Biodegradation **8:** 143–151, 1997.

Water stress effects on toluene biodegradation by *Pseudomonas putida*

Patricia A.Holden*, Larry J. Halverson & Mary K. Firestone
Department of Environmental Science, Policy & Management, Ecosystem Sciences Division, University of California, Berkeley, California 94720, USA (author for correspondence, present address: School of Environmental Science & Management 4666 Physical Sciences North University of California, Santa Barbara, California 93106, USA)*

Accepted 13 May 1997

Key words: matric potential, *Pseudomonas putida*, toluene, water potential, water stress

Abstract

We quantified the effects of matric and solute water potential on toluene biodegradation by *Pseudomonas putida* mt-2, a bacterial strain originally isolated from soil. Across the matric potential range of 0 to – 1.5 MPa, growth rates were maximal for *P. putida* at – 0.25 MPa and further reductions in the matric potential resulted in concomitant reductions in growth rates. Growth rates were constant over the solute potential range 0 to – 1.0 MPa and lower at – 1.5 MPa. First order toluene depletion rate coefficients were highest at 0.0 MPa as compared to other matric water potentials down to – 1.5 MPa. Solute potentials down to – 1.5 MPa did not affect first order toluene depletion rate coefficients. Total yield (protein) and carbon utilization efficiency were not affected by water potential, indicating that water potentials common to temperate soils were not sufficiently stressful to change cellular energy requirements. We conclude that for *P. putida*: (1) slightly negative matric potentials facilitate faster growth rates on toluene but more negative water potentials result in slower growth, (2) toluene utilization rate per cell mass is highest without matric water stress and is unaffected by solute potential, (3) growth efficiency did not differ across the range of matric water potentials 0.0 to – 1.5 MPa.

Introduction

In situ bioremediation is advantageous for mitigating volatile organic compound (VOC) pollution in contaminated soils because, as compared to excavation and offsite treatment, it is generally more cost effective and reduces the risk of releasing pollutants to the atmosphere. Although there is a great deal of interest in *in situ* bioremediation, the factors that may influence *in situ* biodegradation processes are not well understood. Six environmental factors have been identified as possible determinants of successful bioremediation: (1) available soil water, (2) oxygen, (3) redox potential, (4) pH, (5) nutrients, and (6) temperature (Sims et al. 1993). Water availability, specifically the matric and solute components of soil water potential, is a significant determinant of microbial physiology in soil (Harris 1981; Brown 1990). Matric water potential arises from the interaction of soil water with solid surfaces and is the dominant component of total water potential in non-saline soils (Papendick & Campbell 1981). Solute water potential is determined by the interactions of salts with soil water; solute potential may contribute significantly to total water potential when soil water content is low. Through its relationship to water film thickness (Taylor & Ashcroft 1972), matric water potential affects the supply of gas and solution phase nutrients to microorganisms (Papendick & Campbell 1981). Thus, matric water potential can serve as a unifying environmental determinant of unsaturated zone biodegradation.

Given the importance of soil water potential to *in situ* biodegradation, an understanding of the mechanisms through which water potential may affect biodegradation rates is needed. The mass transfer of VOCs through soils as a function of water content is generally understood: at high water contents mass transfer is impeded due to reduced air-filled pore space

and partitioning of VOCs into soil water. At low water contents air-filled pore space is increased yet VOC molecules may be retarded by adsorption onto soil surfaces (organic or mineral) (Petersen et al. 1994). This level of understanding of the effects of water potential on VOC mass transfer in soils is sufficient for incorporation into predictive mathematical models. However, the effects of water potential on intrinsic bacterial physiology during VOC biodegradation are not similarly understood. Therefore, we have examined the effects that water potential has on bacterial growth rates, toluene depletion rates and growth efficiency to provide a better understanding of how water potential may affect biodegradation in the soil environment.

Our approach to examining how water potential affects toluene biodegradation includes studying both matric and solute components of total water potential. In many previous studies of water stress effects on physiology, salts were added to soil slurries to show that, for example, nitrification rates (Stark & Firestone 1995) and 2,4-D degradation rates (Han & New 1994) decreased with decreasing water potential. Adjusting the salt concentration of soil slurries or pure liquid cultures alters the solute component of water potential and may or may not also result in specific ion toxicity. Recently, Schnell & King (1996) reported that methane oxidation rates decreased with decreasing water potential when either salts (KCl or NaCl) or sugar (sucrose) were used to lower the solute or matric water potentials, respectively, of soil slurries. We have adopted a different approach to simulating matric potential: adding high molecular weight polyethylene glycol (PEG), a solute that does not pass bacterial membranes, to liquid cultures (McAneney et al. 1982). PEG is water soluble and its mass in liquid culture media can be varied to simulate matric water potential.

Salts were used in previous studies to simulate total soil water potential in soil slurries because the alternative, that of simply drying soil, resulted in diffusional limitations on the resupply of N, P and K. In whole soil, nutrient limitations at low water content will restrict microbial processes (Stark & Firestone 1995; Schnell & King 1996). In that our goal here was to investigate the physiological effects of water stress on the biodegradation of a VOC, we avoided confounding mass transfer effects by working with well-mixed liquid cultures. Recent studies of methanotrophs showed that water stress affected methane oxidation rates similarly in liquid cultures and in whole soils (Schnell & King 1996). In studies of purified enzyme transformations (Laurent 1971; Andersson & Hahn-Hägerdal 1987) and of bacterial growth (McAneney et al. 1982; Busse & Bottomley 1989), low matric potentials achieved by high PEG concentrations reduced rates. However, a few studies have shown that growth rates (McAneney et al. 1982; Busse & Bottomley 1989) and photosynthetic rates (Potts & Friedmann 1981) are optimal in the presence of a slightly negative matric potential. The composite water stress literature provides some clues as to how water potential may affect microbial physiology during biodegradation processes. However, to our knowledge, an examination of how the solute and matric components of water potential affect the microbial physiology operative in pollutant biodegradation has not previously been performed.

In this report, we have examined the effects of simulated solute and matric components of soil water potential in liquid culture in order to assess, separately from the extrinsic mass transfer limitations that occur in drying soil, the growth and kinetics of toluene utilization by *Pseudomonas putida* mt-2. Our results reveal that (1) a slightly reduced matric potential results in fastest growth rates, (2) toluene depletion rates are fastest when *P. putida* is without matric water stress and (3) carbon utilization efficiency is constant over a range of matric potentials reflective of wet to dry soil.

Materials and methods

Chemicals

Analytical grade toluene, xylene and D-glucose were obtained from Mallinckrodt, Inc. Polyethylene glycol, average molecular weight 8000 (PEG-8000), was obtained from Sigma Chemical Company.

Growth media

The liquid basal medium consisted of 0.5 g NH_4Cl; 1.725 g $Na_2HPO_4 \cdot 7H_2O$; 1.38 g KH_2PO_4; and 10 mL half-strength Hutner's Mineral Base (Smibert & Krieg 1994) per liter of Milli-Q water. Glucose or toluene were provided at equimolar concentrations of carbon (1.28 or 0.563 g per liter, respectively). Based on a Henry's Law constant of 0.27 (gas phase concentration/aqueous phase concentration) (Mackay & Shiu 1981), a liquid culture volume of 100 mL and a total flask volume of 586 mL, the initial toluene concentration in the basal medium solution phase was 250 mg/L. The water potential of the basal medium was – 0.15 MPa. To simulate the solute and matric components of

total water potential, we amended the basal medium with various concentrations of either NaCl or PEG-8000, respectively. To alter the water potential of the basal medium by either – 0.125, – 0.25, – 0.375, – 0.5, – 1.0, or – 1.5 MPa, we added either 1.6 or 70 g, 3.2 or 100 g, 4.8 or 130 g, 6.4 or 150 g, 12.8 or 262 g, and 19.2 or 330 g of either NaCl or PEG-8000 per liter, respectively (Harris 1981; Soroker 1990). All media was filter sterilized (0.2 μm) prior to use. The water potentials we studied represent the range of soil water potential conditions, from near saturation to very dry, that commonly occur in surface soils. For water potentials lower than – 1.5 MPa, microbial metabolism is expected to be severely impaired (Harris 1981) and biodegradation would probably be insignificant.

Because PEG can serve as a carbon source for some bacteria (Haines & Alexander 1975; Cox 1978), we determined in advance that neither PEG-8000 nor ethylene glycol (monomer of PEG) supported growth of *P. putida* by measuring both colony forming units (CFUs) and culture absorbance (660 nm) in cultures unamended with exogenous carbon. Furthermore, because high PEG-8000 concentrations can reduce oxygen solubility (Mexal et al. 1975), we determined in advance that the gas-to-liquid mass transfer of oxygen and toluene was not affected in – 1.5 MPa matric potential adjusted culture media as culture growth rates did not vary across small to large culture volumes.

Bacteria and growth conditions

Pseudomonas putida mt-2 was maintained at – 80 °C and was grown on Luria-Bertani (LB) agar when an inoculum was required for liquid culture. For growth and substrate utilization studies, *P. putida* was first grown in glucose or toluene-amended basal media to early stationary phase. Cells were separated from culture medium by centrifugation (30,000 g; 10 minutes; 5 °C), washed once and resuspended in approximately 1 mL basal medium. This suspension was used to inoculate the PEG or NaCl-amended basal medium and carbon was supplied as either glucose or toluene, corresponding to the carbon source of the inoculum. Cultures were grown in 586 mL triple-baffle side-arm culture flasks (Bellco Biotechnology, Vineland NJ) that were made gas-tight by wrapping threads with Teflon tape and capping the top with a 38 mm Teflon-lined cap and the side-arm with an 18 mm mininert cap (Alltech Associates). Cultures were incubated in an orbital shaker/incubator (150 rpm) at 27 °C. Growth was monitored by measuring changes in culture absorbance at 660 nm.

Gas chromatography

Toluene concentrations were determined by gas chromatography (GC). Headspace samples (60 μL) were analyzed for toluene during bacterial growth using a Hewlett-Packard 5890A gas chromatograph with a flame ionization detector (FID) (modified EPA Method 3810, SW 846). The carrier gas was ultra high purity nitrogen (UHP, Matheson Gas Products) at a flow rate of 30 mL/min. Other operating conditions were: oven and detector temperature: 105 °C; injector temperature: 125 °C.

Toluene standards that were less than 1 mg/L were made by injecting known amounts of saturated toluene vapor into 2-liter static dilution bottles with 24 mm mininert caps (Tekmar Co., Rosemount Analytical Inc.), according to EPA standard operating procedures (Morris et al. 1990). Various volumes of liquid toluene and xylene were combined in gas-tight vials to provide a range of gas-phase toluene standards above 1 mg/L and Raoult's Law was used to calculate the resulting headspace concentrations in these mixtures. All standards and samples were in thermal equilibrium prior to GC analysis.

Henry's Law relationships in water potential-adjusted media

Because PEG is a known cosolvent (Banerjee & Yalkowsky 1988; Morris et al. 1988), we determined Henry's Law coefficients by adding various masses of PEG-8000 or NaCl to a series of gas-tight bottles containing equivalent volumes of basal media. Equivalent volumes of toluene (less than the solubility limit) were added to each vessel, headspace analysis for toluene was performed after the solutions had equilibrated, and a mass balance was used to determine the amount of toluene in solution. NaCl had no effect on toluene gas-liquid partitioning, whereas PEG-8000 did, resulting in the following Henry's Law coefficients: 0.27 for 0.0 MPa, 0.27 for – 0.25 MPa, 0.26 for – 0.50 MPa, 0.20 for – 1.0 MPa, and 0.17 for – 1.5 MPa. These Henry's Law coefficients were then used to determine whether resulting initial solution phase toluene concentrations of 100 to 300 mg/L were toxic. We determined that culture lag phase, growth rate and toluene depletion rates for *P. putida* were unaffected by these initial

toluene concentrations and, thus, the slight variation in Henry's constants had no impact on growth.

Thermocouple psychrometry

The water potential of the culture media was verified before use by thermocouple psychrometry with a nanovoltmeter/thermometer (Models SC10 and NT-3, Decagon Devices Inc., Pullman, WA). Known concentrations of NaCl in deionized, distilled water were used for standards. The water potentials of the standards were calculated using the Van't Hoff relationship (Papendick & Campbell 1981). All measurements were made at 25 °C.

Cellular protein content

Cells were isolated by centrifugation (30,000 g; 20 minutes; 5 °C) within 0.75 to 1.5 hours after the onset of stationary phase, and then solubilized at 90 °C for 10 minutes in 1 N NaOH. Protein content was determined by the method of Bradford (Bradford 1976).

Culturable counts

The number of CFUs within 0.75 to 1.5 hours after the onset of stationary phase was determined by serially diluting the cultures in basal or NaCl-amended media of the same water potential as the culture medium and plating them onto Luria agar. Plates were incubated at 27 °C for 48 hours.

^{14}C-toluene utilization efficiency

The efficiency of ^{14}C-toluene utilization was assessed for matric potentials of 0, – 0.25, – 0.5, – 1.0 and – 1.5 MPa. The inocula was prepared by growing *P. putida* in basal medium to mid-exponential phase with an initial liquid phase toluene concentration of 150 mg/L. Cells were recovered by centrifugation. Triplicate side arm flasks containing 100 mL each of appropriate PEG-amended media were inoculated with 100 μL of the cell suspension and provided with 39 μL of 0.5 μCi/mL ^{14}C-(ring) labeled toluene (Sigma Chemical Co.) for a starting liquid phase toluene concentration of 150 mg/L. The side arm of each flask contained 2 mL of 1 N NaOH to trap ^{14}C-CO_2. The flasks were incubated at 27 °C and shaken at 150 rpm. During growth, NaOH was periodically removed and replaced with fresh NaOH through the side Mininert valve using a 10-inch luer needle attached to a sterile syringe. Removed NaOH was dispensed into a 15 mL scintillation vial and 10 mL of scintillation cocktail (Scintiverse II) was added.

After cultures were grown to maximum optical density (OD), the amount of radiolabel in all pools was determined: NaOH-trapped CO_2, cells, whole culture and culture filtrate. To determine the amount of ^{14}C in the cells and filtrate, 2 mL of early 0.2 μm nylon membrane. The membrane was transferred to a scintillation vial containing 2 mL of 1 N NaOH and heated (90 °C) in a water bath for 10 minutes to lyse cells prior to adding 10 mL of scintillation cocktail. The filtrate was handled in two ways: 1 mL was combined with 1 mL 2N NaOH in a scintillation vial to determine remaining parent compound, intermediates and dissolved ^{14}C-CO_2; the remaining 1 mL was combined with 1 mL of 2 N HCl to drive off dissolved ^{14}C-CO_2 prior to adding scintillation cocktail. The amount of radiolabel in the whole culture was determined by combining 1 mL of the early stationary phase culture with 1 mL of 2 N NaOH in a scintillation vial prior to adding scintillation cocktail. Scintillation counting was done with a Beckman LS9000 instrument.

Results

Influence of water potential on growth rates

We grew *P. putida* with either glucose or toluene as the carbon source so that the effects of reduced water potential (by either NaCl or PEG-8000) on growth rates could be compared between a benign (glucose) and a potentially toxic (toluene) carbon source. In general, we observed that toluene-utilizing cultures grew more slowly than cultures utilizing glucose (Figure 1). When toluene was the carbon source, we observed the fastest ($P = 0.05$, by Fisher's LSD) growth rate at – 0.25 MPa matric potential (Figure 1). Similarly, when glucose was the carbon source, the growth rate at – 0.25 MPa matric potential was significantly faster ($P = 0.05$) than the other matric potential conditions (Figure 1), but it was not faster than the growth rate in basal media. When either glucose or toluene was the carbon source, growth rates decreased with decreasing matric potentials below – 0.5 MPa (Figure 1).

For glucose-grown cultures, growth rates decreased with decreasing solute potential (Figure 1). For cultures grown with toluene, only – 1.5 MPa (NaCl) resulted in a significantly lower growth rate ($P = 0.05$). To determine whether the reduction in growth rate imposed

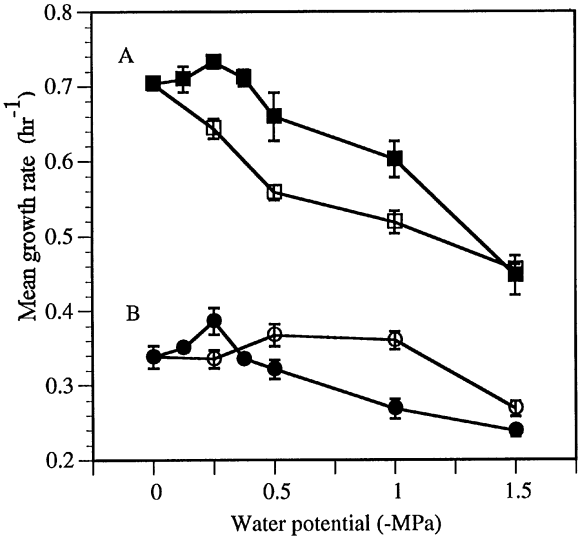

Figure 1. Influence of solute and matric water potential on culture growth rates. A: Glucose; B: Toluene; open symbols = permeating solute (NaCl); closed symbols = non-permeating solute (PEG 8000). Values are the means ± SEM of 3–5 independent replicates.

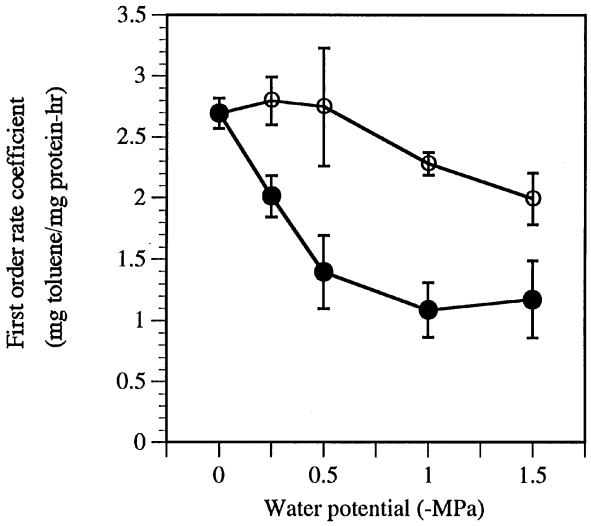

Figure 2. Influence of solute or matric water potential on toluene depletion first-order rate coefficients. Open symbols = permeating solute (NaCl); closed symbols = non-permeating solute (PEG 8000). Values are means ± SEM of 3 independent replicates.

by the solute NaCl was due to either a water potential effect or salt toxicity, we examined the influence of sucrose on growth kinetics. In general, growth rates for the permeating solutes NaCl and sucrose were similar. For example, at – 0.25 MPa, the growth rate (27 °C) with NaCl was 0.339 ± 0.011 hr^{-1} and with sucrose was 0.349 ± 0.005 hr^{-1} when toluene was the carbon source. Growth rate determinations based on either direct counts or CFUs confirmed the patterns shown in Figure 1.

Effect of water potential on toluene utilization rate coefficients

In addition to assessing the influence of water potential on growth rates, we studied the influence of water potential on toluene depletion rate constants. The initial liquid phase concentration of 250 mg/L toluene was well above the Michaelis-Menten half saturation constant for *P. putida* (Holden 1995). Thus our model for describing toluene depletion over time is applicable to reasonably high initial toluene concentrations and is first-order with respect to cell concentration and zero-order with respect to toluene concentration. The differential form of the model is: $dS/dt = - k X$; where S = toluene concentration (mg/L, liquid phase), k = first order rate coefficient (mg toluene/mg cells-time) and X = cell concentration (mg/L). We used culture protein for the cellular mass term, X; thus units for k are: mg toluene/mg-protein-hour. The integrated form of the equation that we used to calculate k for each growth condition was: $k = \mu (\Delta S/\Delta X)$, where μ is the specific growth rate from Figure 1. The calculated rate coefficients plotted against water potential are shown in Figure 2. There was no significant difference (P = 0.05, by Tukey's HSD (Daniel 1991)) between rate coefficients when NaCl was used to reduce the water potential. The rate coefficient was significantly higher for the 0.0 MPa condition as compared to reduced matric potentials. However there was no significant difference in values across the matric potential range – 0.25 to – 1.5 MPa.

Toluene utilization efficiency and cell yield

The growth of microorganisms at reduced water potential conditions may result in the potentially energy-intensive production of compatible solutes (Harris 1981; Brown 1990), which could reduce cell yield. Partly due to our observation that growth rates were fastest at – 0.25 MPa matric potential, we wanted to determine whether matric water potential affected growth efficiency when ^{14}C-toluene was provided as the sole carbon and energy source. The distribution of ^{14}C to the CO_2, cell and filtrate pools was determined by comparing each ^{14}C-labeled pool to the total

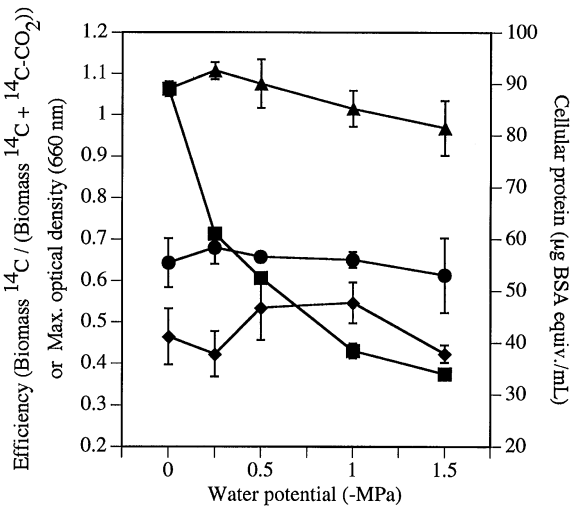

Figure 3. Influence of matric potential on carbon utilization efficiency (●) and cellular protein (◆) and influence of solute potential (▲) and matric potential (■) on maximum optical density. Toluene was the carbon source. Values are means ± SEM of 3–5 independent replicates.

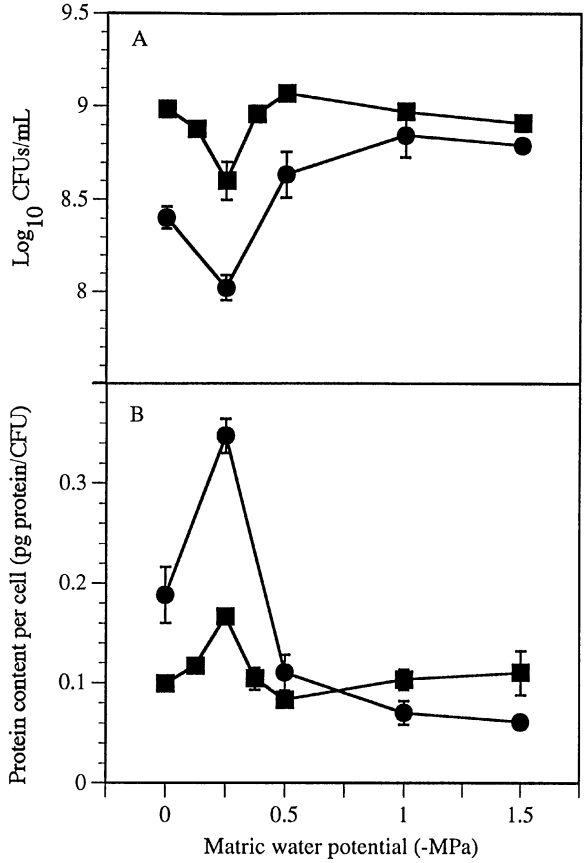

Figure 4. Influence of matric water potential on maximum stationary phase CFUs (*A*) and protein content per cell (*B*); ■ glucose, ● toluene. Values are means ± SEM of 3–5 independent replicates.

amount of radiolabel added. Matric water potential had no effect on the distribution of radiolabel to the three pools studied: cells, NaOH-trapped CO_2, and filtrate. Acidifying the filtrate did not affect total ^{14}C in the filtrate pool; thus ^{14}C-CO_2 was not retained in the culture media and the NaOH trap was an effective sink. Also for all cultures, our mass balance on ^{14}C in the filtrate and cell pools was confirmed by the whole culture ^{14}C values.

The efficiency of toluene catabolism is calculated as the ratio of cell-associated ^{14}C to the combined ^{14}C-CO_2 and cell-associated ^{14}C values, and values are plotted in Figure 3. Using Fisher's LSD (P = 0.05), the conversion efficiencies were statistically indistinguishable across the five tested matric water potential conditions.

As another measure of yield, we determined the cellular protein content of early stationary phase cultures after all toluene was depleted from culture headspace. Regardless of carbon source, neither solute nor matric water potential (Figure 3) had a statistically significant effect (P = 0.05, by Fisher's LSD) on culture protein content.

Finally, maximum ODs for the solute and matric potential-controlled toluene-grown cultures are plotted in Figure 3. Maximum OD means were not significantly different (P = 0.05, Tukey's HSD) across the range of solute potentials tested. However, across the range of matric potentials tested, all maximum OD means were significantly different (P = 0.05) and values decreased with decreasing matric potential.

Cell numbers and size

We examined the effect of water potential and toluene on the numbers of CFUs in early stationary phase. In all cases there was complete utilization of the carbon source and the number of stationary phase CFUs did not change prior to cell harvest. Decreasing solute potential had no statistically significant effect (P = 0.05) on the numbers of early stationary phase CFUs; CFUs with glucose averaged ($\log_{10}$) 8.96 ± 0.02 and with toluene averaged ($\log_{10}$) 8.41 ± 0.03 across the range of solute potentials. The CFUs in the – 0.25 MPa matric potential cultures were statistically fewer (P = 0.05) than either the basal medium or the other matric potential cultures (Figure 4). The

protein content and culturable population data were transformed into protein content per cell (Figure 4). At – 0.25 MPa matric potential the calculated protein content per cell was significantly higher (P = 0.05) than other growth conditions for both carbon sources; otherwise there were no significant differences (P = 0.05) between treatments. The CFU and protein/CFU data (Figure 4) support our visual observations using phase contrast microscopy that, at the time they were harvested for CFU measurements, the cells growing at – 0.25 MPa matric potential were visibly larger than the other water potential conditions. However, by 6 hours after the onset of stationary phase, the number of CFUs in the – 0.25 MPa matric potential treatment increased to levels comparable to the other water potential conditions and the cells were smaller than they were in early stationary phase.

Discussion

In this study, we measured the physiological effects of water potential on *P. putida*. Our goal was to determine the importance of water potential to the biodegradation of a VOC, toluene. The physiological indices that we measured in this study included growth rate, substrate utilization rate, substrate utilization efficiency, yield, cell numbers and size. Our results suggest that VOC biodegradation may be strongly influenced by water potential and by whether the matric or solute component of total water potential is dominant. Further, carbon source appears to influence which component of total water potential, either solute or matric, is most important to microbial physiology. The data in Figure 1 best support these conclusions. Here, we see that for either carbon source, microbial growth rates are highest when the matric potential is slightly negative (– 0.25 MPa). We elaborate on this observation in the next paragraph. Further, with both carbon sources, microbial growth rates decrease with decreasing matric potentials below – 0.5 MPa. Still considering Figure 1, we find that solute potential effects on growth rate are quite different from matric potential effects. Primarily, the effect of solute potential on growth rates differs for the two different carbon sources: growth rates decrease with decreasing solute potential when glucose is the carbon source, yet solute potential doesn't affect growth rate when toluene is the carbon source. At the scale of the biodegrading microorganisms in soil and based on our findings, we might expect that growth rates will decrease when the carbon source shifts from a benign to a toxic (toluene) substrate. Further, we might expect that growth rates would be highest at a slightly negative matric potential, and that reductions in growth rates during soil drying would be attributable more to matric rather than solute effects when a VOC was the carbon source.

In that growth rates were faster at a – 0.25 MPa matric potential than at any other solute or matric water potential we examined, our results suggest that slightly negative matric water potentials may confer physiological advantages to bacteria originating from unsaturated environments. Similar to our results, Busse & Bottomley (1989) previously reported that slightly negative matric water potentials (– 0.25 MPa) can increase the growth rates of *Rhizobium meliloti* in soil. Also, McAneney et al. (1982) found that the growth rate of *Arthrobacter crystallopoietes* was faster at – 0.8 MPa matric potential as compared to either a similar solute potential, the basal medium control, or any other matric or solute potentials that were examined. Finally, the photosynthetic rates of cryptoendolithic cyanobacteria have been shown to be faster at a slightly negative matric water potential (Potts & Friedman 1981). All of these organisms, including *P. putida*, reside in soils and these responses suggest that many soil bacteria may be more physiologically suited to a range of matric potentials that are reflective of the indigenous soil ecosystem.

Our finding that cell yield was constant across a range of matric water potentials that are common in temperate ecosystems is an indication that low matric water potentials may not be as physiologically costly, in terms of maintenance energy requirements, as historically hypothesized (Pirt 1975; Brown 1990). Both Brown (1990) and Pirt (1975) describe the survival of microbes at low water potentials as energy-intensive. Brown (1990) provides the example of *Saccharomyces cerevisiae* that, due to the energy intensive production of glycerol at low matric water potentials, ceases to grow at – 8 MPa. A generalized response to water stress is production of compatible solutes (Harris 1981). Also, production of extracellular polysaccharides in response to matric water stress has been observed (Roberson & Firestone 1992). Responses such as production of compatible solutes or extracellular polysaccharides require cellular energy and should decrease the balance available for cell synthesis. However, for toluene-grown *P. putida* cultures, we found that there was no measurable effect of water potential on cell yield, as determined by measuring the total cellular protein content or ^{14}C incorporation efficien-

cy (Figure 3). Our findings suggest that physiological adjustments to water stress are not necessarily energy-intensive and that a long-standing soil microbiology paradigm warrants further study.

In comparing our findings regarding cellular efficiency to findings published previously, we conclude that certain measures used to indicate efficiency may introduce bias when water potential is the primary culture variable. More specifically, if we had interpreted our measurements of culture maximum OD (Figure 3) as the only index of biomass yield, as others have done (Schnell & King 1996), we would have concluded that matric stress significantly reduces yield. In this case, because the carbon source is completely utilized at the time of measurement, the relationship of yield to water potential can be analogized to efficiency/water potential relationships. We question the use of turbidemitry alone as a tool for indicating biomass yield in water stress experiments because the refractive index of cells is known to change according to culture osmolarity (Pirt 1975). In fact, turbidimetry is the tool often used to monitor cellular morphological changes in response to acute osmotic stress: culture turbidity changes upon exposure to osmolites (Mager et al. 1956; Knowles 1971; Alemohammad & Knowles 1974; Koch 1984). It is interesting that, for our study and Schnell & King's (1996), these types of changes apparently occur for only the non-permeating solute-amended cultures and not for the permeating solute-amended cultures. Changes in maximum OD with changing matric potential could conceivably be attributed to cell size changes. We observed that the cells grown at – 0.25 MPa matric potential were transiently larger at late exponential phase. However, cell sizes at lower matric potential culture conditions were not reported for our studies because, with increasing concentrations of PEG, the refractive index of the culture medium resulted in obscured cell boundaries by phase contrast microscopy. In addition, the other potential effects of water stress on cellular morphology, e.g. plasmolysis or outer membrane changes, could also affect turbidimetric measurements and thus make such measurements suspect as a tool for comparing cell yield across water potentials.

Finally, as with OD, we found that CFUs of late exponential cultures were not a useful measure of total biomass across the range of matric potentials studied (Figure 4). Because of the larger cell size at – 0.25 MPa, maximum CFUs were significantly lower than the other culture conditions (Figure 4A). The cells at – 0.25 MPa, because of their size, had a higher protein content than cells grown at other matric potentials (Figure 4B). Thus, comparing Figures 3 and 4, we see that the interpretation of how water stress affects total biomass is very dependent on the type of biomass measurement made. In our opinion, the cellular protein and carbon utilization efficiency measurements (Figure 3) are more reliable indices of yield because they were not subject to the cellular morphological changes affecting OD and CFU measurements.

In this study we have shown that water potential can affect the physiology of VOC-degrading bacteria. Specifically, we have shown that a slightly negative matric potential (– 0.25 MPa) increased the growth rate of *P. putida* when toluene was the carbon source and that toluene depletion rates and cell yield were practically unaffected by negative water potentials. Thus, the biodegradation of VOCs may be enhanced in slightly unsaturated systems as compared to fully saturated systems. Overall, our findings further define the role of water stress in determining biodegradation rates in the environment. Specifically, our findings can be used to predict how biodegradation in transiently wet and dry environments, such as soil or *ex situ* biofiltration reactors, may be affected by water availability.

Acknowledgements

This study was supported, in part, by the National Institute of Environmental Health Science Superfund Program grant 3P42 ES04705-07, by a graduate assistantship to P.A.H. from Los Alamos National Laboratory and by California AES Project # 6117-H. During the preparation of this manuscript, P.A. Holden's salary was provided through the U.S.E.P.A. Cooperative Agreement No. EPA-CR-832867010. We thank Dr Gary Sayler, University of Tennessee, Knoxville, for providing us with the strain of *P. putida* mt-2 and Dr Steven E. Lindow for access to equipment used in this study.

References

Alemohammad MM & Knowles CJ (1974) Osmotically induced volume and turbidity changes of *Escherichia coli* due to salts, sucrose and glycerol, with particular reference to the rapid permeation of glycerol into the cell. J. Gen. Microbiol. 82: 125–142

Andersson E & Hahn-Hägerdal B (1987) Enzyme action in polymer and salt solutions. II. Activity of penicillin acylase in poly(ethylene glycol) and potassium phosphate solutions in relation to water activity. Biochim. Biophys. Acta 912: 325–328

Banerjee S & Yalkowsky SH (1988) Cosolvent-induced solubilization of hydrophobic compounds into water. Anal. Chem. 60: 2153–2155

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Brown AD (1990) Microbial Water Stress Physiology. Chichester, John Wiley & Sons

Busse MD & Bottomley PJ (1989) Growth and nodulation responses of *Rhizobium meliloti* to water stress induced by permeating and nonpermeating solutes. Appl. Environ. Microbiol. 55: 2431–2436

Cox DP (1978) The biodegradation of polyethylene glycols. Adv. Appl. Microbiol. 23: 173–194

Daniel WW (1991) Biostatistics: A Foundation for Analysis in the Health Sciences. New York, John Wiley & Sons

Haines JR & Alexander M (1975) Microbial degradation of polyethylene glycols. Appl. Microbiol. 29: 621–625

Han SO & New PB (1994) Effect of water availability on degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) by soil microorganisms. Soil Biol. Biochem. 26: 1689–1697

Harris RF (1981) Effect of water potential on microbial growth and activity. In: Parr JF, Gardner WR & Elliott LF (Eds) Water Potential Relations in Soil Microbiology. SSSA Special Publication Number 9 (pp 23–95). Soil Science Society of America, Madison

Holden PA (1995) The effects of water potential on the biodegradation of a volatile hydrocarbon. PhD Dissertation. University of California, Berkeley

Knowles CJ (1971) Salt induces changes of turbidity and volume of *E. coli*. Nat. New Biol. 228: 154–155

Koch AL (1984) Shrinkage of growing *Escherichia coli* cells by osmotic challenge. J. Bacteriol. 159: 919–924

Laurent TC (1971) Enzyme reactions in polymer media. Eur. J. Biochem. 21: 498–506

Mackay D & Shiu WY (1981) A critical review of Henry's Law constants for chemicals of environmental interest. J. Phys. Chem. Ref. Data 10: 1175–1199

Mager J, Kuczynski M, Schatzberg G & Avi-Dor Y (1956) Turbidity changes in bacterial suspensions in relation to osmotic pressure. J. Gen. Microbiol. 14: 69–75

McAneney KJ, Harris RF & Gardner WR (1982) Bacterial water relations using polyethylene glycol 4000. Soil Sci. Soc. Am. J. 46: 542–547

Mexal J, Fisher JT, Osteryoung J & Reid CPP (1975) Oxygen availability in polyethylene glycol solutions and its implications in plant-water relations. Plant Physiol. 55: 20–24

Morris CM, Daughtridge JV & Bumgarner JE (1990) Standard operating procedures for the preparation and use of standard organic mixtures in the static dilution bottle. Atmospheric Research and Exposure Assessment Laboratory, Office of Research and Development, U.S.E.P.A., Research Triangle Park, NC, 27711

Morris KR, Abramowitz R, Pinal R, Davis P & Yalkowsky SH (1988) Solubility of aromatic pollutants in mixed solvents. Chemosphere 17: 285–298

Papendick RI & Campbell GS (1981) Theory and measurement of water potential. In: Parr JF, Gardner WR & Elliot LF (Eds) Water Potential Relations in Soil Microbiology. SSSA Special Publication Number 9 (pp 1–22). Soil Science Society of America, Madison

Petersen LW, Rolston DE, Moldrup P & Yamaguchi T (1994) Volatile organic vapor diffusion and adsorption in soils. J. Environ. Qual. 23: 799–805

Pirt SJ (1975) Principles of Microbe and Cell Cultivation. New York, John Wiley & Sons

Potts M & Friedmann EI (1981) Effects of water stress on cryptoendolithic cyanobacteria from hot desert rocks. Arch. Microbiol. 130: 267–271

Roberson EB & Firestone MK (1992) Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. Appl. Environ. Microbiol. 58: 1284–1291

Schnell S & King GM (1996) Responses of methanotrophic activity in soils and cultures to water stress. Appl. Environ. Microbiol. 62: 3203–3209

Sims JL, Sims RC, DuPont RR, Matthews JE & Russell HH (1993) *In situ* bioremediation of contaminated unsaturated subsurface soils, U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response, Washington, DC

Smibert RM & Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA & Krieg NR (Eds) Methods for General and Molecular Bacteriology (pp 607–654). American Society for Microbiology, Washington, DC

Soroker EF (1990) Low water content and low water potential as determinants of microbial fate in soil. PhD Dissertation. University of California, Berkeley

Stark JM & Firestone MK (1995) Mechanisms for soil moisture effects on activity of nitrifying bacteria. Appl. Environ. Microbiol. 61: 218–221

Taylor SA & Ashcroft GL (1972) Physical Edaphology: The Physics of Irrigated and Nonirrigated Soils. San Francisco, W.H. Freeman and Company