In Situ Determination of Subsurface Microbial Enzyme Kinetics

by J.D. Istok[1,4], J.A. Field[2], and M.H. Schroth[3]

Abstract

The single-well, push-pull test has been used in previous field studies to measure in situ zero- and first-order rates for aerobic and anaerobic microbial respiration in the saturated zone. In this paper we demonstrate that the test can also be used to obtain more generalized descriptions of the kinetics of microbially mediated enzymatic reactions. Laboratory and field tests were performed with the model enzyme substrate *p*-nitrophenyl-β-D-glucopyranoside (PNG). During a push-pull test, injected PNG is hydrolyzed in situ to *p*-nitrophenol (PNP); the rate of PNP production is taken as a measure of the β-glucosidase activity expressed by indigenous microorganisms. Laboratory tests were performed in physical aquifer models packed with natural aquifer sediment; field tests were performed in a shallow unconfined alluvial aquifer at a petroleum contaminated site. The laboratory and field tests demonstrate that it is possible to compute the in situ rate of PNP production as a function of PNG concentration using only data from a single push-pull test. These data can then be used to estimate the Michaelis-Menton kinetic parameters V_{max} and K_m for the hydrolysis reaction. This approach potentially extends the range of applicability of the push-pull test approach for use in determining kinetic parameters for a wide range of microbial processes in situ. These could include the broad class of substituted nitrophenyl substrates used to assay other enzyme systems, as well as microbially mediated redox reactions that occur during contaminant transformations.

Introduction

Quantitative information on subsurface microbial metabolic activity is needed: (1) to improve our understanding of flows of energy, carbon, and nutrients in the subsurface; (2) to quantify rates of transformations of xenobiotic compounds, including a wide diversity of organic and inorganic contaminants; and (3) to design and evaluate the effectiveness of various in situ biotechnologies. Currently, a wide variety of methods are being used to obtain this information including analysis of geochemical data (Lovley and Goodwin 1988); batch, column, and microcosm reactor studies (Wilson et al. 1983); direct observation and culture techniques (Harvey et al. 1984); biochemical marker techniques (Balkwill et al. 1988); molecular methods (Bowman et al. 1993); and ecological modeling (Kelly et al. 1988). The relative advantages and disadvantages of many of these methods are discussed in Chapelle (1993) and Ehrlich (1996). However, it is becoming increasingly apparent that in-situ testing methods will be required to fully characterize subsurface microbial activity, especially in environments that display steep biogeochemical gradients (Madsen 1991, 1998).

A field method for quantifying in situ microbial metabolic activity in the saturated zone of a ground water aquifer, called the single-well, push-pull test, was developed by Istok et al. (1997). Push-pull tests have previously been used (primarily in the petroleum industry) to determine aquifer physical characteristics (Istok et al. 1997). During the injection phase of a push-pull test aimed at determining microbial activity, a prepared test solution containing a noreactive tracer and one or more biologically-reactive solutes (substrates) is injected (pushed) into the saturated zone of an aquifer using an existing monitoring well. During the extraction phase, the test solution/ground water mixture is extracted (pulled) from the same location. Within the aquifer injected substrates are transformed to one or more defined metabolic products by the activity of indigenous microorganisms. A rest phase with no pumping may be included between the injection and extraction phases to allow sufficient time for detectable conversion of substrate to product. Samples collected during the extraction phase are analyzed to prepare breakthrough curves for tracer, substrate and products, which are then interpreted to obtain mass balance and reaction rate information (e.g., Haggerty et al. 1998; Snodgrass and Kitanidis 1998). Previous field applications of the push-pull test have demonstrated the ability of the method to quantify in situ rates of aerobic and anaerobic respiration at sites contaminated with petroleum

---

[1] Department of Civil Engineering, Oregon State University, Corvallis, OR 97331

[2] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR 97331

[3] Swiss Federal Institute of Technology–ETHZ, Institute of Terrestrial Ecology—Soil Biology SGC 19, Grabenstr. 3, CH-8952 Schlieren/ZH, Switzerland

[4] Corresponding author, (541) 737-6838 (voice mail), fax: (541) 737-3099; jack.istok@orst.edu

Received April 2000, accepted October 2000.

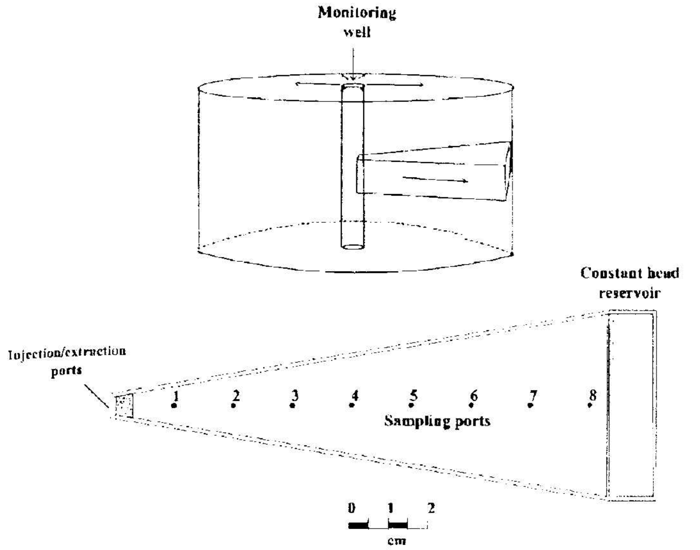

Figure 1. (a) The portion of the flow field near an injection/extraction well represented by physical aquifer models used in laboratory push-pull tests, and (b) plan view of a physical aquifer model.

hydrocarbons (Istok et al. 1997). A comparison of the push-pull test approach with other in situ testing methods (e.g., the closely related in situ microcosm approach) is also presented in Istok et al. (1997). Moreover, the utility of the method for quantifying spatial variability in these processes at the site scale also has been demonstrated (Schroth et al. 1998). In both these studies, microbial activity was expressed as the rate of consumption of an injected electron acceptor in the presence of abundant anthropogenic carbon to serve as the electron donor.

The overall objective of this study was to extend the development of the push-pull test method to allow for the determination of more generalized measures of microbial activity. In particular, we wished to determine if push-pull tests could be used to estimate in situ values for Michaelis-Menton kinetic parameters for a specific microbially mediated enzymatic reaction. For this purpose we utilized a model substrate selected from a class of well characterized substituted-nitrophenyl substrates. Compounds such as *p*-nitrophenyl phosphate, *p*-nitrophenyl sulfate, and *p*-nitrophenyl-β-D-glucopyranoside are routinely used to measure phosphatase, sulfatase, and glucosidase activities in batch soil and water incubation experiments in the disciplines of soil science, limnology, and oceanography (Tabatabai 1994; Miller 1972; Frankenberger and Dick 1983; Eivazi and Tabatabai 1990; Brams and McLaren 1974; Tabatabai and Bremner 1969; Eivazi and Tabatabai 1988; Chrost 1990). These compounds and assays share several characteristics that make them potentially suitable for use in field push-pull tests. In particular, all of the substituted *p*-nitrophenyl substrates and their common hydrolysis product, *p*-nitrophenol (PNP), have high aqueous solubilities and are expected to be only weakly sorbed to aquifer sediments. Microbially mediated hydrolysis rates are typically rapid in environmental systems allowing sensitive detection of PNP production within only a few hours. Moreover, rates of abiotic hydrolysis of the *p*-nitrophenyl substrates are small at typical ground water temperatures (10°C to 15°C).

This paper reports on laboratory and field push-pull tests that were conducted with the model substrate *p*-nitrophenyl-β-D-glucopyranoside (PNG). During a push-pull test, injected PNG is hydrolyzed in situ to *p*-nitrophenol (PNP); the rate of PNP production is taken as the measure of the β-glucosidase activity expressed by indigenous microorganisms. Laboratory push-pull tests were conducted in physical aquifer models packed with natural aquifer sediment; field push-pull tests were conducted in a shallow unconfined alluvial aquifer at a petroleum contaminated site. We demonstrate that it is possible to compute the rate of PNP production for a range of PNG concentrations and, thereby, to determine the Michaelis-Menton kinetics of β-glucosidase activity in situ, using data obtained from only a single push-pull test.

Methods

Laboratory Push-Pull Tests

Laboratory push-pull tests were performed in physical aquifer models (PAMs) constructed in a wedge-shape to approximate flow and transport of injected test solutions near a monitoring well during a field test (Figure 1). The PAMs were constructed of polypropylene with interior dimensions of 5 cm (width at narrow end), 50 cm (width at wide end), 125 cm (length), and 20 cm (height), and a total internal volume of 0.069 m^3 (Figure 1b). The PAMs were packed using the method of Istok and Humphrey (1995) with sediment from the Hanford Formation, an alluvial deposit of sands and gravels of mixed basaltic and granitic origin (Lindsey and Jaeger 1993). The sediment was collected as a single batch from an outcrop at a quarry near Pasco, Washington. The sediment was homogenized by manual mixing, air-dried to a water content between 2 and 3 wt %, and sieved to remove particles > 2 cm in diameter (which were < 0.01% of the original outcrop material). The sieved sediment was a clean sand with approximately 30% fine gravels and less than 5% silt and clay. The sediment contained less than 0.001 wt % organic matter and had a particle density of 2.9 g/cm^3. Batch experiments (data not shown) indicated negligible sorption of PNG and PNP to the seived sediment. The porosity and bulk density of the packed sediment were 0.39 and 1.77 g/cm^3, respectively. Bulk density and porosity were computed for the sediment packs used in laboratory experiments from the mass of sediment packed, the PAM internal volume, and the sediment particle density (as measured by helium pycnometry). During initial water saturation of the sediment pack, wetting fronts were observed to advance with a uniform and symmetrical shape, which indicated that the packing method had created an approximately homogeneous sediment pack. Tap water was used as the synthetic ground water in all laboratory experiments.

After the sediment pack was water saturated, the PAMs were sealed by installing a lid containing eight sampling ports (Figure 1b). Sampling ports were connected to brass well screens that fully penetrated the saturated thickness of the sediment pack. Additional wells were connected to manometers to measure hydraulic head. Experiments were performed under confined conditions. The saturated hydraulic conductivity of the sediment pack was determined periodically from head and pumping rate measurements and was nearly constant ($1.0 \times 10^{-2} \pm 0.6 \times 10^{-3}$ cm/s) during all tests.

Test solutions were injected and extracted using injection/extraction ports located on a vertical plate at the model's narrow end (designed to represent a portion of a monitoring well screen). During the injection phase of a test, flow was directed from the injection/extraction ports toward the model's wide end; during the extraction phase, the flow direction was reversed. A constant head reservoir was connected to the model's wide end to allow pore fluids to leave the sediment pack during the injection phase and to allow tap water to enter the sediment pack during the extraction phase. The volume of test solution was selected to ensure that no injected test solution left the sediment pack through the constant head reservoir.

Injected test solutions consisted of tap water containing 100 mg/L Br- (prepared from KBr) to serve as a conservative tracer. In laboratory test 1, 10 L of test solution containing 133 μM PNG were injected. In laboratory test 2, four separate test solutions were injected sequentially. The volumes and PNG concentrations of the four test solutions were 4 L:548 μM, 3 L:1404 μM, 2 L:2349 μM, and 1 L:3765 μM. In each test, the duration of the rest phase (the time between the end of the injection phase and the start of the extraction phase) was 85 minutes and extraction phase pumping continued until ~ 20 L had been extracted. Injection and extraction pumping rates were constant at ~ 85 mL/min. Water samples were collected from selected sampling ports during the injection, rest, and extraction phases by inserting a stainless steel syringe needle to the mid-depth of the well screens through a septum in the sampling port cap. Additional water samples were collected from the injection/extraction ports during the injection and extraction phases. A 1 mL aliquot of each sample was immediately combined with 0.5 mL of a quench solution in a 2 mL glass vial to prevent additional enzyme-mediated hydrolysis of PNG within the sample containers prior to analysis (Tabatabai 1994). The quench solution consisted of 0.25 M calcium chloride and 0.20 Mtris[hydroxymethyl]amino-methane (Tris) that was adjusted to pH 12 with NaOH. Samples were stored at 4°C until analyzed.

Field Push-Pull Tests

Field tests were performed at a petroleum-contaminated site used in previous push-pull test studies (Schroth et al. 1998). The aquifer at this site was formed in lacustrine deposits of clayey silt and silt, with occasional traces of fine sand, gravel, and peat. The aquifer is unconfined and water table depths range from 0.5 to 1.5 m below land surface. The average ground water (Darcy) velocity at the time of the tests was approximately 10^{-3} m/d. Ten monitoring wells were installed with a hollow-stem auger to a depth of approximately 7 m and were constructed of 5 cm internal diameter polyvinyl chloride well casing and screen. Push-pull tests were performed in monitoring wells MW-1, which is located within the most heavily contaminated and anaerobic portion of the site where Schroth et al. (1998) measured the highest respiration rates. Note that a map showing well locations and measured values for a suite of geochemical indicators are in Schroth et al. (1998).

Injection and extraction phases of the tests were conducted within a 1 m long interval of the well screen using inflatable straddle packers and a peristaltic pump as described in Schroth et al. (1998). Test solutions were prepared from tap water containing 100 mg/L Br^-. In field test 1, 30 L of test solution containing 133 μM PNG were injected. In field test 2, four separate test solutions were injected sequentially. The volume and PNG concentration of the test solutions were 12L (182 μM), 9 L (464 μM), 6 L (869 μM), and 3 L (1335 μM). Field test 3 was conducted to determine the transport behavior and reactivity of PNP formed during tests 1 and 2. In this test, the injected test solution contained 10 μM PNP and no PNG. The field tests were performed approximately one week apart. For all tests, injection and extraction pumping rates were constant at ~ 1 L/min, the duration of the rest phase was 85 minutes, and extraction pumping continued until ~ 60 L had been extracted. Water samples were collected during the injection and extraction phases and handled and stored as described in the previous section. Note that Radakovich et al. (in review) found in lab-

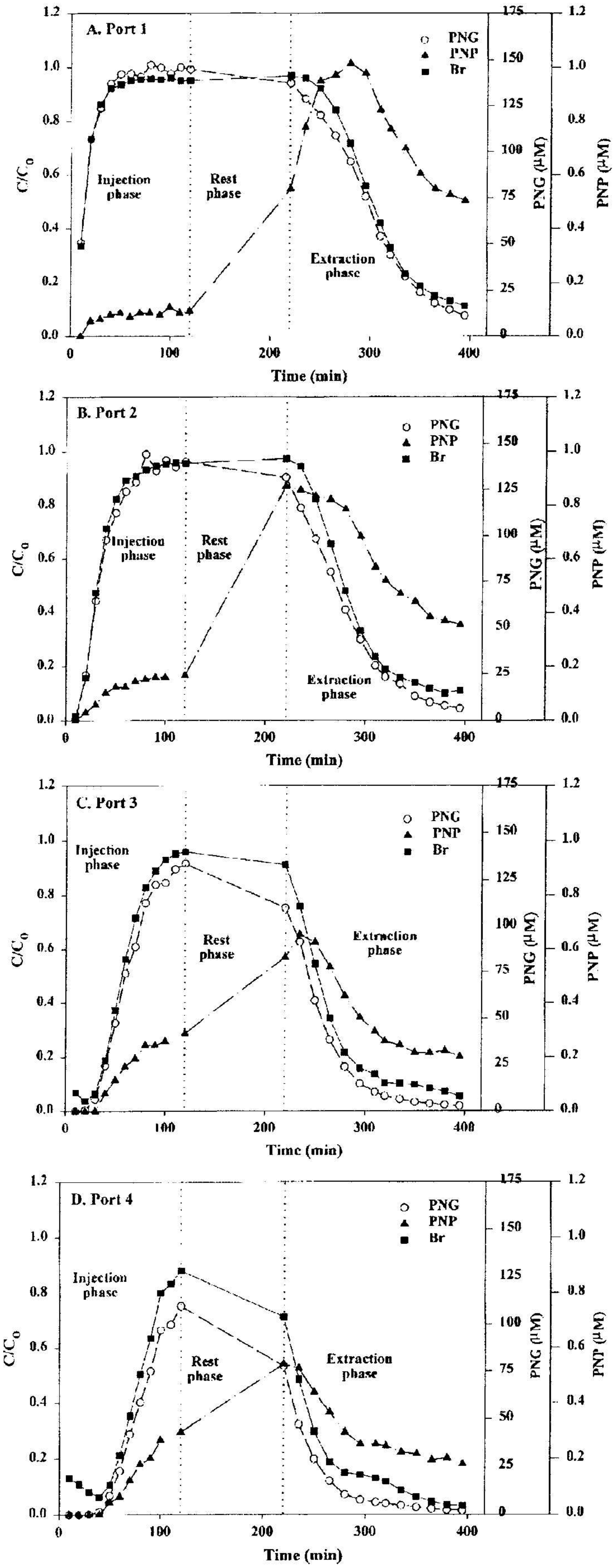

Figure 2. Br^-, PNG, and PNP concentrations measured at sampling ports 1 through 4 during injection, rest, and extraction phases of laboratory test 1. Decrease in PNG concentration and increase in PNP production during the rest phase is attributed to β-glucosidase activity.

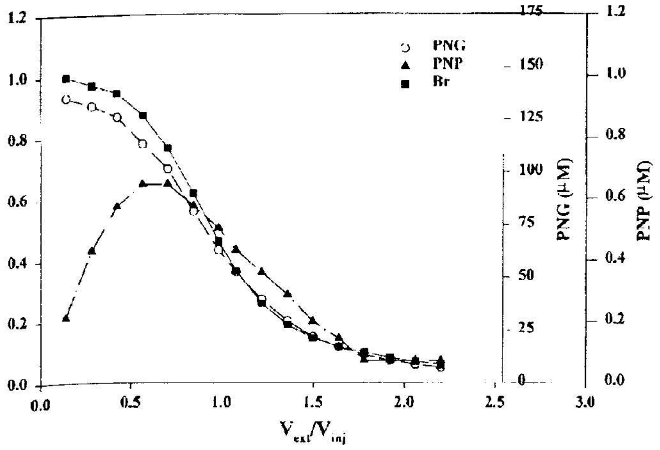

Figure 3. Extraction phase breakthrough curves for Br$^-$, PNG, and PNP for laboratory test 1 showing PNP production attributed to β-glucosidase activity.

oratory studies that abiotic hydrolysis of PNG in ground water from this site resulted in negligible PNP production on the time scale of these experiments.

Analytical Methods

Water samples collected during laboratory and field push-pull tests were analyzed for Br-, PNG, and PNP. Bromide concentrations were determined using a Dionex Model DX-120 ion chromatograph equipped with electrical conductivity detector (Sunnyvale, California). PNG and PNP concentrations were determined using a high performance liquid chromatograph (Alliance 2690, Waters Corp., Milford, Massachusetts) equipped with a ®Luna 5µm C18(2) column (150 mm × 4.60 mm id), and a photodiode array detector. Separation of PNG and PNP was achieved by reverse phase chromatography coupled with gradient elution with a mobile phase mixture of 50 mM phosphate buffer, buffered to pH 2.5, and methanol.

Results and Discussion

Laboratory Push-Pull Tests

Bromide, PNG, and PNP concentrations for sampling ports 1 through 4 during the injection, rest, and extraction phases of laboratory test 1 illustrate the types of data obtained during laboratory push-pull tests (Figure 2). Note that these type of data are not available during field tests (discussed later) and they are included here because they confirm that our interpretation of extraction phase breakthrough curves in both laboratory and field push-pull tests are reasonable. Relative concentrations for Br$^-$ (C/C_0, where C is the Br$^-$ concentration in a sample and C_0 = 100 mg/L is the Br$^-$ concentration in the injected test solution) increased smoothly from zero to one at each port during the injection phase (0 to 120 minutes) as the test solution penetrated further into the sediment pack. Computed Br$^-$ arrival times for the ports were essentially identical with those predicted from the PAM geometry, sediment pack properties, and pumping rates. Bromide concentrations in ports 1 through 3 remained essentially constant during the rest phase (120 to 205 minutes) while those in port 4, which is near the limit of the sediment zone penetrated by the injected test solution, decreased by ~ 12% due to dilution with Br$^-$-free tap water from beyond this zone. Bromide concentrations gradually decreased to zero in all ports during the extraction (205 to 400 minutes) phase as tap water entering the PAM from the constant head reservoir gradually displaced injected test solution from the sediment pack (Figure 2). Br$^-$ concentrations at the injection/extraction ports also gradually decreased to zero during the extraction phase as the test solution was removed from the sediment pack (Figure 3). In Figure 3, V_{ext} is the cumulative volume of water extracted at the time the sample was collected and V_{inj} is the volume of test solution injected. Mass balance calculations indicated that 91.1% of the injected Br$^-$ was recovered during the extraction phase of laboratory test 1.

Similar to Br$^-$, PNG concentrations increased smoothly during the injection phase in all sampling ports (Figure 2). The nearly identical transport behavior of PNG and Br$^-$ observed in ports 1 and 2 indicates that sorption of PNG is negligible in this sediment. However, PNG concentrations did not reach the PNG concentration in the injected test solution (133 µM) at ports 3 and 4 due to the microbially-mediated hydrolysis of a portion of the injected PNG to PNP by microorganisms in the sediment pack. Note that the Br$^-$ and PNG axes in Figure 2 are scaled to allow direct comparison of relative concentrations for Br$^-$ and PNG. Thus, a value of C/C_0 = 1 for Br$^-$ will plot at the same location as a value of PNG = 133 µM. PNG concentrations in ports 1 through 4 decreased (relative to Br$^-$) during the rest phase. Zero-order reaction rate coefficients for PNG hydrolysis, computed from the change in PNG concentration during the rest phase, ranged from 0.337 to 0.577 µM/hr. Note that the results of laboratory batch experiments conducted with autoclaved sediment (results not shown) indicated that abiotic hydrolysis of PNG to PNP is negligible at the temperature and time scale of the laboratory push-pull tests.

PNG hydrolysis was also apparent in the reduced PNG concentrations (relative to Br$^-$) in the injection/extraction ports during the extraction phase. The mass of PNG hydrolyzed during the test is proportional to the area between the plotted Br- and PNG breakthrough curves (Figure 3). Mass balance calculations indicate that 85.6% (0.218 mmoles) of the injected PNG was recovered during the extraction phase compared to 91% for Br$^-$. PNG hydrolysis was also indicated by PNP production observed during the test. It should be noted that a small quantity of PNP (~0.025 µM) was measured in injected test solutions due to its presence as a trace contaminant in the PNG reagent used in this project. However, PNP concentrations increased to levels substantially above 0.025 µM during the injection phase in ports 1 through 4 (Figure 2). PNP production is perhaps most clearly seen in the increase in PNP concentration that occurred in all ports during the rest phase. Zero-order reaction rate coefficients, computed as the increase in PNP concentration during the rest phase, ranged from 0.194 to 0.430 µM/hr. During the extraction phase, PNP concentrations initially increased and then gradually decreased as the injected test solution was gradually displaced from the sediment pack by tap water entering the wide end of the PAM (Figure 2).

PNP production is also seen in the PNP breakthrough curve measured at the injection/extraction ports during the extraction phase (Figure 3). PNP concentrations were initially small, increased to a maximum value of 0.65 µM and then gradually decreased to zero as the test solution was gradually displaced from the sediment pack. In Figure 3, the mass of PNP produced within the sediment pack during the test is proportional to the area beneath the PNP breakthrough curve. Mass balance calculations indicated that 0.016 mmoles of PNP were produced during this test, which is substantially less than the computed mass of PNG hydrolyzed (0.198

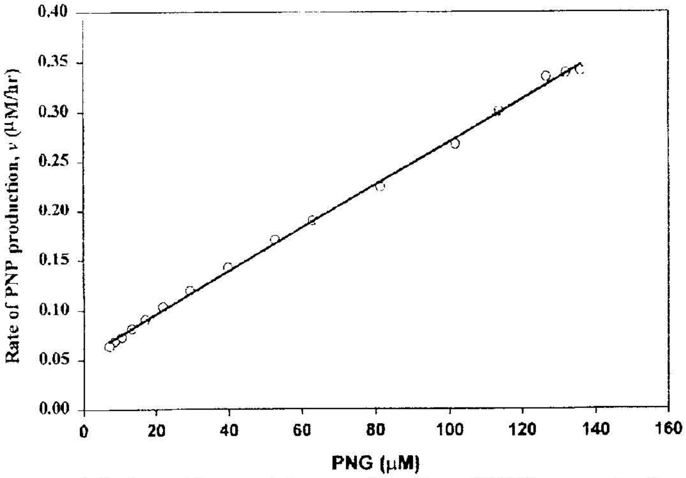

Figure 4. β-glucosidase activity as a function of PNG concentration obtained from the extraction phase breakthrough curves for laboratory push-pull test 1. Lines shows fitted linear model used to estimate first-order reaction rate coefficient for PNP production.

mmoles). It should be noted that abiotic hydrolysis was not a significant factor contributing to PNP production; no increase in PNP concentration was observed in subsamples of injected test solution held at the laboratory temperature throughout the experiment. Radakovich et al. (in review) also reported insignificant rates of abiotic hydrolysis for PNG in a seven-hour period at 25°C.

β-glucosidase activity in soils, sediments, rivers, lakes, oceans, and ground water has been shown to follow Michaleis-Menton kinetics:

$$v = \frac{V_{max}S}{k_m + S} \tag{1}$$

where v is the initial reaction velocity, V_{max} is the maximum rate of substrate utilization, S is the substrate concentration, and K_m is the Michaelis constant, which is equal to the substrate concentration at a reaction velocity equal to one-half the maximum rate of substrate utilization (i.e., $v = V_{max}/2$). When $K_m >> S$, Equation 1 reduces to the linear expression $v = (V_{max}/K_m)S = kS$, where k is a first-order reaction rate coefficient. Similarly, when $S >> K_m$, Equation 1 reduces to $v = V_{max}$ and the reaction velocity is constant (i.e., the reaction is zero-order).

The kinetics of PNG hydrolysis to PNP were quantified through an empirical analysis of the residence time distribution for the injected test solution obtained from the extraction phase breakthrough curve for the Br^- tracer. Recall that in a push-pull test, the last portion of the test solution injected is the first to be extracted and therefore remains in the sediment pack the shortest amount of time. Similarly, the first portion of the test solution injected is the last to be extracted and therefore remains in the sediment pack the longest time. Thus, by considering the transport of the test solution within the sediment pack to be similar to that in a plug flow reactor, we can estimate the residence time for each portion of the test solution sampled during the extraction phase. Let t_i be the elapsed time for extraction phase sample i, where the start of the injection phase is taken as time zero and let M_i be the cumulative mass of Br^- extracted at time t_i. Then the residence time (i.e., the time the sample was in contact with the sediment and thus subject to β-glucosidase activity) t^*_i, can be computed using

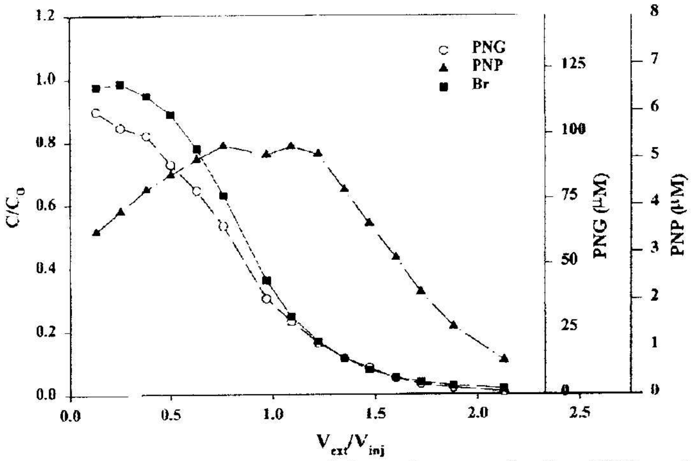

Figure 5. Extraction phase breakthrough curves for Br^-, PNG, and PNP for laboratory test 2 showing PNP production attributed to β-glucosidase activity.

$$t^*_i = t_i - \frac{\left(1 - \frac{M_i}{M_{ext}}\right)}{M_{ext}} T_{inj} \tag{2}$$

where M_{ext} is the total mass of Br^- extracted during the extraction phase and T_{inj} is the duration of the injection phase. Note that Haggerty et al. (1998) and Snodgrass and Kitanidis (1998) used a similar method of computing residence times for interpreting push-pull test data and Haggerty et al. (1998) used this approach to develop procedures for estimating zero- and first-order reaction rate coefficients from extraction phase breakthrough curves for a nonreacting tracer and a co-injected reactive solute. In this study we extended this approach to allow a more generalized description of the kinetics of the PNG hydrolysis reaction, and, by extension any microbial activity assayed by a push-pull test that involves conversion of injected substrate to product. For each extraction phase sample, we computed the residence time using Equation 2 and then computed the initial reaction velocity, v_i, as the rate of PNP production, $v_i = PNP_i/t^*_i$, where PNP_i is the PNP concentration in sample i. By plotting the pairs of values (v_i, PNG_i), where PNG_i is the PNG concentration in sample i, it is possible to examine the effect of substrate concentration on the velocity of the hydrolysis reaction.

Applying this procedure to the extraction phase breakthrough curves for laboratory test 1, the rate of PNP production increased linearly with increasing PNG concentration (Figure 4); the fitted first-order rate coefficient was k = 0.0022 h-1 ($r^2 = 0.998$). Note that although the injected test solution contained a single PNG concentration, a range in PNG concentrations from 0 to 133 µM was created within the sediment pack due to advective-dispersive transport of the injected test solution during the injection and extraction phases of the test.

The observed linear increase in reaction velocity with increasing PNG concentration indicates that the effective Michaelis-constant, K_m, for the β-glucosidase enzyme system of the mixed population of microorganisms within the sediment pack is greater than 133 µM. By increasing the PNG concentration in the injected test solution it is possible to investigate reaction velocities over a wider range in substrate concentrations. However, the relatively high cost of PNG and other substituted-nitrophenyl substrates make it undesirable to inject the total volume of test solution (e.g., 10 L in the laboratory tests and 30 L in the field tests) prepared with a sin-

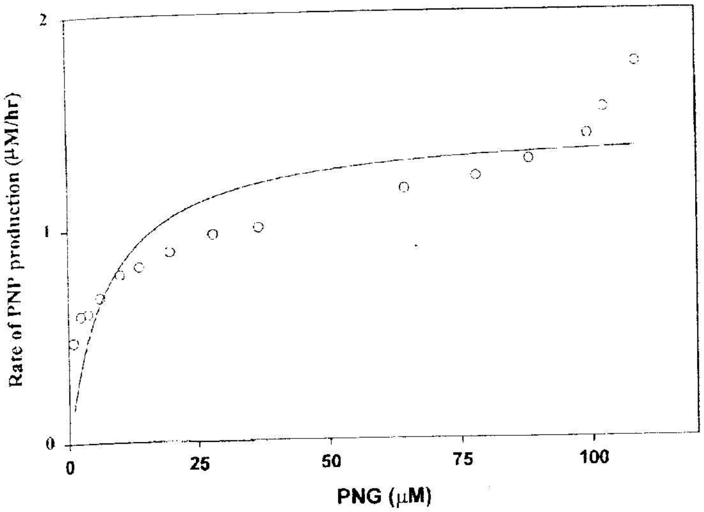

Figure 6. β-glucosidase activity as a function of PNG concentration obtained from the extraction phase breakthrough curves for laboratory push-pull test 2. Line shows fitted Michaelis-Menton equation used to estimate K_m and V_{max} (reaction velocities for PNG < 50 µM were not used to estimate K_m and V_{max}).

gle high substrate concentration $S >> K_m$. Fortunately this is not required, as a similar wide range in PNG concentrations can be created within the sediment pack by sequentially injecting a series of test solutions that vary in volume and substrate concentration.

As an example of this approach, laboratory test 2 was conducted in a fresh sediment pack by sequentially injecting four test solutions that varied in PNG concentrations from 548 to 3765 µM. As expected, higher PNP concentrations were observed than in laboratory test 1 (Figure 5). Note that in this case, the mass of PNG hydrolyzed is not proportional to the area between the Br^- and PNG breakthrough curves because of the varying PNG concentration in the injected test solution. The PNP concentration initially increased to 2.2 µM at $V_{ext}/V_{inj} = 1$, remained constant for $1 < V_{ext}/V_{inj} < 1.8$, and then increased to 2.7 µM at the end of the test. Mass balance calculations indicate that 91% (16.99 mmoles) of the injected PNG was recovered during the extraction phase compared to 89% for Br^-. The computed mass of PNP produced was 0.084 mmoles.

Reaction velocities computed using Equation 2 initially decreased sharply with increasing PNG concentration for PNG < 40 µM and then increased, following the hyperbolic form of the Michaelis-Menton equation (Figure 6). The anomalous high computed reaction velocities at low PNG concentrations are the result of the increase in PNP concentration that occurred during decreasing PNG concentrations at the end of the extraction phase (Figure 5). The same anomalous increase in PNP concentration was observed at all sampling ports (data not shown). The explanation for this increase is unknown but the behavior was replicated in a duplicate test conducted in a fresh sediment pack. Our hypothesis is that the high reaction velocities at low substrate concentrations resulted from induction of the β-glucosidase enzyme system caused by exposure of the microorganisms to the very high PNG concentrations used in this test. Induction of the β-glucosidase system by previous exposure to PNG is well established in the literature and was observed in other laboratory push-pull tests in which replicate experiments conducted in the same sediment pack with the same PNG concentration resulted in increased PNP concentrations and rates of PNP production (data not shown). Although enzyme induction complicates the interpretation of the enzyme kinetics in test 2, it is interesting to note that a single push-pull test was able to detect this effect and to quantify rates of PNP production over a wide range in substrate concentration. If the reaction velocities for PNG concentrations less than 40 µM are not considered, the reaction velocities are approximately described by Equation 1 with $K_m = 43$ µM and $V_{max} = 0.55$ µM/h ($r^2 = 0.90$).

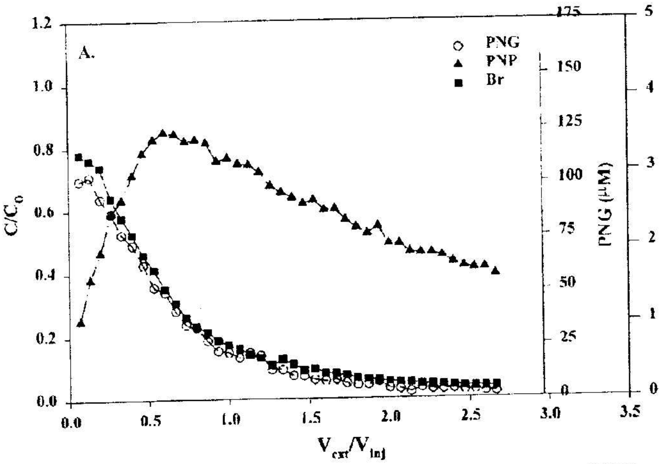

Figure 7. Extraction phase breakthrough curves for Br-, PNG, and PNP for (a) field test 1, and (b) field test 2, showing PNP production attributed to β-glucosidase activity.

Field Push-Pull Tests

In situ hydrolysis of injected PNG to PNP was also observed in field push-pull tests and is attributed to the expression of β-glucosidase activity by indigenous microorganisms in the portion of the aquifer interrogated during the test. The extraction phase breakthrough curves for field test 1 illustrate the type of data obtained (Figure 7a). Extraction phase breakthrough curves for Br^- showed a gradual decline in relative concentration from ~ 0.8 to ~ 0.05 as the injected test solution/ground water mixture was pumped from the aquifer. Mass balance calculations indicated that 60% of the injected Br^- was recovered during the extraction phase of field test 1. PNG breakthrough curves were generally similar to those for Br^- but relative concentrations were slightly less due to hydrolysis of a portion of the injected PNG to PNP. Mass balance calculations indicate that 57% (2.89 mmoles) of the injected PNG was recovered. The computed mass of PNG hydrolyzed (injected mass minus extracted mass, adjusted for Br^- recovery) was 0.76 mmoles.

Evidence for PNG hydrolysis is also clearly seen in the extraction phase breakthrough curves for PNP (Figure 7a). PNP concentrations were initially small, increased to a maximum value of 3.6 µM at $V_{ext}/V_{inj} \sim 0.8$ and then gradually decreased as the test solution/ground water mixture was extracted from the aquifer. Mass balance calculations indicated that 0.20 mmoles of PNP were produced during field test 1. The kinetics of PNG hydrolysis to PNP were quantified using the analysis of the extraction phase breakthrough curves described previously for the laboratory tests. Michaelis-Menton behavior was observed with reaction velocities increasing with increasing PNG concentration (Figure 8A). The data were fit very well by Equation 1 with $K_m = 7.5$ µM and $V_{max} = 0.06$ µM/h ($r^2 = 0.98$).

The value of $V_{max} = 0.06$ µM/h obtained for the Hanford Formation sediment in laboratory test 1 is much smaller than the values of β-glucosidase activity typically reported for field moist surface soils in the field of soil science. For example, Dick et al. (1996) reported values of PNP production in the presence of excess

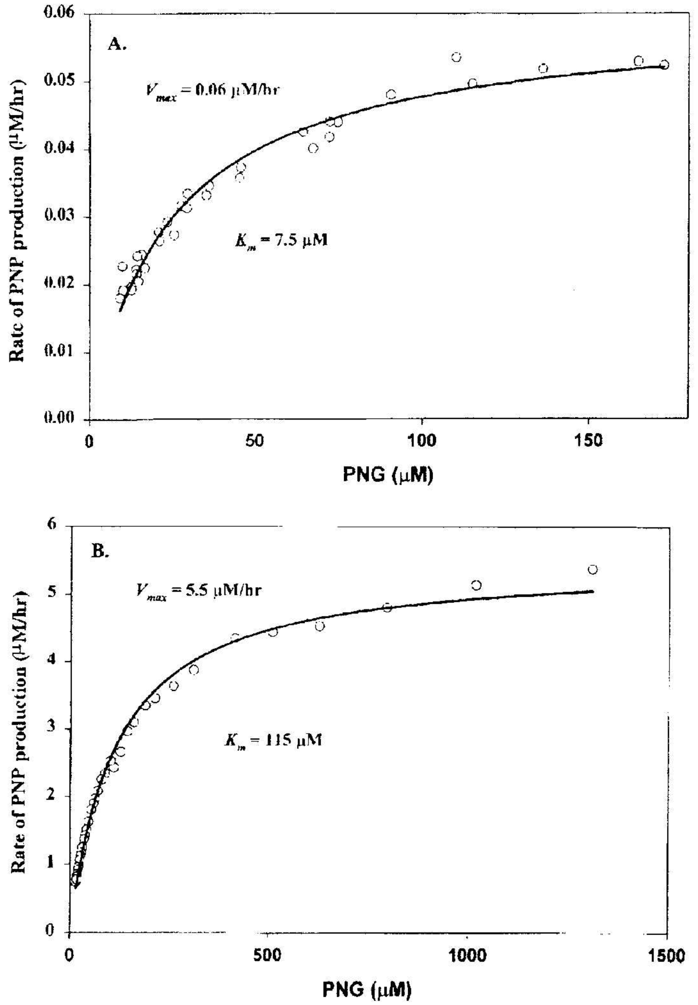

Figure 8. β-glucosidase activity as a function of PNG concentration obtained from the extraction phase breakthrough curves for (a) field test 1, and (b) field test 2. Lines show fitted Michaelis-Menton equation used to estimate K_m and V_{max} for each test.

PNG that ranged from 38 to 720 mg PNP kg^{-1}h^{-1} (Dick et al. 1996). Using a porosity of 0.39 and a bulk density of 1.77 g/cm^3, the computed value of V_{max} in these units for laboratory test 1 is 1.8 × 10^{-3} mg PNP/kg h. The smaller activities measured in our experiments are attributed to the much lower biomass, organic carbon content, and nutrient availability in our sediment compared to surface soils. The ability of the push-pull test method to detect such small rates of PNP production makes it potentially applicable for quantifying β-glucosidase activity in other oligotrophic and low biomass subsurface sediments.

In an earlier study, Radakovich et al. (in review) found that the kinetics of β-glucosidase activity in ground water samples collected from this site varied from one well to the next, which is not unexpected given the large variation in petroleum hydrocarbon levels and in the aerobic and anaerobic respiration rates measured in push-pull tests by Schroth et al. (1998). In this study values of in situ β-glucosidase activity determined by push-pull tests in some wells was extremely high and required much higher injected PNG concentrations to obtain a sufficiently wide range in reaction velocities to allow accurate estimation of K_m and V_{max}. An example is field test 2 conducted in MW-4. In this test, a series of test solutions were injected to create a range in injected PNG concentrations from 182 to 1335 µM. As a result, measured PNP concentrations during the extraction phase of field test 2 were much higher than during field test 1 (conducted in MW-1) and reached a maximum value of 7.59 µM at $V_{ext}/V_{inj} = 0.73$ (Figure 7b). Mass balance calculations indicated that 54% and 47% of the inject Br$^-$ and PNG, respectively were recovered and that 0.349 mmoles of PNP were produced during the test. The kinetics of PNP production during this test showed typical Michaelis-Menton behavior with reaction velocities increasing with increasing PNG concentration (Figure 8b); the data were fit very well by Equation 1 with K_m = 115 µM and V_{max} = 5.5 µM/h ($r_2 = 0.98$).

Conclusions

This study has demonstrated the ability of the push-pull test method to detect and quantify β-glucosidase activity in situ by monitoring the production of *p*-nitrophenol (PNP) from injected *p*-nitrophenyl-β-D-glucopyranoside (PNG). Activity was detected within a few hours in tests conducted in packed sediments in the laboratory and in a petroleum-contaminated aquifer in the field. Using this approach β-glucosidase activity can be easily determined in situ for any defined set of test conditions. Moreover, we have demonstrated that it is possible to compute the rate of PNP production for a range of PNG concentrations and, hence, to determine in situ the apparent Michaelis-Menton kinetic parameters V_{max} and K_m using only data from a single push-pull test. This approach extends the applications of the push-pull test to the in situ determination of kinetic parameters for a wide range of microbial activities and should have numerous applications in the study of subsurface microbial processes.

Acknowledgments

This work was funded by the U.S. Department of Energy Natural and Accelerated Bioremediation Research (NABIR) program; Dr. Anna Palmisano, project manager. Special thanks to Emily Beezhold, Karen Radakovich, Mark Humphrey, and Ralph Reed at Oregon State University for their assistance with the laboratory and field experiments.

References

Balkwill, D.L., F.R. Leach, J.T. Wilson, J.F. McNabb, and D.C. White. 1998. Equivalence of microbial biomass measures based on membrane lip and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. *Microbial Ecology* 16, 73–84.

Bowman, J.P., L. Jimenez, I. Rosario, T.C. Hazen, and G.S. Sayler. 1993. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. *Applied and Environmental Microbiology* 59, 2380–2387.

Brams, W.H., and A.D. McLaren. 1974. Phosphatase reactions in columns of soils. *Soil Biology and Biochemistry* 6, 183–189.

Chapelle, F.H. 1993. *Ground-Water Microbiology and Geochemistry.* New York: John Wiley & Sons Inc.

Chróst, R.J. 1990. Microbial ectoenzymes in aquatic environments. In *Aquatic Microbial Ecology*, ed. J. Overbeck and R.J. Chróst, 47–78. New York: Springer-Verlag.

Dick, R.P., D.P. Breakwell, and R.E. Turco. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In *Methods for Assessing Soil Quality*, Soil Science Society of America Special Publication 49, 247–271. Madison, Wisconsin. SSSA.

Ehrlich, H.L. 1996. *Geomicrobiology*. New York: Marcel Dekker Inc.

Eivazi, F., and M.A. Tabatabai. 1988. Glucosidases and galactosidases in soils. *Soil Biology and Biochemistry* 20, 601–606.

Eivazi, F., and M.A. Tabatabai. 1990. Factors affecting glucosidase and galactosidase activities. *Soil Biology and Biochemistry* 22, 891–897.

Frankenberger, W.T., and W.A. Dick. 1983. Relationship between enzyme activity and microbial growth and activity indices in soil. *Soil Science Society of America Journal* 47, 945–951.

Haggerty, R., M.H. Schroth, and J.D. Istok. 1998. Simplified method of "push-pull" test data analysis for determining in situ reaction rate coefficients. *Ground Water* 36, no. 2: 314–324.

Harvey, R.W., R.L. Smith, and L. George. 1984. Effect of organic contamination upon microbial distributions and heyterotrophic uptake in a Cape Cod, Massachusetts, aquifer. *Applied and Environmental Microbiology* 53, 2992–2996.

Istok, J.D., and M.D. Humphrey. 1995. Laboratory investigation of buoyancy-induced flow (plume sinking) during two-well tracer tests. *Ground Water* 33, no. 4: 597–604.

Istok, J.D., M.D. Humphrey, M.H. Schroth, M.R. Hyman, and K.T. O'Reilly. 1997. Single-well, "push-pull" test method for in situ determination of microbial metabolic activities. *Ground Water* 35, no. 4: 619–631.

Kelly, F.X., K.J. Dapsis, and D.A. Lauffenburger. 1988. Effect of bacterial chemotaxis on dynamics of microbial competition. *Microbial Ecology* 16, 115–131.

Lindsey, K.A., and G.K. Jaeger. 1993. Geologic setting of the 100-HR-3 operable unit, Hanford site, south-central Washington. WHC-SD-EN-TI-132, Rev. 0, Westinghouse Hanford Co., Richland, Washington.

Lovley, R., and S. Goodwin. 1988. Hydrogen concentrations as an indicator of the predominant terminal electron accepting reactions in aquatic sediments. *Geochimica et Cosmochimica Acta* 52, 2993–3003.

Madsen, E.L. 1991. Determining in situ biodegradation—Facts and challenges. *Environmental Science and Technology* 25, 1663–1673.

Miller, J.H. 1972. Assay of β-Galactosidase. In *Experiments in Molecular Genetics*, 352–359. Cold Spring Harbor Press.

Radakovich, K., J. A. Field, and J.D. Istok. In review. β-glucosidase activity in contaminated groundwater. Submitted to *Ground Water*.

Schroth, M.H., J.D. Istok, G.T. Conner, M.R. Hyman, and K.T. O'Reilly. 1998. Spatial variability in aerobic respiration and denitrification rates in a petroleum contaminated aquifer. *Ground Water* 36, no. 6: 924–937.

Snodgrass, M.F., and P.K. Kitanidis. 1998. A method to infer in situ reaction rates from push-pull experiments. *Ground Water* 36, no. 4: 645–650.

Tabatabai, M.A. 1994. Soil Enzymes. In *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America Book Series, No. 5, 775–835.

Tabatabai, M.A., and J.M. Bremner. 1969. Use of *p*-nirophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* 1, 301–307.

Wilson, J.T., J.F. McNabb, D.L. Balkwill, and W.C. Ghiorse. 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. *Ground Water* 21, no.2: 134–142.