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Effects of microcosm preparation on rates of toluene biodegradation under denitrifying conditions

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Microcosms were prepared with subsurface material from two aquifers to examine the effects of preparation methods on rates of toluene biodegradation under denitrifying conditions. In both cases, the data fit a zero-order kinetics plot. However, rates of removal were generally proportional to initial toluene concentrations, resulting in similar half-lives. Increasing the solid/liquid mass ratio resulted in decreased lag times in one aquifer material, although in both cases the specific toluene mass removal rate (g toluene g sediment $^{-1}$ day^{-1}) also decreased. Varying either the initial toluene concentrations or the solid/liquid ratios by two to three orders of magnitude resulted in a half-life variation of only a factor of two, with most of the differences occurring at the extreme ranges of the test variables. These data indicate that similar biodegradation rates might be expected from microcosms prepared with different contaminant concentrations and solid/liquid ratios, which is useful for design of microcosm studies to evaluate biodegradation at field sites.

Keywords: microcosm; aquifer; anaerobic; denitrification; rate; toluene

Introduction

Microcosms can be used to evaluate the potential of subsurface microorganisms to degrade a particular type of organic contaminant under a specific set of conditions, with the goal of using bioremediation as a management strategy for contaminated aquifers. Although studies for these purposes have historically been limited to evaluating aerobic processes [26,29], the interest in engineered anaerobic bioremediation, as well as in the potential for intrinsic microbial processes to reduce the risk of contamination, is promulgating similar evaluations of anaerobic processes [5,6,8,12,13,30]. Microcosms have been used in several cases to evaluate the potential for biodegradation of fuel hydrocarbons under denitrifying conditions [7,9,10,16,27], and in some cases efforts have been made to correlate laboratory rates with field performance [4,11,21]. It is difficult to compare and correlate research results in these cases, since each study uses different types of microcosms, and measured rates of biodegradation may depend on the specific methods of microcosm preparation. Preparation, incubation, and analysis of microcosms involve several variables, and there are few data on the effects of these variables. For example, microcosms are often constructed with a minimal amount of aquifer material (ie, low solid/liquid mass ratio) both to conserve aquifer material and to provide sufficient aqueous solution for chemical and biological analyses. Questions then arise as to whether rates of biodegradation should be corrected for mass of solids or can be compared directly to other studies with different solid/liquid mass ratios. Conversely, using high solid/liquid mass ratios will limit the amount of solution available, which can adversely affect the detection limits for analyses requiring larger volumes, and concentrations of contaminants may have to be increased to account for this as well as greater sorption by aquifer solids. The current study was therefore conducted to evaluate how these two variables, initial substrate concentration and the solid/liquid mass ratio, affected rates of toluene biodegradation under denitrifying conditions in core material from two separate aquifers which had been designated for field studies on nitrate-based bioremediation.

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Materials and methods

Preparation and analysis of microcosms

Aquifer material was obtained from shallow water-table aquifers located in Traverse City, MI, and Park City, KS, USA. These sites had been selected for field demonstration of nitrate-based bioremediation, and aquifer characteristics are reported elsewhere [18,24]. In brief, the Traverse City aquifer is composed of thick glacial deposits; the upper portion is lacustrine in origin. This material was approximately 19% coarse sand, 63% medium sand, and 17% fine sand, with an organic carbon content of 0.024%. Aquifer material was collected from 4.9–9.1 m below ground surface and used for a study involving columns [20], during which the material was exposed to nitrate and aromatic hydrocarbons. The columns were then dismantled and the column material was used for this study. The Park City material was collected from 5.5–6.8 m below ground surface from a heterogeneous, unconsolidated sand and gravel aquifer. This material was approximately 65% coarse sand and gravel, 28% medium sand, and 7% fine sand, with an organic carbon content of 0.062%. Unlike the Traverse City aquifer material, it received no additional treatment. Microcosms were aseptically prepared and incubated in an anaerobic glovebox by adding core material to 12-ml serum bottles as described previously [17]. For the test on the effects of toluene concentration, all microcosms were prepared with

5.0 g wet weight aquifer material. For the test on the effects of solid/liquid ratios, Traverse City microcosms were prepared with 1.0, 5.0, 10.0, and 15.0 g wet weight aquifer material and Park City microcosms were prepared with 0.5, 1.5, 5.0, and 15.0 g wet weight aquifer material. To obtain data on solid/liquid mass ratios, additional microcosms were prepared and then sacrificed to provide weight data on the liquids and the dry solids. Solid/liquid mass ratios are reported as dry mass aquifer solids/mass liquid. Each sample was amended with nutrients to provide solution concentrations of approximately 10–20 mg L^{-1} ammonia-nitrogen, 10–20 mg L^{-1} phosphate-phosphorus, and 10–50 mg L^{-1} nitrate-nitrogen. Poisoned controls were prepared for each treatment group and contained 250 mg L^{-1} mercuric chloride and 500 mg L^{-1} sodium azide as biocides to inhibit microbial growth.

Each microcosm was then spiked with an aqueous stock containing toluene. For the test on the effects of initial toluene concentration, dilutions of aqueous toluene stocks were used to spike the microcosms. Different ranges of concentrations were chosen, generally corresponding to those found at the different sites. The design aqueous toluene concentrations were 25, 250, 2500, and 25 000 μg L^{-1} for the Traverse City microcosms, and 20, 100, 500, and 2500 μg L^{-1} for the Park City microcosms. The initial toluene concentrations measured were generally within 10% of the design concentrations. For the test on the effects of solid/liquid ratios, different volumes of aqueous toluene stock were used in an attempt to obtain similar concentrations in solution, since the test variable was solid/liquid mass ratio and not initial concentration. This was only partially successful, and initial measured toluene concentrations ranged from 1900–3100 μg L^{-1} toluene in the Traverse City microcosms and from 3500–4500 μg L^{-1} in the Park City microcosms.

Once the microcosms were spiked with toluene, they were sealed without headspace using a grey butyl rubber Teflon-coated septum. All microcosms were initially mixed and then incubated under static conditions in the anaerobic glovebox in the dark at 20° C. Two to three replicates from each set were sacrificed at designated times. Each sacrificed microcosm was mixed and centrifuged at $510 \times g$ for 30 min to clarify the supernatant phase, and the supernatant phase was analyzed for toluene by purge-and-trap gas chromatography as described previously [20]. The quantitation limit was 1.0 μg L^{-1}. Supernatant phases were also analyzed for aqueous nitrate, nitrite, ammonia-nitrogen, and phosphate concentrations using standard EPA methods [25]. The detection limit was 0.05 mg L^{-1} for each nutrient. Nitrate removal and nitrite production occurred for all viable treatment groups and did not occur in poisoned controls, indicating denitrifying conditions. Residual microcosm solids were not analyzed.

Studies using radiolabeled toluene

In addition to using poisoned controls, one of the treatments (5.0 g core, 2500 μg L^{-1} toluene) was repeated with the Traverse City aquifer material using uniformly-labeled radiolabeled toluene to verify that toluene removal resulted at least in part from biodegradation rather than from sorption or other losses. As an additional treatment variable, a replicate set of Traverse City microcosms was amended with a combined spike to yield 1000–3000 μg L^{-1} each of benzene, ethylbenzene, and xylenes (BTEX) to assess the effect of additional monoaromatic hydrocarbons on toluene biodegradation. Distribution of the radiolabel was assessed using a modification of the procedure used by Grbic-Galic and Vogel [14]. This assay, which accounts for distribution of the radiolabel in the aqueous phase alone, represents the extent of biodegradation of available soluble substrate and has been described in detail elsewhere [17].

Data analysis

Preliminary examination of the data revealed that removal kinetics were not clearly either first-order or zero-order. This has been observed previously in other cases where subsurface systems were examined for biodegradation of organic compounds under anaerobic conditions [5,15]. Although the data in this study are too few to determine which rate model provides the most reliable representation, most of these data appeared to better fit a linear (mean $r^2 = 0.908$) rather than a logarithmic (mean $r^2 = 0.693$) model. Because the reaction order is unknown, rates of toluene removal were assumed to be pseudo zero order and were calculated using zero-order kinetics as follows:

$$C_o - C = kt$$

where C_o is the initial concentration at the beginning of the selected time period, C is the final concentration at the end of the selected time period, k is the pseudo zero-order rate constant, and t is the time interval. For these data, pseudo zero-order rate constants were obtained using linear regression statistics. Regressions were applied to the linear portions of the graphs, which corresponded to the time intervals shown in Tables 1 and 2.

Because different initial toluene concentrations were used for some of these treatments, it is not possible to compare toluene removal for the various treatment groups using pseudo zero-order rate constants alone. Therefore, pseudo zero-order half-lives were calculated to provide a basis of comparison for toluene removal among the treatment groups. Half-lives were calculated based on the pseudo zero-order rates and the initial concentrations at the beginning of the selected time periods:

$$t_{1/2} = \frac{C_o}{2k}$$

It should be noted that these rate computations are independent of any associated lag periods, and therefore the lag periods do not affect the half-lives for these purposes of comparison. Linear regression statistics were used to generate standard errors and 95% confidence intervals to evaluate the data.

Results

Effect of varying initial toluene concentration

Varying the initial toluene concentration produced different effects in the separate aquifer materials. In the Traverse

Table 1 Effect of initial toluene concentration on pseudo zero-order biodegradation rate under denitrifying conditions for two aquifer cores

Core	Co[a] (μg L^{-1})	Time interval (days)	Rate (mg L^{-1} day^{-1})	Std error	r^2	d.f.	Half-life (95% CI) (days)
Traverse City	27	1.0–3.0	0.0097	0.0007	0.940	11	1.190 (1.060–1.320)
	281	1.0–2.5	0.181	0.0143	0.942	14	0.785 (0.702–0.867)
	2650	3.0–4.0	2.21	0.398	0.814	8	0.630 (0.486–0.775)
	23 600	3.5–4.5	18.6	3.45	0.807	8	0.548 (0.432–0.664)
Park City	22	0.0–0.6	0.0342	0.0019	0.978	8	0.325 (0.297–0.352)
	93	0.0–0.6	0.149	0.0139	0.943	8	0.313 (0.279–0.348)
	489	0.0–0.8	0.561	0.0207	0.987	11	0.439 (0.412–0.465)
	2470	0.0–1.6	1.64	0.0771	0.974	13	0.753 (0.679–0.827)

[a]Initial toluene concentration.

Table 2 Effect of solid/liquid mass ratio on pseudo zero-order toluene biodegradation rate for two aquifer cores

Core	Solid/liquid mass ratio	Co (μg L^{-1})	Time interval (days)	Rate (mg L^{-1} day^{-1})	Std error	r^2	d.f.	Half-life (95% CI) (days)
Traverse City	0.087	1880	2.5–3.0	2.18	0.573	0.783	5	0.385 (0.272–0.498)
	0.490	2080	2.5–3.0	2.01	0.466	0.822	5	0.341 (0.243–0.438)
	1.20	2320	2.5–3.0	2.95	0.450	0.915	5	0.302 (0.237–0.365)
	1.90	3120	2.5–3.5	2.17	0.237	0.923	8	0.594 (0.520–0.669)
Traverse City (respike)	0.087	2620	5.1–5.7	4.48	0.246	0.979	8	0.292 (0.268–0.317)
	0.490	3170	5.1–5.7	5.23	0.344	0.971	8	0.303 (0.278–0.328)
	1.20	3940	5.1–5.7	6.79	0.534	0.956	8	0.290 (0.262–0.318)
	1.90	3700	5.1–5.7	6.38	0.211	0.992	8	0.290 (0.273–0.307)
Park City	0.039	3770	2.0–2.9	2.92	0.242	0.930	12	0.538 (0.493–0.532)
	0.120	4110	1.8–2.9	2.74	0.136	0.971	13	0.533 (0.504–0.562)
	0.420	3450	1.2–2.3	2.34	0.150	0.934	18	0.581 (0.535–0.628)
	1.90	4500	0.7–2.3	2.15	0.160	0.878	26	0.898 (0.815–0.981)

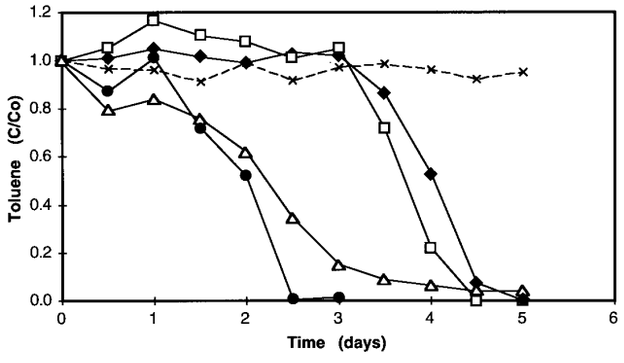

Figure 1 Effect of initial aqueous toluene concentration on toluene removal in denitrifying microcosms prepared with Traverse City aquifer material: 27 μg L^{-1} (—△—), 281 μg L^{-1} (—●—), 2650 μg L^{-1} (—□—), 23 600 μg L^{-1} (—◆—), and (--x--) combined controls. Each value represents the mean of two to three replicates.

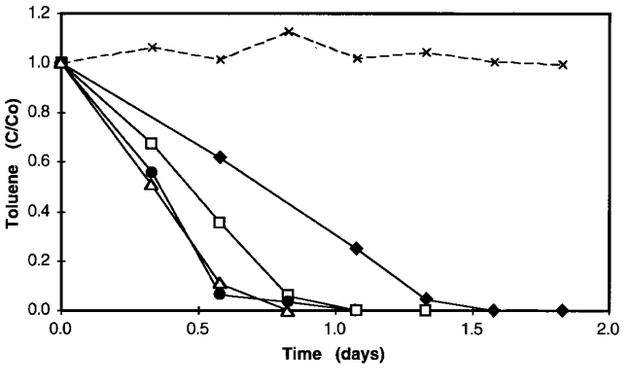

Figure 2 Effect of initial aqueous toluene concentration on toluene removal in denitrifying microcosms prepared with Park City aquifer material: 22 μg L^{-1} (—△—), 93 μg L^{-1} (—●—), 490 μg L^{-1} (—□—), 2500 μg L^{-1} (—◆—), and (--x--) combined controls. Each value represents the mean of two to three replicates.

City aquifer material, increasing the initial toluene concentration generally increased the apparent lag period (Figure 1). There was no discernible lag period with the Park City aquifer material (Figure 2), but toluene removal occurred so rapidly that short lag periods would not have been detected. In comparing pseudo zero-order rates for the different treatment groups, it should be noted that the data in Figures 1 and 2 reflect relative toluene removal rather than absolute concentrations, and therefore the rates of removal can be quite different even though the half-lives are similar. For both aquifer materials, pseudo zero-order removal rates were generally proportional to initial toluene concentration (Table 1). However, different trends were

observed. For the Traverse City aquifer material, the toluene half-life decreased from 1.19 days to 0.548 days as the initial concentration was increased from 27 μg L^{-1} to 24 000 μg L^{-1}, respectively. In contrast, the toluene half-life in the Park City aquifer material *increased* from 0.325 days to 0.753 days as the initial concentration was increased from 22 μg L^{-1} to 2500 μg L^{-1}, respectively (Table 1). Varying the initial toluene concentration by three orders of magnitude therefore resulted in a maximum change in the half-life of only a factor of two, and most of the difference occurred at 25 μg L^{-1}, the lowest concentration tested. This lowest initial toluene concentration was the only case for which toluene removal could be better described by first-order ($r^2 = 0.930$) rather than zero-order ($r^2 = 0.807$) kinetics. Using the first-order model (days 1–4), the rate constant would be 0.983 day^{-1}, and the half-life would be 0.705 ± 0.047 days, not significantly different from those obtained with the other levels.

Effect of varying the solid/liquid mass ratio

Varying the solid/liquid mass ratio had no measurable effect on the lag period for the Traverse City aquifer material, although there were insufficient data collected early enough in the test to resolve minor changes (Figure 3). In contrast, the lag period decreased with increasing solid/liquid mass ratio in the Park City aquifer material (Figure 4). The effects on toluene removal rates were minor (Table 2). In fact, the only significant difference occurred at the highest solid/liquid mass ratio (1.9), where half-lives in both aquifer materials actually increased by 50–70% relative to the other treatments. This observed inhibitory effect with increasing solid/liquid mass ratio becomes more apparent for both aquifer materials (Figure 5) when the pseudo zero-order rates are corrected for differences in liquid volume and sediment mass to provide estimates of specific toluene mass removal rates (μg g^{-1} day^{-1}). Despite the decline in the specific toluene mass removal rate, toluene still disappeared most rapidly in the Park City microcosms with the highest solid/liquid mass ratio, since the inhibitory effect was in part negated by the decreased lag periods (Figure 4). This was not observed in the Traverse City microcosms (Figure 3).

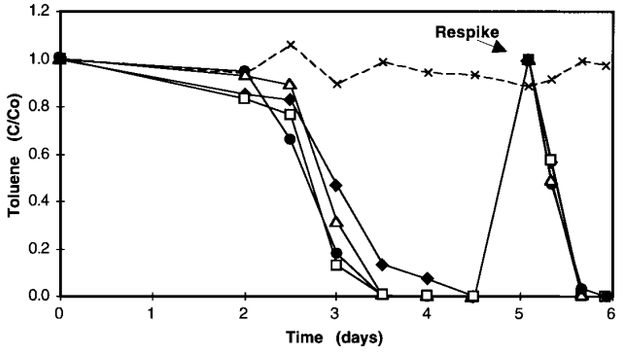

Figure 3 Effect of solid/liquid mass ratio on toluene removal in denitrifying microcosms prepared with Traverse City aquifer material: 0.087 (—△—), 0.49 (—●—), 1.2 (—□—), 1.9 (—◆—), and (--x--) combined controls. Microcosms were respiked with toluene on day 5. Initial aqueous toluene concentrations ranged from 1900–3100 μg L^{-1} and 2600–3900 μg L^{-1} before and after respiking, respectively. Each value represents the mean of two to three replicates.

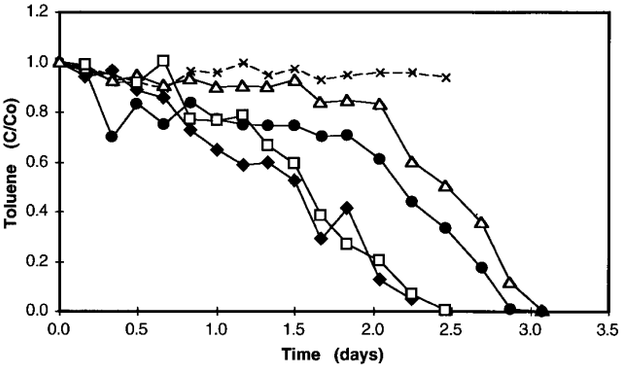

Figure 4 Effect of solid/liquid mass ratio on toluene removal in denitrifying microcosms prepared with Park City aquifer material: 0.039 (—△—), 0.12 (—●—), 0.42 (—□—), 1.9 (—◆—), and (--x--) combined controls. Initial aqueous toluene concentrations ranged from 3800–4500 μg L^{-1}. Each value represents the mean of two to three replicates.

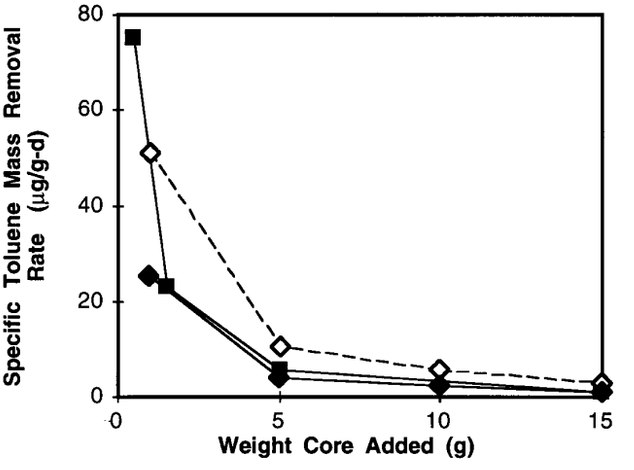

Figure 5 Effect of sediment weight on specific toluene mass removal rate in denitrifying microcosms prepared with Traverse City (—◆—) and Park City (—■—) aquifer material. Data are also presented for Traverse City microcosms after respiking with toluene on day 5 (—◇—).

Respiking the Traverse City aquifer material with toluene, once the initial toluene concentrations had been reduced, resulted in no significant difference in half-lives or lag periods among the respiked treatment groups (Figure 3, Table 1). The specific toluene mass removal rate still decreased with increasing solid/liquid mass ratio, although rates were generally twice those prior to respiking (Figure 5).

Effect of adding other monoaromatic substrates

Addition of other monoaromatic hydrocarbons had no effect on either the removal or extent of biodegradation of toluene in the Traverse City aquifer material (Figure 6). The half-life for toluene, calculated for days 3.8–5.1, was 0.749 ± 0.110 days and 0.739 ± 0.163 days with and without BTEX addition, respectively. The other monoaromatic hydrocarbons in the BTEX-amended microcosms were not

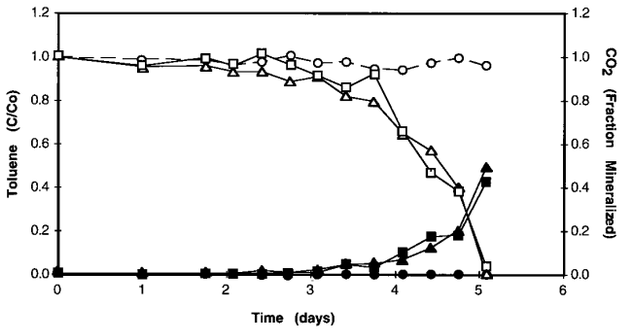

Figure 6 Effect of BTEX addition on toluene removal [no BTEX added (—△—), BTEX added (—□—), controls (--○--)] and toluene mineralization [no BTEX added (—▲—), BTEX added (—■—), controls (--●--)] in denitrifying microcosms prepared with Traverse City aquifer material. Initial aqueous toluene concentrations ranged from 1900–2700 μg L^{-1}, and initial aqueous BTEX concentrations ranged from 1000–3000 μg L^{-1} for each component. Each value represents the mean of two to three replicates.

degraded during this time interval (data not shown). Approximately 43–50% of the toluene was mineralized (Figure 6), with an additional 13–15% of the radiolabel distributed as acid-soluble intermediates (data not shown).

Discussion

Although changes in either the initial toluene concentration or the solid/liquid mass ratio did result in different toluene biodegradation rates in the separate aquifer materials, the effect was not as great as expected. For the Traverse City aquifer material, a change of three orders of magnitude in the initial toluene concentration resulted in a maximum change in the half-life of only a factor of two, with most of the difference occurring at the lowest concentration of 25 μg L^{-1}. Under aerobic conditions, Aelion *et al* [1] found that varying the toluene concentrations from 10–100 ng g^{-1} (corresponding to 42–420 μg L^{-1}) in microcosms prepared with Traverse City aquifer material produced no significant change in the first-order rate of biodegradation, as measured by the respiration of radiolabeled toluene. The reason for the observed inhibition at the higher toluene levels in the Park City microcosms is unclear. Toxicity of the hydrocarbon cannot be discounted, as other research has shown that the basal rate of denitrification in Traverse City aquifer material is inhibited when benzene or *m*-xylene concentrations are increased from 7000 μg L^{-1} each to 20 000 and 16 000 μg L^{-1}, respectively [19]. Also, Jorgensen *et al* [23] observed an inhibition in both the rate of denitrification and toluene biodegradation in denitrifying enrichments prepared from sewage sludge as toluene concentrations were increased from 22 000 to 117 000 μg L^{-1}, although the rates were unaffected down to 6000 μg L^{-1}, the lowest concentration tested. However, these reported inhibitory levels are approximately an order of magnitude higher than the highest level (2500 μg L^{-1}) tested for the Park City aquifer material. Regardless, differences in microbial populations could still account for increased sensitivity to the higher toluene levels. Another possibility is that, unlike the Traverse City aquifer material, the Park City aquifer material had not been previously exposed to nitrate, and this could have exacerbated any toxic effects.

Surprisingly, varying the solid/liquid mass ratio produced only minor effects on toluene removal rates. In fact, the only significant difference occurred for both aquifer materials at the highest solid/liquid mass ratio, where half-lives actually increased. One would expect that increasing the mass of aquifer solids in proportion to the sterilized ground water would provide an initial higher biomass and thus accelerate the rate of removal. However, there are cases where others have found little correlation between measurements of biomass and rates of degradation of organic compounds in anaerobic microcosms prepared with subsurface material [15]. One explanation for this is that the rate of biodegradation may be limited by the bioavailability of toluene sorbed to the aquifer solids [31], despite the fact that these aquifer solids have a relatively low organic carbon content (0.024% and 0.062% for the Traverse City and Park City aquifer materials, respectively). Respiking the Traverse City microcosms with toluene caused the specific toluene mass removal rate to decrease with increasing solid/liquid mass ratio, although rates were generally twice those prior to respiking (Figure 5). This would be expected if sorptive interactions were partially negated by an increase in suspended biomass. This may also account for the observation that, in the Park City aquifer material, the increased aquifer mass resulted in a situation where the decrease in the specific toluene removal rate was accompanied by a larger decrease in the lag period, thus promoting more rapid toluene removal despite sorptive interactions.

Addition of other monoaromatic hydrocarbons had no effect on either the removal or extent of biodegradation of toluene in the Traverse City aquifer material. The other monoaromatic hydrocarbons in the BTEX-amended microcosms were not degraded during this time interval, but some eventually degrade in other microcosm tests prepared with this aquifer material [17]. In aerobic studies with enrichments, multiple substrates have been observed to exert both synergistic and antagonistic effects on benzene biodegradation [3]. With aerobic microcosms prepared from both aquifer slurries and enrichments, Alvarez and Vogel [2] found that the pseudo zero-order rate of toluene biodegradation decreased by 50% when *p*-xylene was present, and by an additional 50% when both *p*-xylene and benzene were present. In this study, both benzene and *p*-xylene were present in the BTEX-amended microcosms, but no effect on the rate of toluene biodegradation was observed.

The data from experiments involving radiolabeled substrate provide evidence that toluene removal in these microcosms is due at least in part to biodegradation, since 56–64% of the radiolabel was transformed to CO_2 and acid-soluble intermediates in the test case. Although mineralization of toluene was incomplete, the data are in agreement with those of other research. In separate aerobic studies with Traverse City aquifer material, researchers have found that 25% of the labeled toluene was respired [1]. The remaining radiolabel was most likely incorporated into biomass. In other studies, Swindoll *et al* [28] observed that uptake into cell biomass represented a large fraction of total

metabolism for many xenobiotic compounds under aerobic conditions, and Jorgensen *et al* [22] reported that 44% of labeled toluene was incorporated into biomass of enrichment cultures under denitrifying conditions.

These data show that changing the initial hydrocarbon concentration or the solid/liquid mass ratio by two to three orders of magnitude generally results in less than 50% difference in the half-life of toluene in these denitrifying microcosms, with a maximum of 100% difference at the extreme ranges of the test parameters. This variability may be minor with respect to that based on sampling location within the same aquifer, but further research is required to ascertain whether this is true. In addition, other parameters (eg, exposure history, nutrients, pH, temperature, mixing, etc) should be similarly investigated with site-specific aquifer material to determine whether the measured rates can adequately predict rates established in active bioremediation systems. It can be argued that the complexities of physical/chemical processes in the field, coupled with subsurface heterogeneities which affect microbial ecology, generally preclude the successful extrapolation of laboratory data to predicting field performance. However, other research has shown that rates of monoaromatic hydrocarbon biodegradation measured in the laboratory under either aerobic or anaerobic conditions can sometimes be successfully correlated with field rates [4,16,21], and additional comparisons are required to determine the optimum methods for predicting field performance.

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