

Quantifying Maintenance Requirements From the Steady-State Operation of a Two-Phase Partitioning Bioscrubber

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Abstract: An innovative method for directly and explicitly quantifying the maintenance energy requirements of pure cultures growing on volatile organic compound (VOC) substrates in a two-phase partitioning bioscrubber is described. Direct evidence of maintenance energy requirements of *Achromobacter xylosoxidans* Y234 is provided both through observed reductions in the macroscopic biomass-to-substrate yield with decreasing specific growth rates, but more remarkably through achievement of steady-state operation. The data conclusively show that maintenance activities do occur in the two-phase partitioning bioscrubber and clearly illustrate the importance of this phenomenon to the operation of this process, and similar bioreactor systems. While benzene was selected as the principal, sole substrate of interest in this study, ethylbenzene degradation experiments were also subsequently performed to illustrate and confirm the general applicability of the proposed technique, as well as the potential capabilities of the two-phase partitioning bioscrubber for the continuous treatment of waste gases containing various VOCs. The proposed method has been shown to generate maintenance energy estimates that are consistent with those obtained while employing more widely recognized estimation strategies, further validating its capabilities. The proposed steady-state mode also offers key operational advantages in terms of decreased disposal requirements in two-phase partitioning bioscrubbers. © 2005 Wiley Periodicals, Inc.

Keywords: maintenance energy; two-phase partitioning bioscrubber; two-phase partitioning bioreactor; VOCs; benzene; ethylbenzene

INTRODUCTION

Maintenance energy has been shown to be required to satisfy a multitude of cellular functions, including motility, maintaining ion gradients, actively transporting molecules, regulating internal concentrations, turning over enzymes and macromolecules (Turner et al., 1989) as well as supporting futile cycles (Tempest and Neijssel, 1984).

Tempest and Neijssel (1984) detail the cellular functions that contribute to the total maintenance demands of a cell. While the complete nature of maintenance energy requirements may not be easily identified, they are likely composed of some combination of each of the above phenomena. Due to difficulties involved in independently distinguishing each of these phenomena they are typically lumped together and accounted for as the total maintenance energy requirements of the organism. Although physiologically distinct, these proposed phenomena are commonly linked by the fact that each can detract from biomass production by redirecting substrate to meet essential needs.

Traditional growth models have accounted for cellular maintenance requirements through either direct consumption of exogenous substrate or endogenous respiration of internal storage materials (which must then be replaced). In terms of model structure, the incorporation of an endogenous respiration term into a growth model is equivalent to the addition of a cellular death term. Although each is physiologically distinct, either maintenance representation produces the same net macroscopic effect: reduction of the net biomass growth yields on substrate. Maintenance energy, therefore, imposes important consequences on the operation of many common biological processes. Consequently, it is not only important to understand the physiological interpretation of this concept, but it is also crucial to be able to reliably estimate maintenance energy requirements to compose adequate models of various biological processes and to generate accurate predictions of their performance. While this work contributes little to a deeper understanding of the physiological notion of maintenance energy, it does present a new method for quantifying maintenance energy requirements using a two-phase partitioning bioscrubber, as well as clearly demonstrating the importance of this phenomena in said process.

BACKGROUND

Perhaps the earliest reproducible physical evidence substantiating the reality of maintenance energy came from

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observations of reduced biomass yields at low specific growth rates in substrate-limited continuous cultures. Among the first to account for this departure from ideality was Herbert (1958) who attributed these effects to the notion of endogenous metabolism, or the oxidation of cellular materials for energy. Soon after, Pirt (1965) would alternatively explain that it was the direct consumption of exogenous substrate used to fuel maintenance functions that was responsible for the disparity in observed yields. Debates over the suitability of each approach continue and can be found in the literature (e.g., Esener et al., 1983; Roels, 1983). In the work presented here, it is assumed that maintenance energy requirements can be best represented by the exogenous substrate consumption model of Pirt (1965). This assumption is thought to be valid when there are no periods of total starvation in which substrate is completely unavailable to the cells, which would result in significant rates of endogenous respiration of cellular storage materials (Roels, 1983).

Pirt proposed a model of batch growth that, therefore, included an exogenous substrate consumption term to account for substrate depletion for purposes other than the production of new biomass:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu \cdot X - m \cdot X \quad (1a)$$

$$\frac{dX}{dt} = \mu \cdot X \quad (1b)$$

where m is the specific rate of substrate consumption for maintenance, S is the substrate concentration, X is the biomass concentration, $Y_{X/S}$ is the growth yield, and μ the specific growth rate of the organism (defined as $\mu = \frac{dX}{dt}/X$). The derivation of Pirt's now-famous linear relationship relies upon two different interpretations of the biomass-to-substrate yield coefficient. The first to be defined is the observed growth yield, $Y_{X/S,obs}$, which predicts the macroscopic amount of biomass production as a result of total substrate consumption, by both growth and maintenance activities, or:

$$Y_{X/S,obs} = \frac{\Delta X}{\Delta S_{growth} + \Delta S_{maintenance}} \quad (2)$$

The second to be defined is the true growth yield, $Y_{X/S}$, which, by definition, accounts only for substrate consumed directly for growth purposes alone according to:

$$Y_{X/S} = \frac{\Delta X}{\Delta S_{growth}} \quad (3)$$

To estimate the true growth yield, maintenance requirements of the culture must be explicitly accounted for in the overall material balance, as in Equation (1a). Pirt's derivations showed that the observed and true growth

yields are related through the maintenance coefficient and the specific growth rate as:

$$\frac{1}{Y_{X/S,obs}} = \frac{1}{Y_{X/S}} + \frac{m}{\mu} \quad (4)$$

Equation (4) is valuable as it constitutes a direct relationship between the maintenance energy requirements of the culture and experimentally observed growth yields. Note that for an assumed constant value of m , Equation (4) predicts that any variation in μ will affect observed values of the growth yield. Analogous observations were also noted by Abbott and Clamen (1973), who saw two- to threefold variations in $Y_{X/S,obs}$ as μ changed between 10% and 100% of its maximum value.

While several attempts of quantifying maintenance energy requirements can be seen throughout the literature, only a few fundamentally derived approaches are available. Pirt (1965) estimated maintenance energy requirements using his linear relationship [Eq. (4)] applied to a chemostat culture. Direct variations of the dilution rate, and therefore the specific growth rate, were performed and the corresponding values of the observed biomass yield were measured. Estimates of m and $Y_{X/S}$ are then easily obtained as the slope and reciprocal of the intercept, respectively, after applying the data to Pirt's (1965) relationship. Bouillot et al. (1990) and Sachidanandham and Kalaiselvi (1998) each performed total biomass recycle by microfiltration of the effluent stream in a chemostat to study the maintenance requirements of *Pseudomonas fluorescens* and *Bacillus sphaericus* 1593M, respectively. Zero specific growth rate was eventually achieved in each case, corresponding to a steady-state where all substrate consumption was reported to be used in support of maintenance functions.

In this article we describe the development of an innovative method for estimating the maintenance requirements of pure cultures consuming toxic volatile organic compounds (VOCs) from a gas feed stream in a two-phase partitioning bioscrubber. This technique also relies upon achievement of a steady-state biomass level by an active culture in a total cell retention system, however without the aid of additional biomass separation equipment. Two-phase partitioning bioscrubbers have been developed as an extension of two-phase partitioning bioreactor technology for the treatment of VOCs, such as benzene and toluene (Daugulis and Boudreau, 2003; Davidson and Daugulis, 2003a, 2003b; Yeom and Daugulis, 2000; Yeom, et al., 2000), from waste gas streams. Two-phase partitioning bioreactors have been the subject of several reviews (Deziel et al., 1999; Malinowski, 2001; Van Sonsbeek et al., 1993) and have been shown to be particularly well suited for the treatment of toxic substrates as they provide and preserve sub-inhibitory conditions in the aqueous phase. Just as for two-phase partitioning bioreactors, two-phase partitioning bioscrubbers are characterized by an aqueous phase containing cells and nutrients, and an organic phase that serves as a sink for high concentrations of toxic substrates. The

dynamic equilibrium established between the two liquid phases, has been shown to be rapidly responsive such that partitioning mass transfer is dictated by the metabolic demands of the cells (Yeom and Daugulis, 2000). Ideal properties of the organic phase, whose selection criteria have previously been the topic of thorough discussion (Collins and Daugulis, 1999), include biocompatibility, non-bioavailability, high partitioning equilibrium, immiscibility in the aqueous phase, and low volatility. Therefore, adding a suitable organic phase to the bioscrubber will promote high levels of bioactivity by ensuring sub-inhibitory aqueous phase substrate concentrations while eliminating adverse solvent-culture interactions and the potential for solvent degradation.

Assuming that substrate mass transfer between the aqueous and organic phases is rapid enough to be considered equilibrated, a simplified model of the liquid phase substrate and biomass concentrations in the two-phase partitioning bioscrubber can be formulated:

$$V_A \cdot \frac{dS_A}{dt} + V_O \cdot \frac{dS_O}{dt} = LR - SR - \frac{1}{Y_{X/S}} \cdot \mu \cdot X \cdot V_A - m \cdot X \cdot V_A \quad (5a)$$

$$V_A \cdot \frac{dX}{dt} = \mu \cdot X \cdot V_A \quad (5b)$$

where V_A and V_O are the aqueous and organic phase volumes, respectively, S_A and S_O are the aqueous and organic phase dissolved substrate concentrations, respectively, LR is the substrate mass loading rate to the bioscrubber, SR is the substrate mass stripping rate from the bioreactor, and the remaining parameters are as previously described. This model has assumed that the maintenance energy of this culture is best represented by exogenous substrate consumption. This assumption has been argued as valid in the absence of periods of complete starvation that could result in significant rates of endogenous respiration of cellular storage materials (Roels, 1983). Supply of sufficient inorganic nutrients and the selection of a conservative substrate loading rate to remove dissolved oxygen limitations will ensure that decreases in the microbial specific growth rate will result solely due to substrate limitations. The predicted steady state of the system can be obtained by setting the derivatives in Equations (5a) and (5b) to zero. At this stationary point, Equation (5a) can be simplified to give:

$$LR - SR = \frac{1}{Y_{X/S}} \cdot \mu \cdot X \cdot V_A + m \cdot X \cdot V_A \quad (6)$$

Since from Equation (5b) it is apparent that $\mu = 0$, Equation (6) further simplifies to predict that at steady state:

$$LR - SR = m \cdot X \cdot V_A \quad (7)$$

Equation (7) implies that at steady state the total rate of substrate supply to the liquid phases (the difference between the substrate loading and stripping rates) is equiv-

alent to the total rate of microbial consumption for the satisfaction of maintenance requirements alone. Therefore, the proposed technique is founded upon the following expression that can be used to estimate the maintenance energy requirements of the culture at steady state:

$$m = \frac{LR - SR}{X \cdot V_A} \quad (8)$$

Thus, when steady-state operation is attained in the two-phase partitioning bioscrubber, the maintenance requirements, reflected through m , are directly estimable.

MATERIALS AND METHODS

Microorganism and Medium

The microorganism selected for this work, *Achromobacter xylosoxidans* Y234 (formerly *Alcaligenes xylosoxidans* Y234), has previously been applied to the degradation of benzene (Davidson and Daugulis, 2003a; Yeom and Daugulis, 2000; Yeom and Daugulis, 2001; Yeom and Yoo, 1999; Yeom et al., 2000). Formulation of the growth medium used for *A. xylosoxidans* Y234 enrichment has previously been developed (Davidson and Daugulis, 2003a) as: 7 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.75 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.6 g/L K_2HPO_4 , 8.42 g/L KH_2PO_4 , 2 g/L sodium benzoate, and 1 mL/L trace elements. Stock trace element solution was prepared as follows: 16.2 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 9.44 g/L CaHPO_4 , 0.15 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 40 g/L citric acid. The growth medium has been designed to achieve pH 6.6, determined to be optimal for this bacterium. Four shake flask cultures were inoculated from frozen stock in 50 mL of medium composed of the above formulation, contained in 125-mL Erlenmeyer flasks prior to incubation at 30°C while being agitated at 150 rpm for 24 h in preparation of to their inoculation in the bioscrubber. Stock cultures previously grown in the above medium were cryogenically preserved at -86°C after combination with 10% (vol/vol) dimethyl sulfoxide.

Chemicals

All nutrients used in the fermentation medium were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Chemicals, specifically benzene and n-hexadecane, were obtained from Alfa Aesar (USA). The purity of benzene and ethylbenzene used in the experiments were both 99% (min., assay) while n-hexadecane was 92% (min., assay).

Reactor Set-Up and Sampling Procedures

The two-phase partitioning bioscrubber experimental apparatus used for the treatment of either benzene or ethylbenzene contaminated air streams is illustrated in

Figure 1. The bioscrubber consisted of a 5-L New Brunswick BioFlo[®] III bioreactor, agitated with two Rushton turbines at 800 rpm and aerated at a total rate of 60 L/h (feed rate plus make-up aeration rate). Although in Figure 1 the bioscrubber is shown to contain two distinct liquid phases, these phases existed as a well-mixed dispersion during operation, ensuring maximum absorption of VOC and oxygen into the liquid phases.

After inoculation, the bioscrubber was not sampled for the first 24 h of operation to allow the culture to recover from its anticipated lag phase of 8 to 10 h (unpublished data) and to enrich the biomass to more significantly quantifiable levels. Conditions were automatically maintained at 30°C, and at pH 6.6 by adding 6M KOH. Dissolved oxygen levels were measured with a polarographic-membrane electrode (Broadley and James Corp., USA). Continuous VOC gas feeds were produced and controlled by passing compressed air, first through a sterile 0.2 µm filter (Millex FG₅₀, Millipore S.A., France), then through a gas rotameter to establish the desired flow rate, and finally, through a submerged gas sparger stone in a 2-L vacuum flask containing approximately 1.5 L of either liquid benzene or ethylbenzene. The VOC flask was maintained at 30 ± 1°C in a water bath. The contaminated air was mixed with sterile make-up air prior to feeding through a sparger to the bottom of the bioreactor. Make-up air was required to maintain high dissolved oxygen levels and its delivery rate was controlled by a separate rotameter. The medium composition in the 2-L aqueous phase of the bioreactor was twice as concentrated as that used in the inoculum shake flasks, described above, but contained no carbon or energy source (i.e., no sodium benzoate). Higher initial nutrient levels were used to avoid

potential nutrient limitations during the experiment. The organic phase consisted of 1 L n-hexadecane, constituting an organic volume fraction of 33%.

The feed and off gases, to and from the bioreactor, respectively, were concurrently sampled for VOC content using gas chromatography (described below). Gas flow rates were verified using a digital flow meter. Ten milliliter liquid samples of the completely mixed reactor contents were periodically drawn into 15-mL centrifuge tubes prior to centrifugation for 15 min at 4°C and 3400 rpm to separate the solid and liquid phases. Liquid phases were separated into aqueous and organic fractions, allowing direct sampling of the VOC content in the organic phase using gas chromatography (described below). Aqueous VOC content was estimated from the experimentally determined equilibrium partitioning relationship for each compound between n-hexadecane and the aqueous phase (not shown). To check for culture purity, liquid samples were periodically investigated using phase microscopy as well as being cultured on solid agar plates composed of the above nutrient concentration but substituted with 2 g/L glucose in place of sodium benzoate for 24 h at 30°C. Cell viability was intermittently assessed in terms of fractional counts of viable cells by preparing samples with the commercial LIVE/DEAD[®] BacLight[™] bacterial viability kit (L-13152, Molecular Probes, Inc., Eugene, OR/Portland, OR) prior to viewing with a fluorescent microscope.

Analytical Methods

Gas chromatography was used to measure benzene and ethylbenzene concentrations directly in the organic and gas

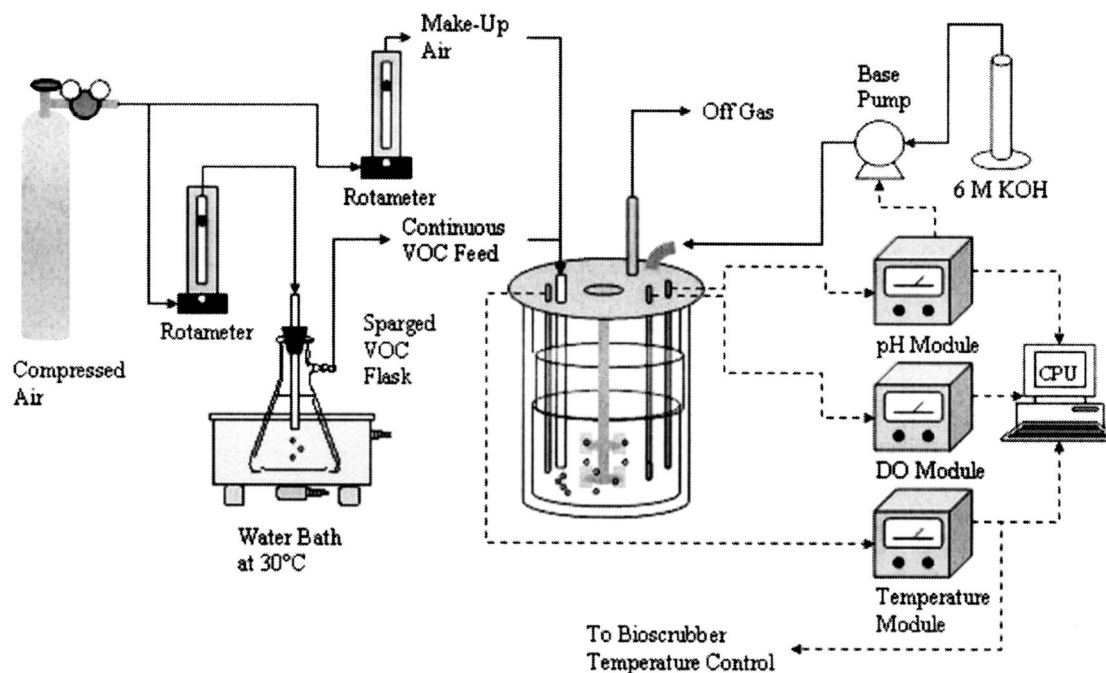


Figure 1. Two-phase partitioning bioscrubber apparatus used for the continuous treatment of benzene contaminated air streams.

phases, and indirectly in the aqueous phase. Samples were analyzed on a Perkin Elmer AutoSystem Gas Chromatograph fitted with a flame ionizing detector and a fused silica capillary column (DB-5, 0.53 mm I.D., 30 m length, 1 μ m film thickness, Model 125-503J, J & W Scientific, Inc., Folsom, CA) designed to be well suited for the analysis of volatile components, particularly BTEX compounds. Helium was used as the carrier gas, flowing at 30 mL/min while injector, column, and detector temperatures were set at 180°C, 150°C, and 250°C, respectively. Output from the gas chromatograph was interpreted via Millennium³² (Workstation Version 3.05.01, Waters Corp., USA) software to perform peak integration and analysis and compared to external standards.

Biomass concentration was determined through optical density measurements. After centrifugation, liquid supernatant was discarded from the sample tube to remove excess salts and coloration from the suspension, leaving behind only biomass. The biomass pellet was then resuspended to its original concentration in deionized water. The optical density of the resuspended sample was measured using a Biochrom Ultraspec 3000 UV/Visible Spectrophotometer (Biochrom, Ltd., UK) at 650 nm after serial

dilution to ensure that samples were measured in the linear range of the instrument.

RESULTS

The proposed procedure for estimating maintenance energy requirements was first applied to the degradation of benzene by *Achromobacter xylosoxidans* Y234 in a two-phase partitioning bioscrubber. The dynamic profiles of biomass accumulation, organic and aqueous phase benzene concentrations, and the benzene mass loading and stripping rates to and from the bioscrubber are depicted in Figure 2a through 2d for an average initial constant benzene loading of 334 ± 29 mg-benzene/h. A step change in the benzene mass loading rate was then performed at 314 hours to an average of 558 ± 31 mg-benzene/h to provide a second set of data for computation of the cellular maintenance requirements, as well as to determine if there was any loss of bioactivity by the culture during the first steady-state growth phase. Note that errors are reported at one standard deviation throughout. Over the course of the entire first loading level, average values of the elimination rate and elimination capacity of 331 ± 32 mg-benzene/h and

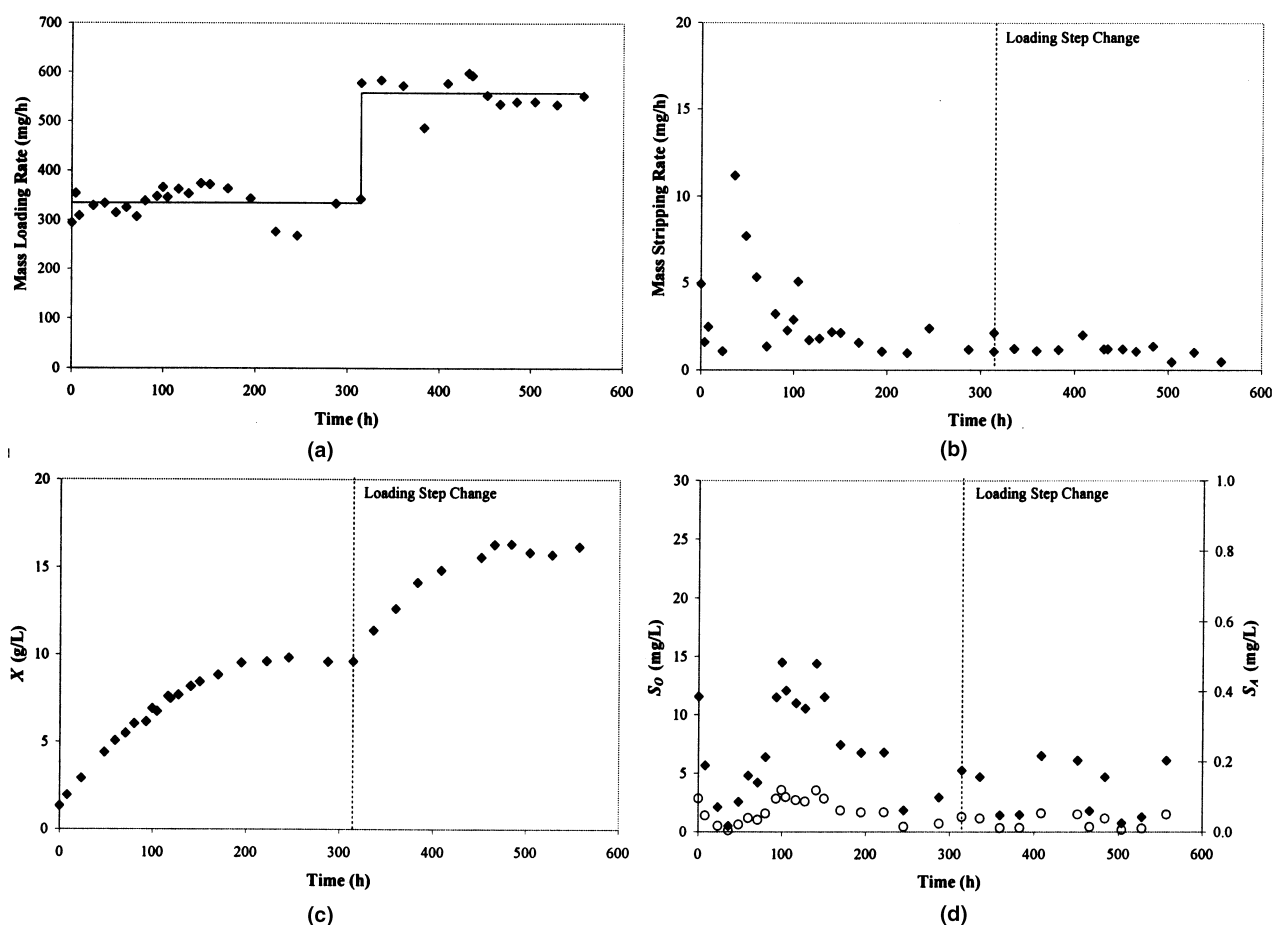


Figure 2. (a) Mass loading rates of benzene applied to the bioscrubber before and after the imposed step change. Average loading rates of 331 mg/h and 557 mg/h are also shown (solid line). (b) Mass stripping rate of benzene from the bioscrubber. (c) Biomass accumulation in the aqueous phase. (d) Accumulation of benzene in the aqueous (○), and organic (◆) phases.

110 ± 10 mg-benzene L h, respectively, were obtained while over the whole second loading level an average elimination rate and elimination capacity of 557 ± 32 mg-benzene/h and 186 ± 10 mg-benzene L h were produced, respectively. From Equation (8), the maintenance energy requirements have been calculated as 0.0166 ± 0.0011 mg-benzene mg-cells h and as 0.0168 ± 0.0003 mg-benzene mg-cells h between 222 h and 314 h at the first loading rate and between 465 h and 557 h at the second loading rate, respectively. Thus, each set of steady-state data (observed to begin at 200 h and 450 h) comprises 92 h of continuously high rates of substrate consumption without the accumulation of additional biomass. Since these two estimated values of the maintenance coefficient are not statistically different at the 95% confidence level, the average value of m for *A. xylosoxidans* Y234 growth on benzene is 0.0167 ± 0.0014 mg-benzene mg-cells h. Observation of identical maintenance demands at each mass loading rate suggests that this is an estimate of the characteristic maintenance requirements of *A. xylosoxidans* Y234 on benzene under the bioscrubber conditions studied. Note that any variation in any such physical conditions as ionic strength, pH, or temperature of the growth medium can lead to variations in the estimated maintenance requirements of the culture. Heijnen and Roels (1981) and Roels (1983) serve as perhaps the most comprehensive collections of estimated values of maintenance energy coefficients available, providing numerous references that typically range between 0.02 mg-substrate mg-cells h and 0.2 mg-substrate mg-cells h.

From Figure 3, it can be seen that the specific growth rate on benzene diminishes throughout the dynamic phase of each loading, approaching levels of zero at each of the observed steady states. This is consistent with the observation of zero net growth, as was shown in Figure 2c. After the step change is performed in the benzene mass loading rate, additional substrate immediately becomes available for the production of new biomass, as indicated by the quick step increase in the specific growth rate. The

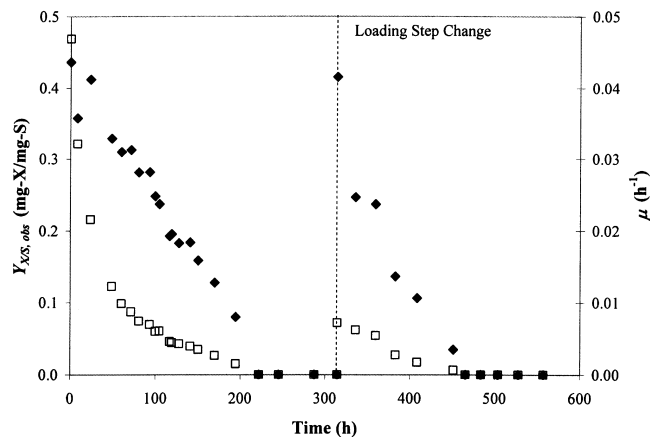


Figure 3. Decline of experimentally observed specific growth rates (□) and observed growth yields (◆) throughout the dynamic phase towards steady state for the two benzene mass loading rates performed.

system responds to the excess substrate through biomass proliferation, however, the total maintenance requirements gradually dominate substrate consumption, again sending the specific growth rate to zero and a second steady state is attained. The greatest value of the specific growth rate measured in these experiments was 0.047 h⁻¹ and occurred at the initial sample during the first benzene mass loading where biomass levels were their lowest. The culture was not observed to be growing exponentially during the dynamic periods, but rather the system was absorption mass transfer limited, as indicated by the characteristic linear increase in biomass that is approached initially in Figure 2c. Previous measurement of the maximum specific growth rate of *A. xylosoxidans* Y234 on benzene has been reported as 0.58 h⁻¹ by Yeom and Daugulis (2000). The observed growth yield on benzene throughout dynamic and steady-state operation have also been plotted in Figure 3 for both loading rates performed. The observed growth yields also begin at maximal values and diminish throughout the dynamic phase at each loading rate performed, approaching values of zero as the system approaches each steady state. Previous estimates of the growth yield of *A. xylosoxidans* Y234 on benzene were reported to be constant parameters valued at 0.46 mg-cells/mg-benzene by Yeom and Daugulis (2000) and 0.56 mg-cells/mg-benzene by Davidson and Daugulis (2003a). Both values compare well with the range of observed yields obtained here. The constant values of the previous estimates are representative of the narrow range of specific growth rates examined in those studies.

Pirt's relationship [Eq. (4)] was also examined via application to the data presented here to obtain an estimate of the true benzene growth yield of *A. xylosoxidans* Y234, and simultaneously, an estimate of m to be used for comparison of the capabilities of this popular estimation method with the one presented in this work. From Figure 4, after applying linear least-squares regression to the entire data set, the Pirt equation provides an estimate for m of 0.0173 ± 0.0004 mg-benzene mg-cells h. However,

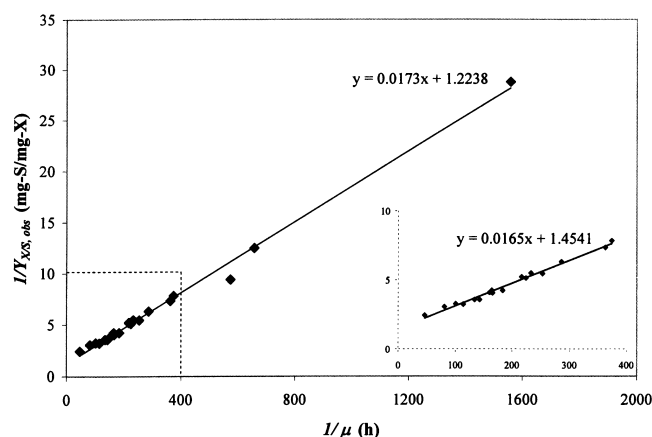


Figure 4. Estimation of m and the $Y_{X/S}$ for benzene by Pirt's (1965) method using linear least-squares regression over the entire data set. Inset shows the same estimation over a limited region.

although a linear trend is apparent over the entire range of data, the obvious uneven distribution of data may have unduly influenced the parameter estimates. The data in question are those that correspond to biomass concentrations on the verge of achieving steady-state levels. To investigate the effect of these data on the parameter estimates, Pirt's relationship was reapplied to the more closely distributed data of Figure 4, found for $1/\mu$ values of up to 400 h. Over this limited data range, shown as an inset in Figure 4, Pirt's equation provides an estimate for m of 0.0165 ± 0.0005 mg-benzene mg-cells h. The two m estimates obtained using Pirt's relationship are in fact found to be statistically different from each other at the 95% confidence level. Furthermore, while Pirt's relationship predicts m estimates that are statistically different from the proposed method using the entire data set, the limited data set yields consistent estimates at the 95% confidence level. Estimates of the maximum, or true, growth yield of *A. xylosoxidans* Y234 on benzene have been obtained as 0.78 ± 0.11 mg-cells/mg-benzene and 0.69 ± 0.06 mg-cells/mg-benzene using the entire and limited data sets, respectively. These values are also different at the 95% confidence level. An estimate of the maximum theoretical aerobic growth yield of typical bacteria on benzene is calculated as 1.21 ± 0.04 mg-cells/mg-benzene by the method of Shuler and Kargi (1992). The significance of the true growth yield, $Y_{X/S}$, however, has been called into question by Tempest and Neijssel (1984) who argue that it exists only as a mathematical constant with no real physiological importance due to the inconsistent observations of non-constant maintenance requirements.

Steady-state conditions arise in this system as the total maintenance energy requirements of the culture are amplified by an increasing biomass level to the point where satisfying the total culture maintenance demands requires the entire exogenous substrate supply, sparing no additional substrate for new biomass production. The resultant shift in the distribution of benzene consumption for the primary purposes of growth at high specific growth rates to maintenance purposes at low specific growth rates for this experiment is illustrated well in Figure 5 using the experimental data and the estimated value for m . These results suggest that the processes of catabolism and anabolism have indeed become completely decoupled at the steady state.

Subsequent to the successful application of the proposed estimation method with the continuous treatment of a benzene-contaminated waste gas stream, a second substrate was selected to demonstrate the broad potential of the technique. Preliminary experiments (not shown) identified ethylbenzene as an ideal alternative substrate due to both its high degradation potential by *A. xylosoxidans* Y234 and its favorable physical properties, specifically high volatility. The benzene experiment was then repeated, however, only at a single ethylbenzene loading rate of 69 ± 8 mg-ethylbenzene/h. No step change in loading rate was performed in the interest of brevity. Correspondingly, the analysis of the data will also be much more concise. The

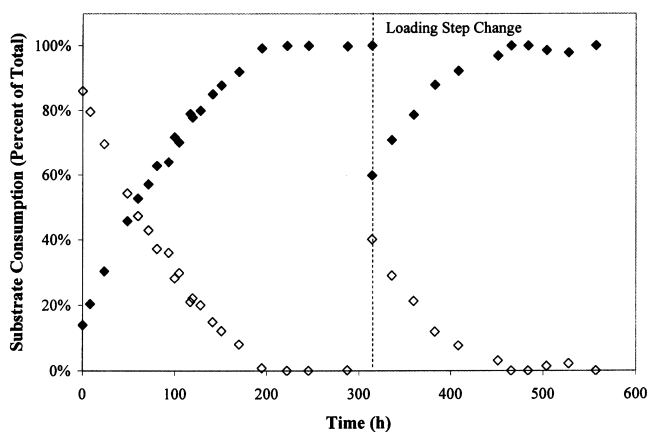


Figure 5. Experimentally estimated distribution of benzene consumption between growth (\diamond) and maintenance (\blacklozenge) functions throughout dynamic and steady-state operation for both mass loading rates performed.

dynamic profiles of biomass accumulation, organic and aqueous phase ethylbenzene concentrations, and the mass loading and stripping rates to and from the bioscrubber are depicted in Figure 6a through 6d. Averages obtained over the length of the experiment of the elimination rate and elimination capacity were 68 ± 8 mg-ethylbenzene/h and 23 ± 3 mg-ethylbenzene L h, respectively. The maintenance energy coefficient for growth of *A. xylosoxidans* Y234 on ethylbenzene has been calculated from Equation (8) as 0.0194 ± 0.0019 mg-ethylbenzene mg-cells h between 72 h and 154 h at the obtained stationary state. These data cover 82 h of high elimination rates without an increase in biomass. Application of Pirt's equation to the ethylbenzene data using linear least-squares regression again yields satisfactory concurrence with the method proposed here within, as can be seen in Figure 7. From Pirt's equation, m has been estimated as 0.0170 ± 0.0002 mg-ethylbenzene mg-cells h. As before, the effect of the data obtained as the process came within reach of steady state on the parameter estimates was investigated by focusing in on $1/\mu$ values of up to 200 h. From this limited data set, shown as an inset in Figure 7, m has been estimated as 0.0194 ± 0.0011 mg-ethylbenzene mg-cells h. As was the case with benzene, the two m estimates obtained using Pirt's relationship are again found to be statistically different from each other at the 95% confidence level. Furthermore, while Pirt's relationship predicts m estimates that are statistically different from the proposed method using the entire data set, the limited data set yields consistent estimates at the 95% confidence level. The true growth yield has also been estimated for *A. xylosoxidans* Y234 growth on ethylbenzene as 1.10 ± 0.17 mg-cells/mg-ethylbenzene and 1.30 ± 0.20 mg-cells/mg-ethylbenzene over the entire and limited data sets, respectively. These values are not different at the 95% confidence level. The theoretical aerobic growth yield of typical bacteria on ethylbenzene can again be calculated as 1.24 ± 0.04 mg-cells/mg-ethylbenzene (Shuler and Kargi, 1992).

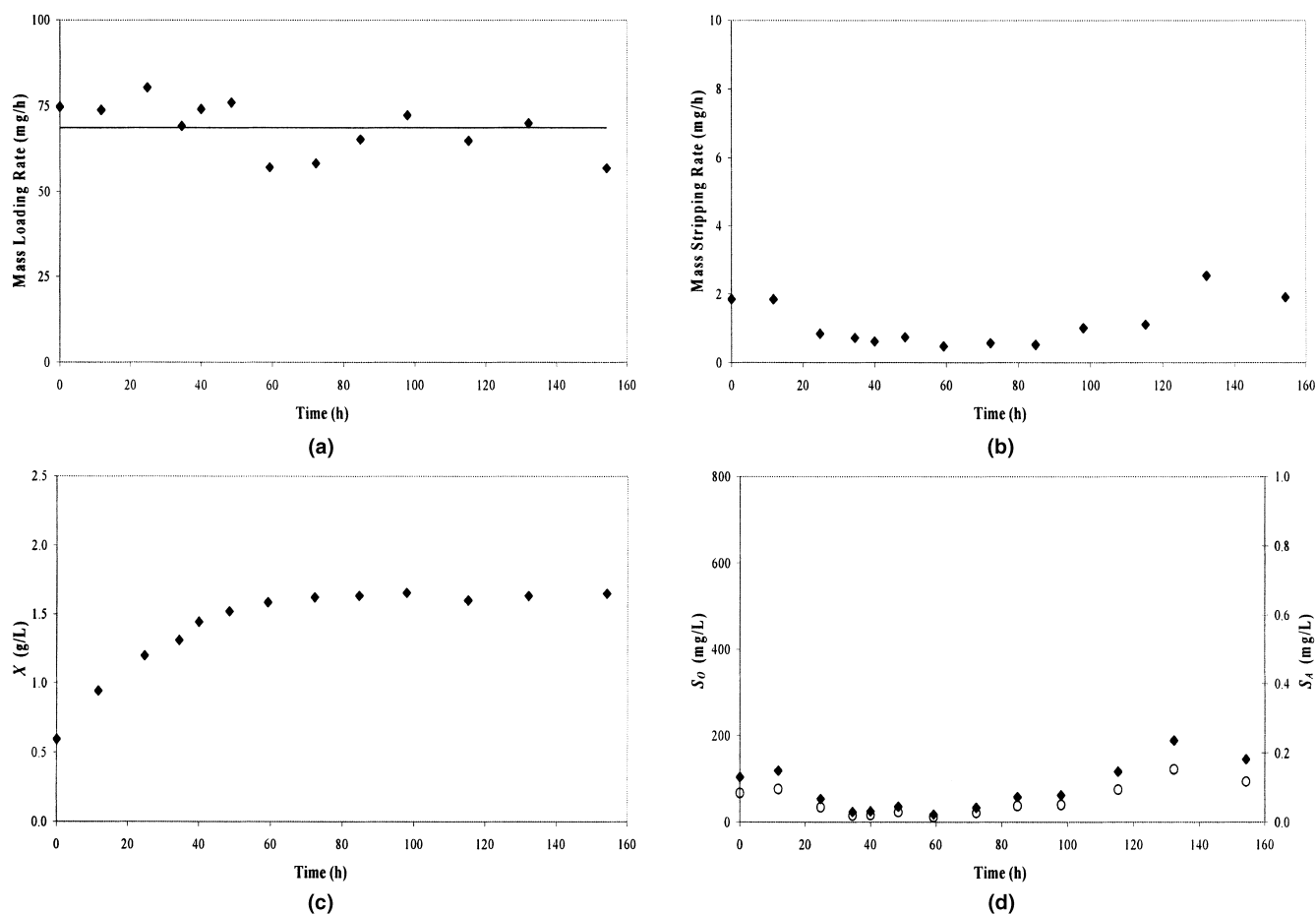


Figure 6. (a) Mass loading rate of ethylbenzene applied to the bioscrubber. Average loading rate of 69 mg/h is also shown (solid line). (b) Mass stripping rate of ethylbenzene from the bioscrubber. (c) Biomass accumulation in the aqueous phase. (d) Accumulation of ethylbenzene in the aqueous (O), and organic (◆) phases.

Viability assessment was conducted to search for evidence of cell death during the course of the experiment. However, while the LIVE/DEAD[®] BacLight[™] bacterial viability kit was successful at identifying both live and dead cells, results of the tests were inconclusive at identifying a discernible trend of dead cell accumulation throughout the dynamic and steady-state growth stages. Therefore, since no evidence was available to support or refute that cell death was the dominant process responsible for the occurrence of zero net growth rates at the observed steady state, the assumed exogenous substrate consumption model was retained.

Purity tests conducted throughout each of the experiments gave no indication that contamination was present in the culture at anytime.

DISCUSSION

Agreement between the results of the proposed method and the widely used equations of Pirt (1965) speaks well of the capabilities of this approach for obtaining reliable estimates of maintenance energy requirements. In this application, parameter estimates obtained using Pirt's relationship were

found to be quite sensitive to the data obtained at exceptionally low specific growth rates. Values of m estimated using Pirt's relationship after excluding these data were found to be in much better agreement with those obtained using the proposed method. However, exclusion of these data from the estimation could potentially narrow the region of applicability of the parameter in future simulations. Discrepancies between the two methods could be overcome in future studies by increasing the sampling frequency as the two-phase partitioning bioscrubber approaches the steady state. Additional data from this important region would then be available to estimate parameters by Pirt's method that would be more characteristic of the entire range of operation. Nevertheless, agreement between the two methods, supported by the linear trends seen in Figures 4 and 7, indicates that the assumption of a constant specific rate of exogenous substrate consumption for maintenance was indeed valid and adequately captured the dependence of the macroscopic biomass growth yield on the specific growth rate in this study. The relative similarity between the maintenance coefficient estimates for *A. xylooxidans* Y234 on benzene and ethylbenzene is not surprising, given the chemical similarity between these two compounds.

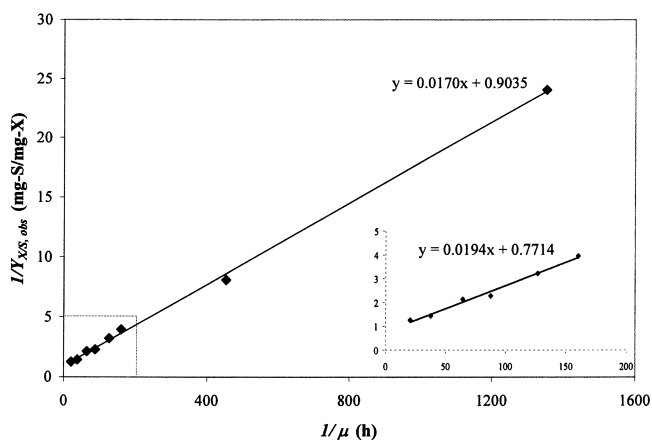


Figure 7. Estimation of m and the $Y_{X/S}$ for ethylbenzene by Pirt's (1965) method using linear least-squares regression over the entire data set. Inset shows the same estimation over a limited region.

It should be noted that although structural differences would be present in models that account for maintenance by either exogenous or endogenous (death) terms, each phenomenon will elicit the same macroscopic effect in this process, namely the reduction of net biomass yields on substrate. As such, the proposed method could be easily modified to also estimate the specific rate of endogenous respiration (or death rate) by a second, simple algebraic expression after Equations (5a) and (5b) are appropriately altered to include the terms of Herbert (1958), rather than those of Pirt (1965), and the same assumptions and simplifications are made.

The method proposed in this article, while unique, is similar in concept to a method previously presented to estimate maintenance requirements in chemostat cultures by Bouillot et al. (1990). The characteristics operation of the two processes is similar in that biomass was fully retained in the bioreactor throughout the experiment. In the chemostat this was achieved through filtration of the effluent stream, while with the bioscrubber this is automatically performed as the liquid contents are never exchanged. In each case, the continual increase in biomass results in a sufficiently high cell density culture that the total maintenance demands consume the entire exogenous substrate supply to each vessel. Both techniques are superior to the traditional use of chemostat cultures, such as that used by Pirt (1965) as they allow for the actual attainment of conditions corresponding to a zero specific growth rate, rather than simply approaching them. This is because it is under the conditions of substrate-limited growth that the most reliable, and statistically significant, estimates of the maintenance coefficient will be obtained. Under substrate-limited conditions the maintenance requirements will appear as their most significant due to resultant low specific growth rates. The macroscopic effects of maintenance requirements are less observable near the maximum specific growth rate because the corresponding maximum specific substrate consumption rate for growth

is generally several orders of magnitude greater than m . Roels (1983) even suggests that maintenance typically does not become noticeable until below specific growth rates of 0.1 h^{-1} . Furthermore, unlike the method proposed by Bouillot et al. (1990), the proposed method leads to a simpler operation as it requires no additional biomass separation equipment. The proposed method is also preferable when studying VOC substrates as volatile losses due to stripping are directly accounted for.

Solvent addition has fulfilled its primary objective in the two-phase partitioning bioscrubber, helping to maintain low aqueous substrate concentrations throughout each of the experiments (Figs. 2d and 6d). The organic solvent phase ensured that substrate inhibition at higher loading rates was of no concern. Any increasingly negative influences that either VOC may have had on *A. xylosoxidans* Y234 were relieved and not permitted to affect the characteristic estimates of m . The effect of substrate inhibition on maintenance requirements has previously been documented by Chen and Johns (1996) who, while investigating acetate consumption by *Chlamydomonas reinhardtii*, observed increasing estimates of m at higher substrate concentrations. Substrate inhibition is expected to lead to higher maintenance energy requirements as a result of interference with such cellular functions as active transport, as well as requiring more energy to maintain an intact cell structure (Maiorella et al., 1983).

High levels of bioactivity were clearly maintained throughout the entire duration of each experiment, during both the dynamic and steady-state growth phases, as evidenced by several notable performance criteria. In each set of experiments, equivalently high elimination capacities were observed for each loading rate throughout indicating that no loss in metabolic activity was observed between the two modes of operation for either compound. Continuous dissolved oxygen consumption and base consumption throughout each of the dynamic and steady-state modes also supports this conclusion. From the benzene experiments it is seen that the stationary culture (operating at the first steady state) was immediately able to respond to an increased benzene loading rate at the step change, suggesting that no bioactivity was lost in the culture as a result of steady-state operation. Therefore, an active culture can be retained, even after prolonged periods of operation (in excess of 92 h) in which maintenance demands accounted for all of the metabolic activity. Steady biomass levels did not occur due to inorganic nutrient limitations as seen by the continually high substrate elimination rates throughout the entire operation, as well as by the ability of the process to rapidly increase the biomass levels as a result of the introduction of excess substrate in the benzene experiment.

This study does not constitute the first observation of the existence of a biological equilibrium, herein referred to as the steady state, in biological processes. Turner et al. (1989) found that the growth profiles of constant fed-batch cultures of *Klebsiella oxytoca* on various substrates were

shown to approach biological equilibrium as substrate consumption for non-growth-associated processes, accounted for as maintenance requirements, consumed increasingly significant quantities of substrate as growth rates diminished. Diks et al. (1994) also attributed the occurrence of biological equilibrium in their trickling biofilter system treating dichloromethane contaminated gas streams to maintenance requirements. The existence of a biological equilibrium has many important process implications in biological systems. In fact, promoting maintenance requirements has been of interest to several researchers attempting to minimize biomass production. Sakai et al. (1992) were able to sustain a continuously fed activated sludge process for 30 days without the production of excess biomass using cell retention, maintaining a constant biomass concentration. In continuous activated sludge treatments, Low and Chase (1999) found that the total amount of sludge wasted could be reduced by 44% by increasing the reactor biomass concentration from 1.7 g/L to 10.3 g/L. Therefore, by operating at higher biomass concentrations, the total maintenance requirements of the culture can be enhanced and the amount of new biomass production, as well as its associated disposal costs, can be greatly decreased. While previous work has been performed on benzene treatment in the two-phase partitioning bioscrubber system by Davidson and Daugulis (2003a), past operation was never permitted to approach the predicted steady state of the system, the critical condition required for the direct estimation of the maintenance energy requirements by the proposed method. Rather, in the past work, medium exchanges were performed intermittently to control the biomass level within a desired concentration range, replacing up to 50% (by volume) of the reactor contents with sterile medium and n-hexadecane in appropriate volume fractions. No such exchanges were performed in the present work; rather, the liquid reactor contents remained closed. Although the final waste product that will be obtained in the two-phase partitioning bioscrubber after adopting the proposed mode of steady-state operation will consist of a much higher mass of cells, the total quantity of biomass wasted will be drastically lower than an operation incorporating medium exchanges over the lifetime of the treatment.

The results of this experiment also demonstrate the notable capabilities of the two-phase partitioning bioscrubber as a waste-gas treatment method. High removal efficiencies of at least 98% were obtained for both benzene and ethylbenzene over 557 h and 154 h of continuous operation, respectively. Past experimentation with this system is noted to have achieved similarly high benzene removal efficiencies (95%) for up to 140 h, while operating at an elimination capacity of 133 mg L h (Davidson and Daugulis, 2003a). While elimination capacities of only 110 mg L h and 186 mg L h were obtained at each loading level in this study, these were intentionally maintained at low levels compared to the theoretical maximum to eliminate the potential influence of dissolved oxygen limita-

tions on maintenance energy requirements. No prior work has been conducted with ethylbenzene.

CONCLUSION

The effect of maintenance energy has been dramatically shown in the two-phase partitioning bioscrubber. Not only has a traditional maintenance indicator, reduced growth yield, confirmed its significance, but so too has the striking achievement of steady-state operation during long-term, continuous substrate loading. Such a response by the process is impossible to ignore and provides irrefutable evidence of the macroscopic effects of maintenance energy. As can be seen, the existence of a steady-state biomass concentration was not only achieved, but was reproduced at two different benzene mass loading rates, as well as a single ethylbenzene loading rate. The reproducibility of benzene m estimates obtained at each steady state suggests that a characteristic value of the maintenance energy requirements of the culture has been obtained with this novel method. Translation of the proposed method to an additional VOC, ethylbenzene, suggests the potential for broad applicability of this approach.

Operation of the two-phase partitioning bioscrubber to allow for continuous biomass accumulation represents a previously unreported application. Such a mode of operation represents a significant improvement over previous policies as it capitalizes on diminishing observed biomass growth yields and can lead to lower total amounts of biomass production, and associated disposal requirements, over the course of a treatment. Adoption of this strategy in future two-phase partitioning bioscrubber application is, therefore, underway.

NOMENCLATURE

LR	loading rate of substrate to the bioscrubber	mg/h
m	rate of maintenance by exogenous substrate consumption	mg-S mg-X h
S_A	aqueous phase substrate concentration	mg/L
S_O	organic phase substrate concentration	mg/L
SR	stripping rate of substrate from the bioscrubber	mg/h
t	time	h
V_A	aqueous phase liquid volume	m^3
V_O	organic phase liquid volume	m^3
X	aqueous phase biomass concentration	mg/L
$Y_{X/S}$	true growth yield	mg-X/mg-S
$Y_{X/S, obs}$	observed growth yield	mg-X/mg-S

Greek letters

μ	specific growth rate	h^{-1}
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