) 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 air stream to saturation. The second air stream continuously supplied ethylbenzene using a syringe pump (kd Scientific, Model KDS200, Holliston, MA). The two air streams were mixed in a mixing chamber prior to entering the biofilter. In this manner, ethylbenzene was provided continuously to the biofilter column at an inlet concentration of 100 $ppm_v$ throughout the duration of the experiment except when the shutdown experiments were conducted. A total gas flow rate of 14.5 L/min entered the biofilter yielding an empty bed contact time of 1 min. The inlet ethylbenzene mass loading rate to the biofilter remained relatively constant at $26.3 \pm 3.6$ g/($m^3$ h). Negligible leachate amounts ranging from 10 to 20 mL/day were generated throughout the experimental period.

Biofilter operation was divided into a startup phase, which lasted 18 days, followed by a series of chemical feed as well as shutdown experimental phases. The startup phase was defined to be the time period required to achieve steady state and consistently remove 100% of the inlet ethylbenzene loading across the entire biofilter. The biofilter was operated for a total of approximately 70 days. During each short-term chemical feed experiment, a regulatory substrate was provided to the biofilter in addition to ethylbenzene for a period of three days. The concentration of the regulatory compound being investigated was increased every 24-h period (Table I) during each chemical mixture test. For the purpose of this research, regulatory compounds are defined as those which have different effects on the expression of homogentisate-1,2-dioxygenase (*ElHDO*) in the fungus *E. lecanii-corni*. The regulatory substrates which were used in this study are: methyl propyl ketone, *o*-xylene or phenylacetate. These compounds repress, do not affect or induce *ElHDO* expression in batch reactors, respectively (Gunsch et al., 2006). The regulatory substrates were mixed with the ethylbenzene and provided simultaneously to the column using the syringe pump during each substrate mixture experiment.

**Table I.** Conditions during each chemical feed experiment.

| Time period (h) | Target inlet ethylbenzene concentration[a] ($ppm_v$) | Target inlet regulatory substrate concentration[b] ($ppm_v$) |
|---|---|---|
| 0(baseline condition) | 100 | 0 |
| 0–24 | 100 | 50 |
| 24–48 | 100 | 100 |
| 48–72 | 100 | 200 |

[a]Throughout the experimental phase, the measured ethylbenzene concentration was $101.6 \pm 13.5$ $ppm_v$.

[b]The average measured inlet concentrations were within 10% of the target for each regulatory substrate and the standard deviation was 10.6, 6.0 and 9.7 $ppm_v$ for methyl propyl ketone, *o*-xylene and phenylacetate, respectively, over the length of each short-term experiment.

In all cases, ethylbenzene degradation profiles as well as biomass samples in the gas-phase biofilter test section were collected immediately before the introduction of the regulatory substrates and used as the time zero, baseline measurement in our analyses. The mixture experiments were conducted in the following order: (1) methyl propyl ketone and ethylbenzene (days 19–22 of operation); (2) *o*-xylene and ethylbenzene (days 25–28) and; (3) phenylacetate and ethylbenzene (days 32–35). Following each mixture test, the feed of the regulatory substance was discontinued and the biofilter was provided ethylbenzene only (100 $ppm_v$) for a period of 4 days to re-establish steady state, baseline conditions prior to the next experiment. During each shutdown test, the ethylbenzene supply to the column was discontinued for a period of 1 and 7 days. Following the starvation period, *ElHDO* expression was monitored over a period of 3 days upon re-introduction of the chemical feed. Similar to the chemical feed experiments, ethylbenzene and biomass sampling was performed immediately prior to the shutdown. These measurements were used as the time zero baseline values. Moist air was provided using a fine-mist nebulizer during the shutdown periods. In all phases of this research, ethylbenzene at a target steady inlet concentration of 100 $ppm_v$ (measured as $101.6 \pm 13.5$ $ppm_v$) was used as the baseline condition. This concentration was chosen because it is representative of typical industrial inlet concentrations (Elmrini et al., 2001; Groenestijn and Hesselink, 1993).

## Analytical Methods

Biofilter performance was monitored by measuring the inlet and outlet concentration of ethylbenzene and the regulatory compounds for each of the three bioreactor sections. Duplicate gas-phase samples were collected from each section and analyzed by GC/FID as previously described (Gunsch et al., 2006). Packing media samples were collected from each section along the biofilter for biomass and nutrient characterization (Fig. 1B). Biomass was removed from the packing media by placing packing pellets in 10 mL of deionized water, sonicating for 3 min and vortexing for 3 min. Biomass accumulation in each section was monitored by determining the chemical oxygen demand per unit packing weight. Inorganic nitrogen in the form of ammonium ($NH_4^+$), nitrite ($NO_2^-$) and nitrate ($NO_3^-$) was also analyzed in the biofilm to monitor the nutrient levels in

the bioreactor column. $NH_4^+$ and $NO_3^-$ levels were determined using an Orion ammonium/nitrate combination selective probe (Thermo Orion, Beverly, MA) while the $NO_2^-$ level was determined using the diazotization method (Clesceri et al., 1998). 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. Samples were 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 (Thermo Orion, Beverly, MA).

### Gene Expression Analysis

To quantify changes in *ElHDO* expression as a function of regulatory substrate concentration, it was crucial to maintain a constant ethylbenzene loading to the system while systematically varying the regulatory substrate concentration. For this reason, only gene expression and VOC loading data from the inlet section are presented herein because this is the only section where a constant ethylbenzene load is maintained. The loading to the remaining biofilter sections is solely dependent on the ethylbenzene removed in previous sections and this varies substantially depending on the regulatory substrate provided in a given experiment. Similarly, biomass levels in bioreactors tend to stratify with the greatest quantities near the inlet (Song and Kinney, 2000), and this trend was observed in this bioreactor system. To minimize the impact of this variable on gene expression measurements, biomass samples were collected from a relatively narrow segment of the inlet bioreactor [15–20 cm packed bed depth].

Biomass samples collected immediately prior to time zero were used as the baseline measurement for each short-term experiment. 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 I. Composite samples consisting of three packing pellets were collected at regular time intervals (4, 12 and 24 h after the introduction of each regulatory substrate). To minimize the overall disturbance to the biofilm in the bioreactor, only one composite sample consisting of three packing pellets was collected per time point from the sampling port (Fig. 1B). The packing pellets were always obtained from the sampling port located 10 cm from the bottom of the top packed bed section. Each biomass sample consisted of two packing pellets from the middle and one from the side. While some inherent sampling location variation existed from one sampling event to another, the variation due to sampling location did not significantly alter the gene expression levels. The extent of gene expression variation was calculated during a preliminary qRT-PCR experiment conducted with packing pellets collected from another gas-phase biofilter that was setup and run identically to the one used in this study. This preliminary experiment demonstrated that *ElHDO* expression varied by a maximum of ±3.3% depending on the exact sampling location within the packing.

Biomass was extracted by placing the packing pellets in 10 mL of deionized water, sonicating for 3 min and vortexing for 3 min. Each biomass extract from the composite sample was divided into two equal samples and used in separate total RNA extractions for duplicate analysis. Total RNA extraction was performed using RNAqueous® kits (Ambion, Austin, TX). cDNA was synthesized in triplicate from each total RNA sample using reverse transcription reagents from New England Biolabs® Inc. (Beverly, MA). The cDNA was used in subsequent qRT-PCR amplification using an Applied Biosystems 7700 Real Time PCR machine (Foster City, CA) as previously described (Gunsch et al., 2006). Because gene expression from a given sample was found to vary by a maximum of ±2.8% during the preliminary experiment, PCR inhibition and DNA extraction efficiency were assumed to be equal for all samples. *ElHDO* expression was measured by calculating $T_N$, the relative target gene expression number, as calculated in Equation (1):

$$T_N = \frac{X_{N,q}}{X_{N,cb}} = (1+E)^{-\Delta\Delta C_T} \quad (1)$$

(Gunsch et al., 2006) where $X_{N,q}$ is the normalized number of target molecules in the experimental sample q (*ElHDO* transcript number under experimental conditions), $X_{N,cb}$ the normalized number of target molecules in the calibrator sample (*ElHDO* transcript number under baseline conditions), $E$ the PCR efficiency, and $\Delta\Delta C_T$ is the difference between the PCR cycle number when the target (*ElHDO*) and endogenous (*18S rRNA*) molecules reach the threshold fluorescence level in the experimental and calibrator samples (Gunsch et al., 2006). $X_{N,q}$ and $X_{N,cb}$ both include normalization to the *18S rRNA* endogenous control gene (Gunsch et al., 2006). For each treatment condition, the reported $T_N$ value represents the average $T_N$ value obtained for the three cDNA samples from mRNA Extract 1 (Fig. 1B). The cDNA from the replicate experiment (mRNA Extract 2) was used to confirm the reported $T_N$ value. In all cases, this value was within 10% of the reported $T_N$ value. Using the methodology described above, gene expression experimental levels can be compared to baseline gene expression levels. Samples with $T_N$ values $>1$ have gene expression levels higher than the baseline condition, and those with $T_N$ values $<1$ have expression levels lower than the baseline condition. $X_{N,cb}$ was found to be relatively steady from one short-term experiment to another varying by less than 10% throughout the experimental phase. For the purpose of these experiments, the baseline condition is defined as the *ElHDO* expression immediately preceding time zero (beginning of



DOI 10.1002/bit

each chemical feed or shutdown experiment). In all cases, the baseline condition consisted of ethylbenzene at a steady inlet concentration of 100 $ppm_v$ (measured as $101.6 \pm 13.5$ $ppm_v$) into the first gas-phase biofilter section. To determine if a specific $T_N$ value was significantly different from the baseline $T_N$ value, *t*-tests with 95% confidence intervals were used.

## Results and Discussion

### Biofilter Operation

Low ethylbenzene removal rates were observed in the top section during the beginning of the startup phase (5–13% removal). These low removals were subsequently determined to be associated with a biofilm moisture content drop to 12% between days 6 and 9. Once the moisture content was increased by adjusting the air flow into the nebulizer to stabilize the moisture content at levels ranging from 20 to 30%, moisture content no longer limited biofilter performance. Ethylbenzene removal also increased in the top section (to 30–40%) and 100% removal across the biofilter was achieved. The pH in the biofilter leachate remained at approximately 5 throughout the experiment. The pressure drop across the biofilter column increased from 0.05 to 0.1 kPa between the beginning and the end of the experiments. A high level of nitrogen on the order of 4 g N/g COD was observed during the startup phase when biomass levels were low. However, as the biomass levels increased in the biofilter, nitrogen availability stabilized at approximately 0.04 g N/g COD in the first section. Nitrogen concentrations in excess of 0.03 g N/g COD are considered non-limiting in gas-phase biofilters (Song et al., 2003). All available nitrogen detected in the bioreactor was in the form of ammonium. Nitrate levels were below the detection limit (2.3 mg N/kg dry media) throughout the 70-day experiment and nitrite levels were insignificant compared with the ammonium levels. The likely reason most nitrogen was found in the form of ammonium is that nitrogen was supplied as ammonium sulfate.

### Substrate Interactions

Several biofiltration studies have examined substrate interactions and have analyzed the impact of one compound's presence on the other's degradation potential (Chang et al., 2001; du Plessis et al., 2001; Strauss et al., 2004; Zhang et al., 1995). These analyses, however, have not looked at how the expression of a specific gene is modified in response to the introduction of another compound in the mixture in a gas-phase biofilter. In the current study, the effect of substrate mixtures on *ElHDO* expression was quantified. Overall, the biofilter gene expression results are consistent with trends observed in a batch reactor study previously described (Gunsch et al., 2006). As in those batch studies, increased *ElHDO* expression was observed in the presence of phenylacetate, whereas no effect on $T_N$ was observed with *o*-xylene and a decrease in *ElHDO* expression was observed when methyl propyl ketone was present.

#### *Ethylbenzene and Methyl Propyl Ketone*

Low levels of methyl propyl ketone (50 $ppm_v$) did not appear to significantly impact overall ethylbenzene removal rates (Fig. 2A) when compared to the baseline measurement at time zero when no methyl propyl ketone was present. However, as the concentration of methyl propyl ketone increased, a delayed effect was observed. Overall, the removal of ethylbenzene in the first section decreased by 41% in the presence of 100 $ppm_v$ methyl propyl ketone and by an additional 36% in the presence of 200 $ppm_v$ methyl propyl ketone, 24 h after the second loading change. This decrease was not observed immediately. A delay of more than 12 h was observed after the introduction of 100 and 200 $ppm_v$ methyl propyl ketone in the biofilter feed prior to the decrease in ethylbenzene removal rate. Methyl propyl ketone was completely removed in the top gas-phase biofilter section at the lowest loading and partially removed at the two highest loadings (Fig. 2B). Interestingly, at the microscale, the $T_N$ values dropped off immediately upon the introduction of even low levels of methyl propyl ketone (Fig. 2A). Average *ElHDO* expression decreased by 73% during the first 24 h of operation and then by another 65% four hours after the next incremental increase in the methyl propyl ketone feeding rate. Beyond that point (i.e., for the 200 $ppm_v$ case), however, it becomes difficult to draw any direct conclusions from the $T_N$ values because the values are statistically not significantly different ($\alpha = 0.05$). The $T_N$ values in the biofilter remained suppressed (and relatively constant if one considers the error bars) throughout the remainder of the methyl propyl ketone experiment.

Responses were observed more rapidly at the microscale (i.e., gene expression) than the macroscale (i.e., ethylbenzene removal rate) and, thus, indicate that the $T_N$ values act as a leading indicator in the case of down-regulation with the potential to predict the imminent failure of a biofilter. The routine measurement of gene expression in biofilters may be a useful complement to VOC removal data. However, for these data to be useful in industry, real-time automated monitoring tools will need to be developed. The $T_N$ values can function as a leading indicator due, in large part, to the fact that $T_N$ values are directly related to the transcription control (regulation of mRNA synthesis) rather than translational control (regulation of protein synthesis) (Brown and Johnson, 2001). Gene expression in eukaryotic systems involves a number of sequential steps including transcription, RNA processing, transport, translation and mRNA turnover (Brown and Johnson, 2001; Cao and Parker, 2001; Ryeom et al., 2003). 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

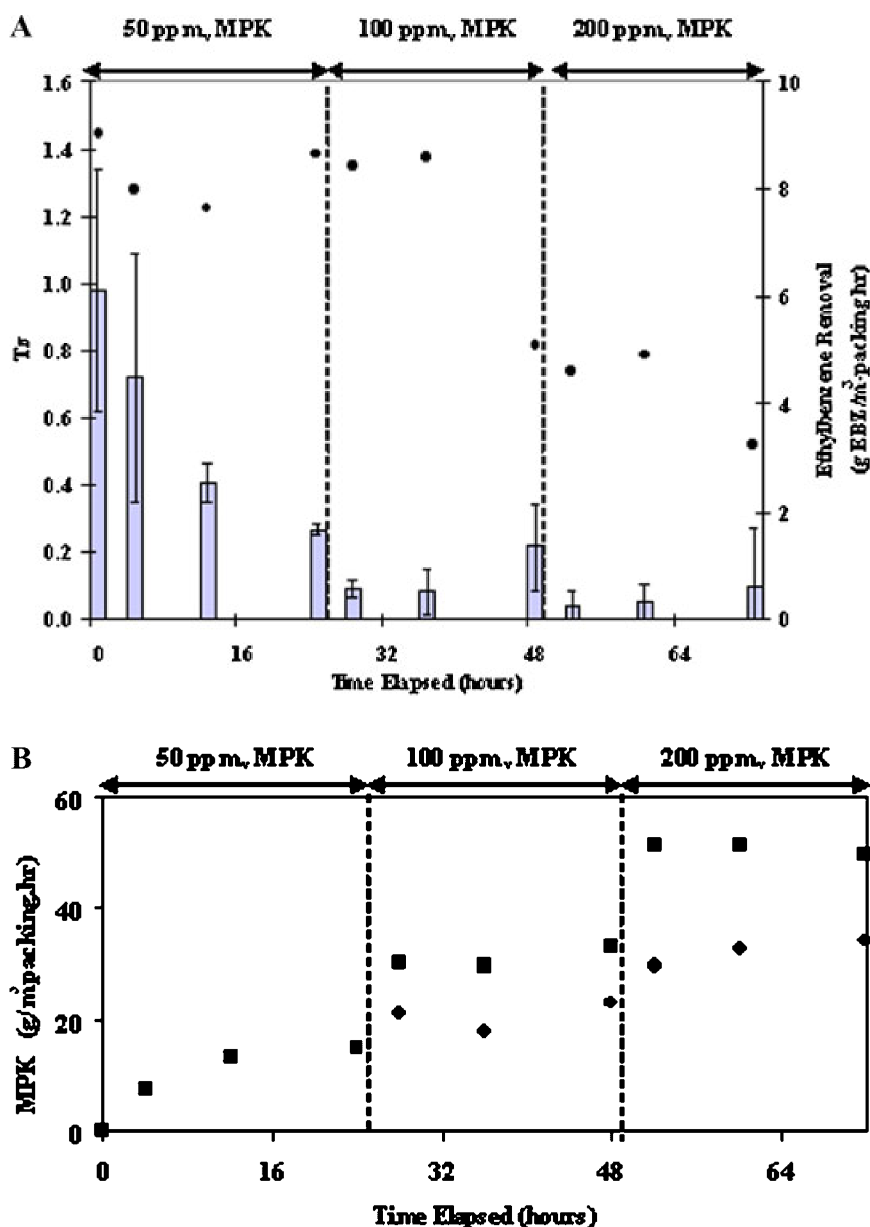


**Figure 2.** **A**: Effect of methyl propyl ketone (MPK) on ethylbenzene (EBZ) degradation in the inlet biofilter section: ethylbenzene removal (●) and relative target expression number (bars). Samples collected immediately prior to MPK introduction were used to define the baseline *ElHDO* expression and EBZ removal levels. Error bars represent one standard deviation. **B**: Methyl propyl ketone loading (■) and removal (◆) in the inlet biofilter section. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

may not be instantaneously correlated but rather correlated over a period of time. The time period will vary dependent on the biological and genetic system studied. Although the repression effect caused by the introduction of methyl propyl ketone into the biofilter 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.

### *Ethylbenzene and* o*-Xylene*

The introduction of *o*-xylene into the biofilter feed had little to no effect on $T_N$ values as compared to the baseline level at the beginning of the experiment (Fig. 3A) even though some *o*-xylene removal was observed in the biofilter (Fig. 3B). Ethylbenzene removal varied during this experiment mostly due to fluctuations in the inlet concentrations. However the fluctuations had little to no effect on $T_N$ values and the $T_N$ values were not statistically significantly different from the baseline (time zero) $T_N$ value (Fig. 3A). Average $T_N$ values



DOI 10.1002/bit

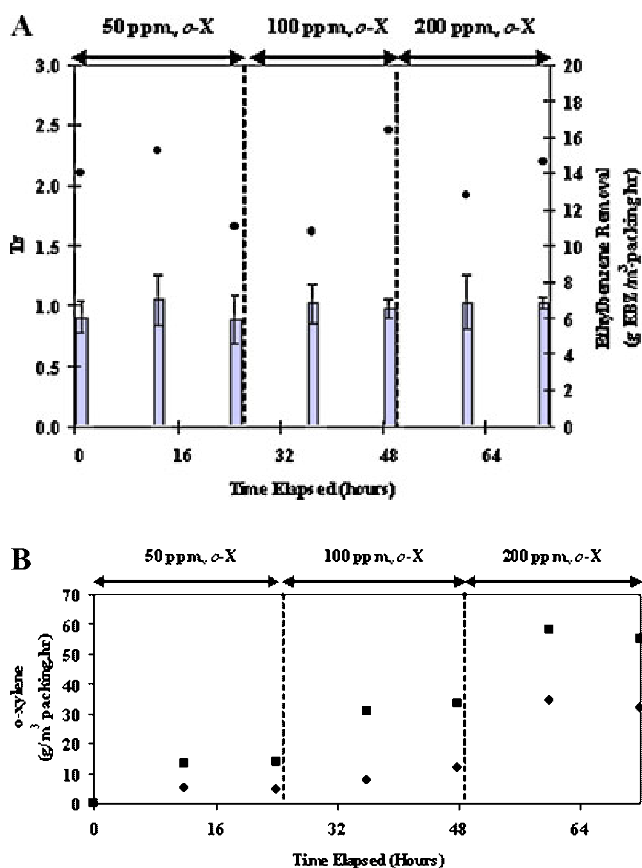


**Figure 3.** **A**: Effect of *o*-xylene (*o*-X) on ethylbenzene (EBZ) degradation in the inlet biofilter section: ethylbenzene removal (●) and relative target expression number (bars). Samples collected immediately prior to *o*-X introduction were used to define the baseline *ElHDO* expression and EBZ removal levels. Error bars represent one standard deviation. **B**: *o*-Xylene loading (■) and removal (◆) in the inlet biofilter section. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

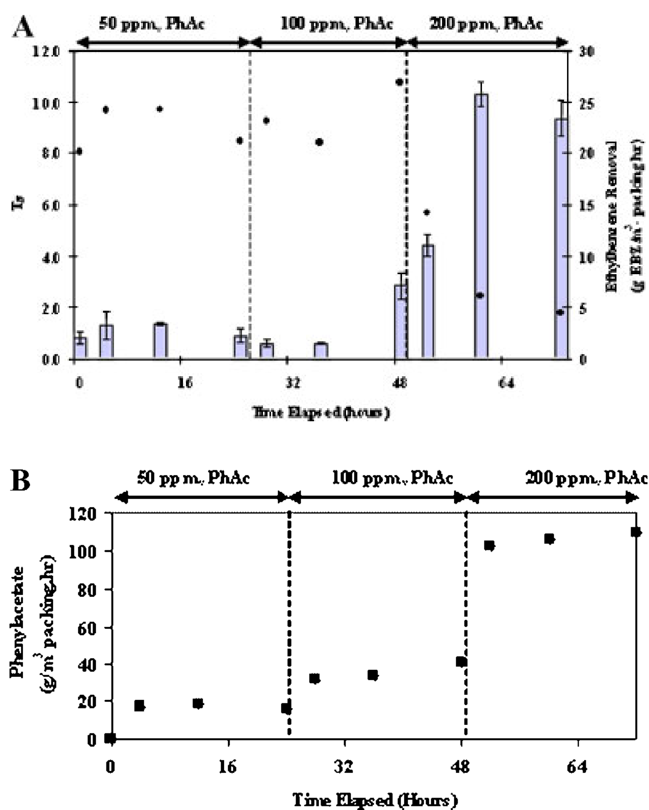


**Figure 4.** **A**: Effect of phenylacetate (PhAc) on ethylbenzene (EBZ) degradation in the inlet biofilter section: ethylbenzene removal (●) and relative target expression number (bars). Samples collected immediately prior to PhAc introduction were used to define the baseline *ElHDO* expression and EBZ removal levels. Error bars represent one standard deviation. **B**: Phenylacetate loading (■) and removal (●) in the inlet biofilter section. Loading and removal are both shown. However, removal data points cannot be seen because they are superimposed with loading data points. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

oscillated between 0.89 and 1.03 implying that the gene expression levels under these conditions are the same as under the baseline condition. This result was expected since our previous results showed that *o*-xylene does not affect *ElHDO* expression in batch reactors (Gunsch et al., 2006).

### *Ethylbenzene and Phenylacetate*

$T_N$ values remained approximately constant at their baseline, time zero level until 48 h after phenylacetate was introduced into the feed (i.e., $T_N$ close to 1.0). In all cases, phenylacetate was fully removed (Fig. 4B). The response to the 100 ppm$_v$ phenylacetate loading was delayed as compared to the methyl propyl ketone response. The effect on $T_N$ was observed 24 h after the second loading increase in the presence of phenylacetate (Fig. 4A) as compared to 4 h in the presence of methyl propyl ketone (Fig. 2A). Over the same time period, ethylbenzene removal rates remained relatively constant (Fig. 4A). The evidence indicates that, for phenylacetate-influenced *ElHDO* $T_N$ values, a certain threshold level exists. This threshold effect has been previously reported for other substrates and is generally thought to be the result of a protein mediated regulatory mechanism (Ryeom et al., 2003; van Hoof and Parker, 2002).

During the last phase, at the highest phenylacetate loading, the expression increased almost 10-fold from that at the beginning of the experiment (Fig. 4A). However, the ethylbenzene removal simultaneously decreased sharply. The most likely explanation for the decrease in ethylbenzene consumption is a result of catabolite repression. In fact, transcriptional level catabolite repression has been observed in several fungal systems (Scherens et al., 2006; Tate et al., 2006; Usaite et al., 2006). Because phenylacetate is a likely intermediate in the ethylbenzene degradation pathway in *E. lecanii-corni* (Gunsch et al., 2005), hypothetically, cells would have to expend less energy to degrade phenylacetate as compared to ethylbenzene (phenylacetate is the fourth intermediate in the proposed ethylbenzene degradation pathway). Therefore, it is likely

that if cells were already degrading the substrates as efficiently as possible and two substrates were available, then they would continue to degrade phenylacetate, thereby leaving the ethylbenzene untouched. Repeating this experiment with an *ElHDO* inducer which is not in the same pathway as the primary substrate (i.e., ethylbenzene) may provide some additional insights as to the utility of gene expression quantification in biofilters under up-regulation conditions. This additional test would be useful for determining the relative contributions of both the inducer and the primary substrate to the overall gene expression.

## Shutdown/Startup Tests

Although biofilters have been shown to be reliable for removing a variety of organic compounds, their long-term performance can still be unreliable especially when they are subjected to dynamic loading conditions. The time required for the biomass to regain full degradation capacity in the case of a shutdown or the ability for the microbial population to degrade the additional substrate, when a feed concentration change occurs, varies with each study depending on the experimental setup, the microbial population, the length of shutdown and the type of substrate (Dickinson and Schweizer, 1999; Groenestijn and Hesselink, 1993; Martin and Loehr, 1996;). The time needed to return to pre-shutdown degradation levels is generally measured by contaminant degradation profiles rather than microbial activity measurements and thus, does not provide any indication as to the nature of the microbial recovery (i.e., new biomass growth or transcriptional control). In the current study, we hypothesized that transcriptional control rather than biomass death was occurring. As such, 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 re-introduction of ethylbenzene in the biofilter feed. Results obtained for the 1-day and 7-day shutdown experiments follow this trend.

During the 1-day shutdown experiment, *ElHDO* expression gradually decreased over the 24-h period following the removal of ethylbenzene from the inlet air stream (Fig. 5A). The expression decreased by 32, 72 and 93% in the first 4, 12 and 24 h, respectively. Upon re-introduction of ethylbenzene, the gene expression levels recovered to pre-shutdown levels within 4 h and remained at approximately the same level over the subsequent 3-day monitoring period. During the 7-day shutdown experiment, a similar trend was observed. *ElHDO* expression gradually decreased once the ethylbenzene supply was shut off (Fig. 5B). Expression levels were 54, 70 and 80% lower after 4, 12 and 24 h, respectively. Upon re-introduction of the carbon source, the expression level returned to pre-shutdown values within 4 h and remained at those levels. Overall, when comparing *ElHDO* expression levels after restart between the 1- and 7-day shutdown experiments, $T_N$ values were not statistically significantly ($\alpha = 0.05$). In both

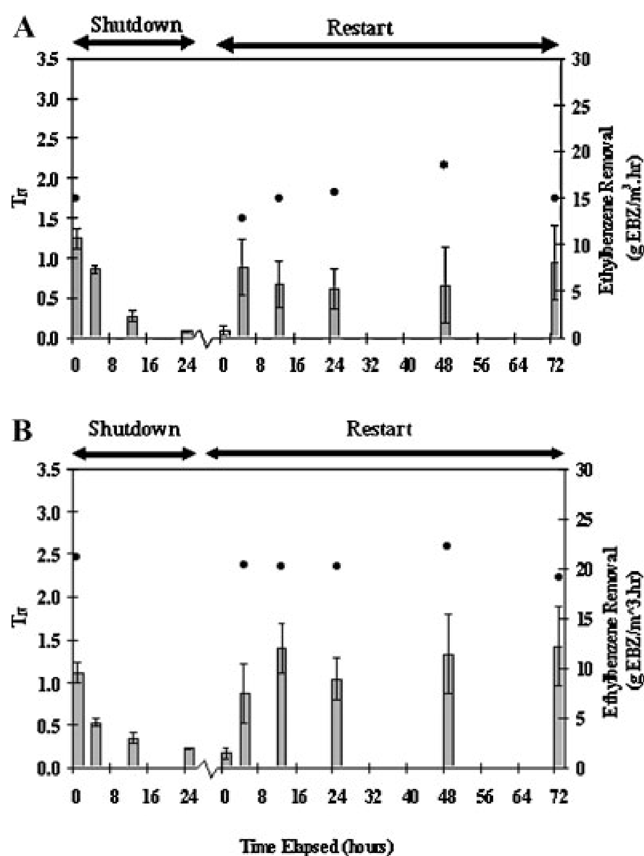


**Figure 5.** **A**: Effect of shutdown on ethylbenzene degradation in the inlet biofilter section[0] during the 1-day shutdown experiment. Ethylbenzene removal (●) and relative target expression number (bars). Samples collected immediately prior to shutdown were used to define the baseline *ElHDO* expression and EBZ removal levels. Error bars represent one standard deviation. **B**: Effect of shutdown on ethylbenzene degradation in the inlet biofilter section[0] during the 7-day shutdown experiment. Ethylbenzene removal (●) and relative target expression number (bars). Samples collected immediately prior to shutdown were used to define the baseline *ElHDO* expression and EBZ removal levels. Gene expression was monitored for the first 24 h following shutdown and 72 h following startup. Error bars represent one standard deviation.

cases, the results at the macro- and micro-scale indicated a similar pattern in that ethylbenzene removal recovered within four hours after column restart as did the *ElHDO* expression levels. These data indicate that qRT-PCR is sensitive enough to analyze biofilm gene expression patterns and suggests that, in this case, degradation recovery is linked to gene expression and, thus, transcriptionally controlled.

Fungal recovery periods after carbon starvation are significantly shorter than those observed in bacterial systems (Jin et al., 2007; Woertz et al., 2001). In this research, a 4-h time window was sufficient to regain full degradation capacity regardless of the length of starvation. Other biofiltration research in bacterial systems has shown that 9–48 h have been required to allow the microbial population to recover (Groenestijn and Hesselink, 1993; Martin and Loehr, 1996). The faster recovery of gene expression in the case of *E. lecanii-corni* is consistent with previous published



DOI 10.1002/bit

results (Woertz et al., 2001). Fungal cells have been shown to be resilient to starvation and adverse environmental conditions (e.g., survival over wide ranges of pH and moisture content), thus demonstrating their high adaptability potential (Cox et al., 1996; Woertz et al., 2001). This may be due to the greater amounts of endogenous reserves stored by fungi as compared to bacteria (Johnson et al., 2005; Thomsson et al., 2005).

### Limitations of Relative Gene Expression Measurements

While relative gene expression quantification may be useful in some instances such as for detecting down-regulation of a biodegradation gene in response to changes in environmental conditions, this tool has several limitations, particularly when applied to gas-phase biofilters operating under field conditions. First, relative gene expression quantification requires prior knowledge of the genetic sequences relevant to a specific biodegradation pathway. As more and more microorganisms become sequenced, this barrier will be reduced. However, until more sequences are known, this method is most likely to be used as a supplemental measure of biofiltration performance to complement the traditional macroscale measurements.

An important difference between the present study and the majority of other published studies that quantified gene expression in natural and engineered environmental systems is that relative rather than absolute quantification was used (Devers et al., 2004; Han and Semrau, 2004; Holmes et al., 2004; Johnson et al., 2005; Lee et al., 2006). Relative quantification does not require the use of standard curves and as such might be easier to perform (Livak and Schmittgen, 2001). This becomes an especially important factor when developing tools which could eventually be common practice in industry and be performed by relatively untrained personnel. However, caution should be exercised when selecting relative over absolute quantification since it cannot be universally applied. Care must be taken to select appropriate endogenous and target genes with similar PCR efficiencies, as well as an endogenous gene which is not affected by experimental treatments (Livak and Schmittgen, 2001).

The interpretation of relative gene expression data becomes limited when dealing with down regulated genes if very accurate quantification is needed because of the low sensitivity at small $T_N$ values ($<0.25$). Even though the standard deviations obtained in this study are consistent with other published results obtained using the comparative method (Schmittgen and Zakrajsek, 2000; Semighini et al., 2002), these standard deviations may be too large in some instances to understand gene expression patterns for down regulated genes. For up-regulated genes, 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, the same standard deviation (i.e., 0.2–0.5) becomes very large in a statistical sense. Therefore, although 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. This case was encountered when methyl propyl ketone was introduced into the bioreactor. Under these conditions, the macroscale level measurement (although delayed) is a better indicator of change. Thus, it is likely that relative gene expression quantification should be used to supplement information gained from macroscale measurements.

The final limitation identified in this study was that although ethylbenzene removal rate and $T_N$ values were generally found to be linked in short-term experiments (Figures. 2A,3A and 4A), no direct relationship was apparent. In most cases there was a lag between changes in $T_N$ and ethylbenzene removal rate. In some cases, when $T_N$ went down, the ethylbenzene removal rate did not decrease until 24 h later (Fig. 2A). Our result is similar to that obtained by Lee et al. (2006) who examined reductive dehalogenase expression. In that study, there also was no apparent direct relationship between dehalogenase transcript numbers and rates of dechlorination activity. Thus, it is likely that additional factors other than mRNA transcript numbers play an important role in controlling substrate utilization rates. Nonetheless, because of the inherent complexities of a gas-phase biofilter system operating in the field, it is probable that quantitatively linking gene expression to pollutant removal rates will remain a challenge. However, if a mathematical correlation could be established between transcript numbers and substrate utilization rates, gene expression information could be incorporated into predictive performance models for gas-phase biofilters.

## Conclusions

The main conclusions which may be drawn from this research are:

- Relative gene expression changes can be detected in response to short-term changes in chemical feed conditions at contaminant concentrations that may be encountered in the field.
- Gene expression can act as a leading indicator of gas-phase biofilter failure under short-term down-regulation conditions.
- Relative gene expression quantification of small down-regulation events is limited by the sensitivity of the qRT-PCR measurement. Thus, it is difficult to assess relative gene expression changes when $T_N$ values are small ($<0.25$).
- The relationship between $T_N$ and removal rate in experimental gas-phase biofilters is complex. No direct

correlation was found between substrate utilization rate and relative gene expression levels in the biofilm during the short-term experiments. Although the $T_N$ and ethylbenzene removal rate trends followed expected results based on previous batch reactor studies, the responses were not immediate and varying lag periods were observed.

This material is based upon work supported by the National Science Foundation Grant No. 9875881 and a grant from the Texas Advanced Technology Program (ATP-314). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or the Advanced Technology Program. Dr. Gunsch would like to thank the National Science Foundation for supporting her research through a Graduate Research Fellowship and Katy Moreira for helping with sample analysis.

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