

Quantification of homogentisate-1,2-dioxygenase expression in a fungus degrading ethylbenzene

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Abstract

A quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) assay was utilized to quantify the expression of *EIHDO* in the fungus *Exophiala lecanii-corni* during the biodegradation of ethylbenzene and other volatile organic pollutants. The assay was applied to measure the impact of pollutant mixtures on *EIHDO* expression relative to that of a housekeeping gene (*18S rRNA*). Three compounds were tested in mixtures with ethylbenzene: methyl propyl ketone, phenylacetate and *o*-xylene. These chemicals repressed, induced, or had no effect on ethylbenzene degradation, respectively. The results demonstrate that the gene target expression value (T_N) is a useful parameter for evaluating the effect of pollutant mixtures on gene expression. T_N was found to reflect macroscopic changes in ethylbenzene utilization rates although these two parameters were not related in a linear fashion for all compounds. The assay was log-linear over 5 orders of magnitude of RNA concentration and reproducible between samples (the largest T_N standard deviation was 20%). The comparative qRT-PCR assay used in this research represents a viable alternative to absolute quantification methods to monitor in situ fungal gene expression in natural and engineered environmental systems.

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1. Introduction

Recent application of real time polymerase chain reaction (PCR) to environmental engineering systems has led to a better understanding of the distribution of

microbial populations in the environment (Dionisi et al., 2002; Mesearch et al., 2004). While these studies have demonstrated the potential of this technology for quantifying key microbial populations, one major disadvantage is that they quantify information at the DNA level only rather than at the RNA level. As a result, these studies do not provide any direct information about the relative microbial activity of specific microorganisms present in environmental samples. In contrast, a combination of reverse transcription (RT) and real time PCR

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can be used to monitor the abundance of in situ mRNA molecules and thus provide an assessment of specific cellular activity occurring in a given environmental system. RT-PCR has become increasingly popular to monitor in situ gene expression because of its high sensitivity and specificity (Meckenstock et al., 1998; Wilson et al., 1999). However, most environmental studies conducted to date have relied on semi-quantitative techniques to monitor gene expression changes even though several quantitative RT-PCR (qRT-PCR) techniques have been developed for clinical research (Révillion et al., 1997; Latil et al., 2003).

The few published environmental studies which make use of qRT-PCR have mostly focused on bacterial systems (Han and Semrau, 2004; Devers et al., 2004) despite the importance of fungal species in the environment. Fungi have been successfully used in groundwater bioremediation applications (Fournier et al., 2004) and have been shown to be capable of degrading a wide range of volatile organic pollutants (Woertz et al., 2001). *Exophiala lecanii-corni*, a dimorphic fungus, is of particular interest because of its ability to degrade a variety of volatile organic compounds including toluene, ethylbenzene and methyl propyl ketone (Woertz et al., 2001; Qi et al., 2002). *E. lecanii-corni* is capable of removing toluene loadings 2–7 times higher than similar bacterial systems, and is more resilient to adverse treatment conditions such as low pH and nitrogen limitations (Woertz et al., 2001).

Waste streams requiring treatment often contain a mixture of organic pollutants. For instance, a biofilter designed to control emissions from a paint manufacturing facility may need to simultaneously remove at least 12 different volatile organic compounds including ethylbenzene. Because enzymes are ultimately responsible for contaminant degradation, it would be useful to investigate the link between macroscale biodegradation rates and microscale gene expression levels for each substrate in a chemical mixture. The *E. lecanii-corni* homogentisate-1,2-dioxygenase gene (*EIHD0*) was previously shown to be up-regulated in the presence of ethylbenzene and may be responsible for the ring cleavage step in the degradation pathway (Gunsch et al., 2005). Here, we describe an assay that combines a comparative quantification method with real time RT-PCR to monitor fungal gene expression patterns in environmental systems. The overall goal of this research was to determine if the relative target gene expression value (T_N) could be used as a simple measure to track expression changes as a function of ethylbenzene degradation of a target gene (*EIHD0*) relative to that of a housekeeping gene (*18S rRNA*). We report on

the use of a real time qRT-PCR method targeted for the quantification of changes in fungal gene expression levels in batch reactors as a result of interactions between a primary substrate (ethylbenzene) and secondary compounds.

2. Materials and methods

2.1. Strains, growth conditions and RNA extraction

E. lecanii-corni (CBS 102400) was used as the parent strain for all experiments. Four different sets of batch reactors were prepared using the setup and inoculation methods previously described in Gunsch et al. (2005). The substrate treatment conditions examined included: 1) ethylbenzene alone; 2) ethylbenzene with methyl propyl ketone; 3) ethylbenzene with *o*-xylene and; 4) ethylbenzene with phenylacetate. For each treatment condition, batch reactors were prepared in triplicate. To ensure that compound depletion was not due to abiotic losses, one triplicate set of killed controls was prepared for each substrate treatment condition.

For the ethylbenzene alone condition, ethylbenzene was added to each reactor at 20 μ M (liquid phase concentration). For each substrate mixture condition, ethylbenzene was added to each reactor at 20 μ M (liquid phase concentration) along with 30 μ M (liquid phase concentration) of each secondary compound (methyl propyl ketone, *o*-xylene and phenylacetate). Once the first 20 μ M dose of ethylbenzene was consumed (as determined by gas chromatography), a second 20 μ M aliquot was provided, and cells were harvested 24 h later. In all cases, both ethylbenzene and the secondary compound(s) were still present in the reactor at harvesting time. In the case of the methyl propyl ketone, the aforementioned protocol was modified because a diauxic degradation pattern was observed (i.e. methyl propyl ketone was entirely consumed prior to ethylbenzene consumption). In that case, methyl propyl ketone was added at 30 μ M (liquid phase concentration) aliquots at the same time as the second ethylbenzene aliquot.

The entire cell suspension (20 mL) in each of the three batch reactors for each treatment condition was equally divided into two centrifuge tubes. Cells were pelleted by centrifugation and supernatant removed by pipetting. Cells were disrupted by freezing/grinding in liquid nitrogen for 1 min prior to RNA extraction. Total RNA was extracted from each reactor sample using RNAqueous kits (Ambion, Austin, TX). The final elution volume used was 40 μ L. RNA concentration and quality were verified by gel electrophoresis and

spectrophotometrically using a standard conversion factor of one absorbance unit at 260 nm per 40 µg RNA/mL (Ausubel et al., 1989). Only samples with A_{260}/A_{280} ratio greater than 1.8 were used. In this way, total RNA was extracted from each of the three batch reactors at each treatment condition. Total RNA concentration varied from 0.3 to 0.5 µg/µL amongst all samples. RNA concentration was adjusted by dilution with sterile DI water prior to cDNA synthesis. The amount of total RNA used for cDNA synthesis varied from 0.25 pg to 1 µg for the standards and was fixed at 250 ng for all other experiments. RNA samples were stored at -80°C .

2.2. DNase treatment and first-strand cDNA synthesis

The efficiency of DNA removal during RNA extraction was evaluated by comparing PCR yields from samples subjected to Ambion's DNA-free™ reagents and samples not receiving this treatment. cDNA was synthesized in triplicate for each total RNA sample using reverse transcription reagents from New England Biolabs® Inc. (Beverly, MA). Since biomass and total RNA were extracted independently from each of the three separate batch reactors and the cDNA synthesis reactions were performed in triplicate from each total RNA extract, a total of 9 cDNA synthesis reactions were performed for each treatment condition. The following mixture was used for the cDNA synthesis step: 0.25 pg–1 µg total RNA, 1.875 µM dN₉, 2.5 mM dNTPs, 2 µL of 10× reverse transcriptase buffer, 10 units RNase inhibitor and 25 units M-MuLV reverse transcriptase. The reaction was carried out in a 20 µL volume using a MWG Biotech PCR Thermal Cycler (High Point, NC). Samples were later diluted to 50 µL using sterile water and stored at -20°C .

2.3. Real time PCR

In order to quantitatively measure the expression of the *EIHDO* gene, amplification of an endogenous control gene

(i.e., one involved in “housekeeping”) was performed simultaneously, and the relative expression level between *EIHDO* and the endogenous control gene was assessed. The *18S rRNA* was used as the housekeeping gene in this assay. The primer/probe combinations were designed by Applied Biosystems (Foster City, CA) through their Assays-by-DesignSM service. Probes were synthesized using 6-carboxyfluorescein [6-FAMTM] and 6-carboxy-tetra-methyl-rhodamine [TAMRA] labels at the 5' and 3' ends, respectively. The primer/probe combinations are shown in Table 1. An Applied Biosystem 7700 Real Time PCR apparatus (Foster City, CA) was used to perform the quantitative expression study. Each sample consisted of 18 µL from the cDNA reaction described above, 20 µL 2× Taqman® Universal PCR Master Mix and 2 µL of 20× primer/probe mixture. The reaction protocol consisted of one cycle at 50 °C for 2 min, followed by one cycle at 95 °C for 5 min, followed by 40 cycles of a 15-s denaturation step at 95 °C followed by a 1-min annealing/extension step at 60 °C. Each amplification reaction was setup in triplicate. The following three negative control reactions were carried out with each set of samples analyzed: (1) no RNA-template but reverse transcriptase and polymerase provided; (2) RNA and polymerase provided but no reverse transcriptase; and (3) RNA and reverse transcriptase provided but no polymerase.

2.4. Specific ethylbenzene utilization rates

Three secondary compounds (phenylacetate, methyl propyl ketone and *o*-xylene) with varying regulatory effects on the ethylbenzene utilization rate were identified for this study. For the purposes of this research, regulatory compounds are defined as those chemicals which affect the ethylbenzene utilization rate (mass ethylbenzene consumed per unit time per unit biomass weight). Reactor setup and inoculation methods were previously described in Gunsch et al., 2005. All chemicals were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). Each secondary compound was tested in triplicate batch reactors at three different initial concentrations (10, 20 and

Table 1
Primer/probe combinations used for real time qRT-PCR assay

Description	Sequence
<i>18S rRNA</i> forward primer sequence	5'-CGGCGATGGTTCATTCAAATTTCTG-3'
<i>18S rRNA</i> reverse primer sequence	5'-CCTTGGATGTGGTAGCCGTTT-3'
<i>18S rRNA</i> probe	5'-CTCAGGCTCCCTCTCCG-3'
<i>EIHDO</i> forward primer sequence	5'-GGTGTGTGCGACTGTTTGG-3'
<i>EIHDO</i> reverse primer sequence	5'-TTCATCGAAGACCACGAGAATACC-3'
<i>EIHDO</i> probe	5'-CTCTACGGCAAGTTTG-3'

40 μM liquid phase concentration) in the presence of 20 μM ethylbenzene (liquid phase concentration). A similar harvesting scheme was used as described earlier. Cells were harvested 24 h after a second 20 μM ethylbenzene addition. The repressor compound (methyl propyl ketone) was added in 10, 20 or 40 μM (liquid phase concentration) aliquots at the same time as the second ethylbenzene aliquot. Specific utilization rates (SURs) for ethylbenzene were determined for each condition using Eq. (1).

$$\text{SUR} = \frac{\text{Cumulative Mass of Ethylbenzene Consumed (g)}}{(\text{Active Degradation Period (h)} \times \text{Cell Mass (g dry weight)})} \quad (1)$$

The active degradation period represents the amount of time fungal cells were actively degrading ethylbenzene as determined by gas chromatography.

2.5. Analytical methods and dry weight measurement

The depletion of ethylbenzene and the secondary compounds (phenylacetate, methyl propyl ketone and *o*-xylene) was monitored by gas chromatography as described in Gunsch et al. (2005). The biomass produced in each incubation bottle at the end of the experiment was determined by filtering each culture by vacuum through dried Whatman filter paper (0.2 μm pore size), placing the filter paper in aluminum planchettes and drying at 105 $^{\circ}\text{C}$ for 4 h. Filters and planchettes were cooled to room temperature in a desiccator and weighed on a four-place balance.

2.6. Relative quantification

In order to quantify *EIHDO* gene expression, the comparative threshold cycle (C_T) method was used for relative quantification of the target gene of interest (*EIHDO*) with respect to an endogenous control (*18S rRNA*) (Livak and Schmittgen, 2001). Using this method, the normalized quantity of target molecules present in a sample (X_N) can be calculated. In the qRT-PCR technique, the number of cycles required to reach a threshold fluorescence level for the endogenous control gene and the target gene of interest can be determined and used to determine X_N for a particular sample.

$$X_N = K \frac{(1 + E_R)^{C_{T,R}}}{(1 + E_X)^{C_{T,X}}} \quad (2)$$

Where: E_X and E_R are the PCR efficiencies for the target and the endogenous molecules, $C_{T,X}$ and $C_{T,R}$ are the

number of PCR cycles at which the target and endogenous molecules reach their threshold fluorescence level and K is a constant, respectively.

The PCR efficiencies for the target and the endogenous molecules are calculated using Eq. (3):

$$E = (10^{-1/s} - 1) \quad (3)$$

where E and s represent PCR efficiency and the standard curve slope (Fig. 1A and B), respectively. Efficiencies of 0.99 ± 0.01 and 0.98 ± 0.02 were obtained for *18S rRNA* and *EIHDO*, respectively. Because E_X and E_R are approximately equal, these efficiencies will simply be referred to as E .

A comparison of X_N values between a baseline or calibrator condition (i.e., where a single substrate is present) and a different treatment condition (i.e., when multiple chemicals are present) can be accomplished by dividing the normalized amount of target molecules (X_N) for any sample q by the X_N for the calibrator sample (cb) as shown in Eq. (4). In this study, the calibrator sample was chosen as one that represented *EIHDO* expression levels at the baseline treatment condition (i.e., when ethylbenzene is provided as the sole substrate).

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K(1 + E)^{-\Delta C_{T,q}}}{K(1 + E)^{-\Delta C_{T,cb}}} \quad (4)$$

where $X_{N,q}$ is the normalized number of target molecules at a given treatment condition, $X_{N,cb}$ the normalized number of target molecules at the baseline conditions, K is a constant, and $\Delta C_{T,q}$ is equal to the difference in the threshold cycles for the target and endogenous molecules ($C_{T,X} - C_{T,R}$).

For efficiencies close to 1 (i.e., $E_X = E_R = 1$), Eq. (4) can be further simplified (Livak and Schmittgen, 2001) to obtain a simple expression for the target gene expression number, T_N :

$$T_N = \frac{X_{N,q}}{X_{N,cb}} = (2)^{-\Delta \Delta C_T} \quad (5)$$

where $\Delta \Delta C_T$ is the difference in ΔC_T for sample q and the calibrator ($\Delta C_{T,q} - \Delta C_{T,cb}$), and T_N is a unitless value hereby referred to as the relative target gene expression number.

T_N represents the change in target gene expression due to a given treatment condition relative to that observed at the baseline treatment condition. Samples with T_N values > 1 have gene expression levels higher than the baseline condition, and those with T_N values < 1

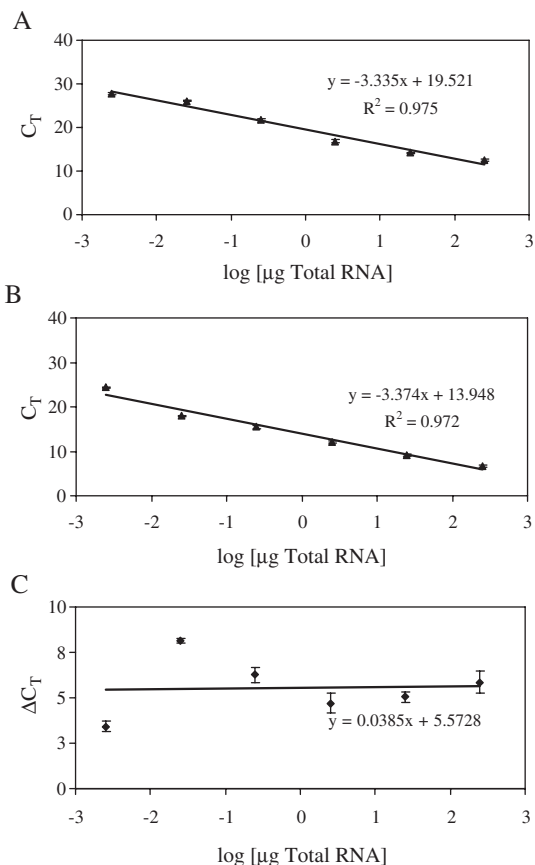


Fig. 1. Real time qRT-PCR standards over 5 orders of magnitude. A) *EIHD0* target amplification, B) *18S rRNA* target amplification and C) real time qRT-PCR efficiency comparison. Error bars representing one standard deviation are shown but some are too small to be visible.

have expression levels lower than the baseline condition. However, for these calculations to be valid, the efficiencies of the target and endogenous amplifications must be approximately equal. To ensure that this was the case, standard curves were generated to assess the variation of ΔC_T with total RNA dilution. Standard curves covering 0.25 pg to 1 μg total RNA were used for this purpose.

3. Results

3.1. *EIHD0* and *18S rRNA* efficiency analysis

As shown in Fig. 1A and B, the coefficient of determination (R^2) values relating C_T to $\log [\text{RNA}]$ values were >0.97 for both targets over the entire range tested (0.25 pg to 1 μg total RNA). This result indicates that for the given real time qRT-PCR setup and cycling conditions, RNA amounts between 0.25 pg and 1 μg are acceptable for relative comparison of *EIHD0* and *18S rRNA* expression levels. Subsequent experiments were

carried out using 250 ng total RNA to ensure that conditions always fell within the linear range. Furthermore, to be able to use the comparative threshold cycle analysis method, the amplification efficiencies for both targets must be similar over the RNA concentration

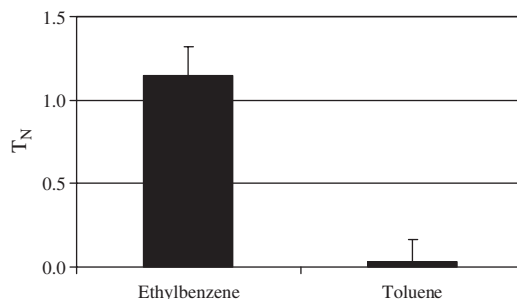


Fig. 2. Relative target expression numbers (T_N) for toluene and ethylbenzene cells grown in a bubbler system as described in Gunsch et al. (2005). Data presented are averages \pm one standard deviation ($n=4$).

range used (Livak and Schmittgen, 2001). For this to be true, a plot depicting the difference between the C_T values for *EIHD0* and *18S rRNA* (ΔC_T) as a function of $\log [\text{RNA}]$ should have a relatively flat slope (e.g., <0.1). Fig. 1C shows that, over the range analyzed (0.25 pg to 1 μg total RNA), the ΔC_T values remain essentially constant. The slope of the curve is 0.0385, which indicates that the comparative threshold cycle analysis can be used to compare *EIHD0* and *18S rRNA* expression levels in *E. lecanii-corni*.

3.2. Validation experiment

To validate the real time qRT-PCR method, *EIHD0* expression levels for *E. lecanii-corni* cells grown on toluene and ethylbenzene were compared. Previously, Northern blot analysis showed that toluene-grown cells did not express *EIHD0* at levels observed in cells grown on ethylbenzene (Gunsch et al., 2005). As shown in Fig. 2, *EIHD0* expression was clearly lower in toluene-grown cells as compared to ethylbenzene-grown cells. In addition, DNase treatment did not have a statistically significant effect on T_N values indicating that there was negligible DNA contamination after the total RNA extractions (results not shown). For this reason, a DNase treatment step was not included in the subsequent cDNA synthesis reactions. As expected, no amplification was observed in any of the negative controls.

3.3. Ethylbenzene utilization rate study

Three regulatory compounds were identified using these guidelines: 1) Intermediates in the proposed ethylbenzene degradation pathway (Gunsch et al., 2005) might induce *EIHD0*; 2) Compounds not metabolized by *E. lecanii-corni* would have no effect on *EIHD0* and; 3) Compounds more easily degradable than ethylbenzene might repress *EIHD0*. Phenylacetate, *o*-xylene and methyl propyl ketone were identified, tested and found to induce, not affect and repress ethylbenzene degradation, respectively. SURs were calculated for ethylbenzene in the presence and absence of each chemical as shown in Fig. 3. As expected, in the absence of regulatory compounds, the SURs are statistically similar ($\alpha=0.05$). A change in the specific ethylbenzene utilization rate was observed in the presence of the repressor at the medium and high concentrations. In the presence of methyl propyl ketone, the SUR of ethylbenzene decreased $\sim 45\%$ 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

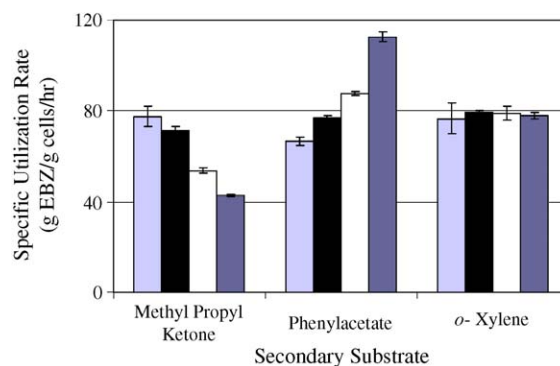


Fig. 3. Ethylbenzene Specific Utilization Rate (SUR) in the presence of methyl propyl ketone, *o*-xylene and phenylacetate. \square , \blacksquare , \square , and \blacksquare represent zero, low, medium and high concentrations of secondary compounds (0, 10, 20 and 40 μM respectively). Data presented are averages \pm one standard deviation ($n=3$ for each treatment condition).

increased, the effect on the specific ethylbenzene utilization rate was amplified. In the presence of the inducer, phenylacetate, the ethylbenzene utilization rate increased by $\sim 69\%$ at the highest concentration as compared to cells which had not been exposed to phenylacetate. In the presence of *o*-xylene, the ethylbenzene utilization rate was statistically similar to that without *o*-xylene. These compounds clearly have different effects on ethylbenzene degradation and thus were used in the subsequent *EIHD0* gene expression experiments.

3.4. Real time qRT-PCR study with mixtures

As shown in Fig. 4, a similar trend was observed for T_N and specific ethylbenzene utilization rates in the presence of phenylacetate, *o*-xylene and methyl propyl ketone. 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 those observed in the baseline growth sample (also grown only in the presence of ethylbenzene). In the presence of the repressor, methyl propyl ketone, *EIHD0* expression was reduced. The average T_N value decreased by $\sim 81\%$. In the presence of the inducer, phenylacetate, the expression level increased by $\sim 52\%$. Cells that were grown in the presence of ethylbenzene and *o*-xylene expressed *EIHD0* in quantities similar to those observed when only ethylbenzene was present. While the T_N value for this case was slightly lower than that with ethylbenzene alone, these two values are not statistically different ($\alpha=0.05$). The target expression numbers, therefore, follow the expected trends and reflect the results obtained for the specific ethylbenzene

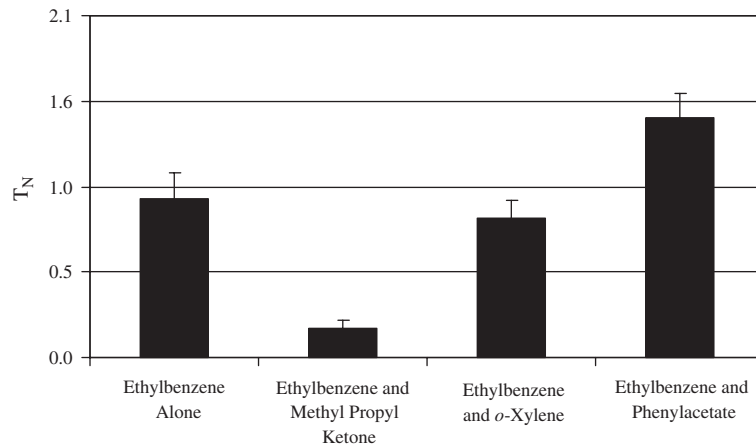


Fig. 4. Normalized target expression numbers for ethylbenzene alone (20 μ M initial concentration) and for ethylbenzene (20 μ M initial concentration) in the presence of other organic compounds (30 μ M initial concentration) in a batch reactor. Data presented are averages \pm one standard deviation ($n=9$ for each treatment condition).

utilization rates in the presence of the three regulatory compounds.

4. Discussion

Real time qRT-PCR was successfully applied to quantify *EIHD0* gene expression relative to *18S rRNA*. This method could potentially be applied to many different biological environmental systems to measure gene expression if a suitable endogenous control is available. Relative quantification has been used in several studies where β -actin, glyceraldehyde-3-phosphate dehydrogenase and *18S rRNA* served as housekeeping genes (Livak and Schmittgen, 2001; Schmittgen and Zakrajsek, 2000). Recently, *18S rRNA* has been validated for normalized expression levels by qRT-PCR analysis under a number of experimental conditions and has been found to be more reliable than other housekeeping genes for normalization (Goidin et al., 2001; Schmittgen and Zakrajsek, 2000). For these reasons, *18S rRNA* was used as the housekeeping gene despite reservations regarding changes in expression levels and potential imbalances in rRNA and mRNA fractions between different samples. However, the PCR efficiency study and the individual standard curves for both target and housekeeping genes allayed these concerns and suggested they were not significant for this study. A known quantity of RNA was also used in the cDNA synthesis step rather than a normalized number of cells, because the most critical factor for successful relative quantitative analysis was previously shown to be the use of normalized RNA (Schmittgen and Zakrajsek, 2000). For this reason, a normalized quantity of total RNA and

the *18S rRNA* gene was used in this comparative gene expression level study.

As shown in Fig. 2, a significant difference in *EIHD0* expression was observed between *E. lecanii-corni* cells grown on ethylbenzene and those grown on toluene ($T_N=1.15\pm0.17$ and 0.03 ± 0.22 , respectively). The results from this experiment indicate that the comparative threshold cycle method can be used to analyze *EIHD0* gene expression relative to *18S rRNA*. The amount of *EIHD0* gene expression when ethylbenzene is not present is negligible and indicates that this gene is not constitutively induced (Gunsch et al., 2005). Real time qRT-PCR has several advantages to more conventional semi-quantitative tools such as Northern blots which have been used to quantify gene expression in environmental systems (Meckenstock et al., 1998; Wilson et al., 1999). First, the quantification sensitivity is greatly increased. Oleson et al. (2003) found that real time qRT-PCR could be used to evaluate gene expression changes over 5 orders of magnitude as compared to 4 or less with standard semi-quantitative RT-PCR. Second, gene expression quantification data is obtained immediately at the end of a PCR cycle, thereby shortening the time from sampling to analysis. Hence, an entire experiment can be performed in 4–5 h as opposed to several days with Northern blot analyses. In addition, because of the minimal amount of sample manipulation, increased reproducibility and high throughput are possible (Grekova et al., 2002). The third potential advantage is that the expression of several genes can be analyzed simultaneously. Although multiplex reactions were not used in this study, multiplex real time qRT-PCR is available. One final advantage to this

method is that it requires fewer reactions, which may decrease sample analysis costs. However, caution must be exercised to ensure that the relative efficiencies of all genes are similar (Boivin et al., 2004).

Compounds that might affect *EIHDO* gene expression were identified and the nature of their effects determined in this research. Because contaminant streams rarely, if ever, contain a single chemical, it is of interest to understand how the presence of other compounds affects microbial degradation by quantifying the overall effect. To coordinate their numerous metabolic activities, cells must regulate the catalytic activities of enzymes in response to changes in the environment. Enzymatic expression levels are expected to vary according to a variety of parameters such as changes in nutrient concentration, level of cell stress (extreme pH or temperature) and nature of compound. Cells adapt to their environmental surroundings by a series of regulatory actions aimed at controlling enzyme level and activity. Thus, it is logical to suspect that a comparison between macroscale measurements of degradation potential (specific ethylbenzene utilization rate) and microscale measurements (gene expression — T_N) should provide some insights as to the magnitude of the effect of secondary compounds on primary metabolism.

In this substrate mixture study, two observations were made with significant implications for microbial degradation studies. First, the real time qRT-PCR method is reproducible. 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 T_N standard deviation was approximately 20%. The second important observation is that T_N values did follow the specific ethylbenzene utilization rate trends. T_N values and SURs remained constant with the introduction of *o*-xylene. In addition, the rates decreased in the presence of methyl propyl ketone and increased in the presence of phenylacetate. The increase in T_N relative to SURs was proportional in the case of phenylacetate but not in the case of methyl propyl ketone. In the presence of phenylacetate, T_N increased by 52% (comparing 0 and 30 μ M phenylacetate) and the utilization rate increased by 69% (comparing 0 and 40 μ M phenylacetate). With methyl propyl ketone, T_N decreased by 81% (comparing 0 and 30 μ M methyl propyl ketone) and the utilization rate decreased by 45% (comparing 0 and 40 μ M methyl propyl ketone). It is thus important to note that while these two parameters are related, their change is not necessarily proportional. It is possible that the degree to which changes in utilization rate and T_N are linked is related to

the complex mechanisms used by cells for up and down-regulation. It is likely that different post-transcriptional and post-translational regulatory mechanisms are used and further research is necessary to elucidate the relationship between T_N and SUR.

Although real time qRT-PCR has become increasingly popular for clinical applications, relatively few studies have been performed using this technique for environmental applications (Han and Semrau, 2004; Devers et al., 2004). Studies incorporating the use of real time PCR have mostly been DNA-based experiments (Mesearch et al., 2004). The main drawback of these techniques is that they do not provide any information about microbial activity. Because 16S rDNA has been found to be stable in dead cells, the presence of a specific organism in a sample does not imply that it is actually responsible for a specific degradative activity (Lindahl, 1993).

By contrast, RNA-based tools specifically target the active fraction of biomass in a sample. RNA-based methods are the most appropriate to describe biofilm level activity. 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. The expression level in each sample is then determined by comparing the quantities of unknown and known products after amplification using capillary electrophoresis. The post-PCR analysis of the products is generally not automated and thus its use in high-throughput applications such as in the screening of numerous environmental samples may be limited.

Relative and absolute quantification are two common methods which are used to analyze quantitative PCR data. Absolute quantification measures the absolute transcript copy number for a specific gene, and has been used in a number of clinical studies (Schmittgen and Zakrajsek, 2000; Iyer et al., 1999). One disadvantage of absolute quantification is that standards must be run on all PCR plates and involves a large number of wells, thereby limiting the number of samples which can be simultaneously analyzed. By contrast, relative quantification does not require standards for each plate, once it has been determined that efficiencies of the endogenous control and gene of interest are the same. Thus, when absolute transcript numbers are not needed, relative quantification may be sufficient. For instance, in experiments comparing the effect of various environmental treatments on biodegradation efficiency, absolute quantification may provide more information than necessary.

Recently, Devers et al. (2004) used comparative quantification to analyze bacterial atrazine catabolism gene expression using SYBR™ Green dye. One potential advantage of Taqman® probes as compared to SYBR™ Green is that the possibility of false positives is reduced. To obtain a fluorescence signal using Taqman® probes, both primers and probe must anneal to the target nucleic acid sequence. With SYBR™ Green-based real time PCR, only the primers must anneal. While both SYBR™ Green and Taqman® probe based real time PCR can be successfully used to analyze environmental systems performance, the Taqman® probe system may be better suited for environmental analysis because of the additional degree of specificity in Taqman® probe assays and the multiplex potential. A simplified analysis of relative changes in T_N values coupled with a multiplex Taqman® PCR assay could be a powerful tool for understanding gene expression changes resulting from system modifications in more complex environmental applications.

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References

- Ausubel, F.M., Bent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), 1989. Current Protocols in Molecular Biology. Wiley, New York.
- Boivin, G., Côté, S., Déry, P., De Serres, G., Bergeron, M.G., 2004. Multiplex real-time PCR assay for detection of influenza and human respiratory syncytial viruses. *J. Clin. Microbiol.* 42, 45–51.
- Devers, M., Soulas, G., Marin-Lauren, F., 2004. Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. *J. Microbiol. Methods* 56, 3–15.
- Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. *Appl. Environ. Microbiol.* 68, 245–253.
- Fournier, D., Halasz, A., Spain, J., Spanggard, R.J., Bottaro, F.C., Hawari, J., 2004. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanol by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 70, 1123–1128.
- Goidin, D., Masmessier, A., Staquet, M.J., 2001. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal. Biochem.* 295, 17–21.
- Greková, M.C., Slaerno, K., Mikkilineni, R., Richert, J.R., 2002. Sp3 expression in immune cells: a quantitative study. *Lab. Invest.* 82, 1131–1138.
- Gunsch, C.K., Cheng, Q., Kinney, K.A., Szaniszló, P.J., Whitman, C.P., 2005. Identification of a homogentisate-1,2-dioxygenase gene in the fungus *Exophiala lecanii-corni*: analysis and implications. *Appl. Microbiol. Biotechnol.* 68, 405–411.
- Han, J.-I., Semrau, J.D., 2004. Quantification of gene expression in methanotrophs by competitive reverse transcription-polymerase chain reaction. *Environ. Microbiol.* 6, 388–399.
- Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C.F., Trent, J.M., Staudt, L.M., Hudson Jr., J., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., Brown, P.O., 1999. The transcriptional program in the response of human fibroblasts to serum. *Science* 283, 83–87.
- Latil, A., Bieche, I., Chene, L., Laurendeau, I., Berthon, P., Cussenot, O., Vidaud, M., 2003. Gene expression profiling in clinically localized prostate cancer: a four-gene expression model predicts clinical behavior. *Clin. Cancer Res.* 9, 5477–5485.
- Lindahl, T., 1993. Instability and decay of the primary structure of DNA. *Nature* 362, 709–715.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408.
- Meckenstock, R., Steinle, P., van der Meer, J.R., Snozzi, M., 1998. Quantification of bacterial mRNA involved in degradation of 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 from liquid culture and from river sediment by reverse transcriptase PCR (RT-PCR). *FEMS Microbiol. Lett.* 167, 123–129.
- Mesearch, M.B., Nakatsu, C.H., Nies, L., 2004. Bench-scale and field scale evaluation of catechol-2,3-dioxygenase specific primers for monitoring BTX bioremediation. *Water Res.* 38, 1281–1288.
- Oleson, L.H., Norgaard, J.M., Pallisgaard, N., Bukh, A., Hokland, P., 2003. Validation and clinical implication of a quantitative real-time PCR determination of MDR1 gene expression: comparison with semi-quantitative PCR in 101 patients with acute myeloid leukemia. *Eur. J. Haematol.* 70, 296–303.
- Qi, B., Moe, W.M., Kinney, K.A., 2002. Biodegradation of volatile organic compounds by five fungal species. *Appl. Microbiol. Biotechnol.* 58 (5), 684–689.
- Révillion, F., Hornez, L., Peyrat, J.-P., 1997. Quantification of *c-erbB-2* gene expression in breast cancer by competitive RT-PCR. *Clin. Chem.* 43, 2114–2120.
- Schmittgen, T.D., Zakrajsek, B.A., 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods* 46, 69–81.
- Wilson, W., Bakermans, C., Madsen, E.L., 1999. In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl. Environ. Microbiol.* 65, 80–87.
- Woertz, J.R., Kinney, K.A., McIntosh, N.D.P., Szaniszló, P.J., 2001. Removal of toluene in a vapor-phase bioreactor containing a strain of the dimorphic black yeast *Exophiala lecanii-corni*. *Biotechnol. Bioeng.* 75, 550–558.