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Alternative method for rapidly screening microbial isolates for their potential to degrade volatile contaminants

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SUMMARY

A method is described for rapidly screening the metabolic potential of bacteria to oxidize semivolatile and volatile compounds as a sole carbon source. The method is based on an automated system that utilizes Microplates™ manufactured by Biolog, Inc. (Hayward, CA, USA). This system detects bacterial respiratory activity from the oxidation of a carbon source introduced in volatile form. This is in contrast to the original design, which is based on inoculating a carbon source directly into each well. The 96-well (MT) microtiter plates contain nutrients and a tetrazolium dye. When a bacterial species is capable of oxidizing a volatile carbon substrate, the dye turns purple, and a spectrophotometric plate reader quantifies the response. As a test of this method 150 isolates, including isolates known to degrade some of the test compounds and negative controls were evaluated for their potential to oxidize carbon tetrachloride, toluene, and *o*-xylene. Thirty-seven isolates (25%) were qualitatively identified as contaminant oxidizers, and thirteen of these (35%) showed significant degradation capabilities for both toluene and *o*-xylene.

INTRODUCTION

Methods of isolating bacteria capable of degrading contaminant compounds employ enrichment techniques and growth on selective media [2,8,12]. These techniques are labor-intensive and costly. Furthermore, enrichment assays are developed for a particular contaminant and are not necessarily adaptable to other contaminants. There are two aspects to screening bacterial populations that are commonly considered in biodegradation/remediation activities. Bacteria may be tested as individual isolates or as mixed cultures (consortia) for their ability to degrade specific compounds or groups of compounds [2,8,11]. A number of procedures have been developed for these screening assays [3,14,15]. Our work initially focused on identifying isolates that were capable of degrading specific compounds. To do this we modified a commercially available system that can quickly and efficiently evaluate large numbers of isolates for their potential to degrade volatile carbon compounds that are commonly found at hazardous waste sites. This method was developed to assay contaminants added in a volatile form. A similar method has been developed by Gorden et al. [10] who used volatiles added directly into the wells. Ultimately this technique could be applied to a wide variety of compounds/contaminants.

The method is suitable for use with semivolatile and volatile compounds using a commercially available system that detects carbon oxidation based on respiratory activity (Biolog, Inc., Hayward, CA, USA) [4,5]. The Biolog system was originally developed as a tool that identifies microbial isolates by examining their ability to oxidize various carbon sources [6,7]. Briefly, this microplate system is based on the addition of an aqueous bacterial suspension to a 96-well microtiter plate. Each well contains a complex nutrient medium, a redox tetrazolium dye and the potential carbon source. Oxidation of the substrate forms NADH, which donates electrons to the electron transport chain, thereby reducing the redox dye (tetrazolium violet) from its colorless form to an irreversible purple formazan. Thus, when a bacterial species oxidizes the carbon substrate supplied, the indicator dye turns purple. A spectrophotometric plate reader is used to read the plates, which automatically subtracts the background density (color) from blank wells and generates a numerical value.

We have modified the original design to permit screening of numerous isolates for their ability to oxidize volatile carbon compounds. This screening technique identified within 24–72 h isolates which had the potential to oxidize the carbon compounds of interest. This procedure minimized the need to perform detailed and laborious analyses on all isolates. Once identified, only those isolates with the greatest potential to metabolize the carbon compound were quantitatively assayed for degradation kinetics, optimization of contaminant and nutrient concentrations as well as characterization of genetic material.

This project was initially developed and implemented as a method for screening bacterial isolates obtained in the Department of Energy's (DOE) Office of Health and

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Environmental Research (OHER), Deep Subsurface Microbiology Subprogram. The DOE Subsurface Science Program has resulted in the isolation of over 4500 bacterial isolates [1,9]. Although these isolates represent a potential source of bacteria and/or genetic material for the degradation of contaminants, their ability to metabolize unusual and refractory substrates has not been fully examined. This paper describes our system, the method of cell preparation, and the incubation regime.

MATERIALS AND METHODS

Chemical compounds

The volatile carbon compounds chosen for this study (carbon tetrachloride, toluene and *o*-xylene) were based on their interest to DOE and their compatibility with the plastic microtiter plates. Other volatile compounds such as TCE, chloroform and methylene chloride were considered; however, the 96-well Microplates were not compatible with these vapors: dissolution and deformation of the plates occurred within 24 h. We have identified alternative plates which are compatible with these organic vapors; they can be custom filled by Biolog with the respiratory indicator mixture.

The volatile carbon sources were introduced into the wells by vapor diffusion. Microtiter plates incubated with the bacteria were placed in a desiccator containing an open beaker of contaminant. The desiccator was sealed, permitting the vapors to saturate the atmosphere and diffuse into the aqueous phase. The estimated volatile concentration within the wells was determined not to exceed the solubilities of 800 parts per million (p.p.m.) for carbon tetrachloride, 515 p.p.m. for toluene and 175 p.p.m. for *o*-xylene [17], based on the assumption that the system reached equilibrium.

Bacterial cultures

Microbial isolates were obtained from numerous sources. The negative control isolate which was assumed not to degrade these compounds was *Escherichia coli* obtained from E. Garrison (Oak Ridge National Laboratory, TN, USA), the positive isolates known to degrade toluene and *o*-xylene were *Pseudomonas putida* mt-2 (from R. Burlage, Oak Ridge National Laboratory) for toluene and *P. putida* KC-3 and KC-4 (from K. Chapatwala, Selma University, Alabama, USA) for *o*-xylene. There is currently no known isolate for aerobic utilization of carbon tetrachloride.

Two sets of microbial cultures were used in these experiments. One set was obtained from the DOE/OHER subsurface microbial culture collection (Subsurface Microbiology Culture Collection, D. Balkwill, Florida State University, USA). The isolates discussed here were intially isolated from the Idaho National Engineering Laboratory (INEL) (those denoted with the prefix 'I'). A total of 150 isolates were screened as described below. Representative positive and negative results are provided for IO163, IO116, IO277, IO097, IO093. A second set of cultures originated as enrichments from drilling in the DOE Subsurface Science Program at Yakima Barricade, Hanford, Washington, USA (S27, S25, S18a).

In the enrichments approximately 2 g of soil were aseptically added to 100 ml minimal salts media (MSM) [13]. Volatile carbon compounds (carbon tetrachloride, toluene and *o*-xylene) were added from water-saturated solutions to a calculated final aqueous concentration of 10 p.p.m. Bottles were capped with Teflon^{R}-lined septa and incubated at room temperature on an orbital shaker. Growth was usually visible within 2 weeks or not at all. At monthly intervals 100-μl aliquots of bacterial suspensions were transferred to fresh MSM and volatiles were added to a final concentration of 10 p.p.m. (aqueous phase). To isolate the bacteria into pure culture (following three consecutive MSM transfers) 0.1 ml of the suspension was plated onto PTYG plates (Difco peptone, 5.0 g; Difco tryptone, 5.0 g; Difco yeast extract, 10.0 g; glucose, 10.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; agar, 15.0 g; distilled water, 1.0 L) and incubated at room temperature for 1–7 days. Each morphologically-distinct colony was subsequently screened for its ability to oxidize carbon tetrachloride, toluene and *o*-xylene as described below.

Screening procedure

Following the guidelines developed for the Biolog plates, bacterial cultures were grown on appropriate nutrient plates (generally PTYG) for 48–96 h at 25–30 °C. From these plates suspensions were made to equal an optical density of 0.3 or 0.6 (590 nm) in phosphate buffered 0.85% NaCl. Cultures were held overnight at room temperature to minimize false positives by allowing time for utilization of stored nutrient. Each bacterial isolate was inoculated into one row (eight wells at 150 μl per well) of a 96-well MT™ plate. These MT plates contained only tetrazolium dye and trace amounts of nutrients. The first row was a control or blank containing only sterile saline, the absorbance of which was subtracted from all other absorbance readings. The second row was inoculated with *E. coli* as a negative control. When appropriate positive control cultures were available the third row contained the positive control (toluene and *o*-xylene degraders). The remaining 9–10 rows were inoculated with the isolates under evaluation.

Each bacterial isolate was inoculated into four separate 96-well plates. One plate was incubated in a plastic bag to minimize desiccation, containing only atmospheric air and no additional carbon source. This enabled us to identify false positives. The other three plates were placed in separate desiccators containing the beaker filled with either toluene, *o*-xylene or carbon tetrachloride. The plates were read via an automatic vertical spectrophotometer Series 750 Microplate Reader (Cambridge Technology, Inc., Watertown, MA, USA) after 0, 12 and 24 h. This system optically reads each well (absorbance of 590 nm), quantifies the absorbance change relative to the eight wells inoculated with sterile saline only and records the data as a specific absorbance. Oxidation of the substrate is read by the change in color from an opaque solution to a purple color.

Statistical analyses

This screening procedure is a qualitative measure of carbon utilization; therefore, the Student *t*-test ($P < 0.05$) was used only to confirm identification of previously known degraders (Fig. 1). For the actual screening procedures it was arbitrarily decided that a change in the optical density greater than 0.05 from time zero to time 12 h identified a potential candidate for further analysis.

RESULTS AND DISCUSSION

A color change (increase in absorbance) theoretically represented oxidation of the volatile carbon source. In general, exposure of the inoculated plates to the contaminant vapors produced a visible color change if the bacterial isolate was capable of utilizing the carbon source, whereas no color change indicated non-utilization. Initial evaluation of the Biolog system's capability to estimate oxidation of volatile compounds was confirmed by inoculating the plates with *P. putida* mt-2, KC-3 and KC-4 (Fig. 1). Samples were incubated in the presence of toluene or atmospheric air. Each mean and standard deviation was calculated from the absorbance value of eight replicate wells.

The control cultures gave the expected results based on our previous knowledge of their metabolic capabilities. *E. coli* (negative control) indicated a slight increase in color after a 24-h incubation in toluene (0.21 ± 0.01) over air (0.20 ± 0.01). The three volatile degrading isolates mt-2, KC-3 and KC-4 all showed a significant absorbance increase in the toluene treatment over air. Thus, this system has the capability of identifying degraders for which the carbon source was initially supplied as a vapor. Potential explanations for the increase in absorbance for the air incubation are discussed below.

In order to screen numerous isolates efficiently while maximizing identification of potential degraders and minimizing false positives, we optimized the incubation time and initial inoculation concentration. Plates were incubated in air, carbon tetrachloride, toluene or *o*-xylene vapors and read at 0, 6, 12 and 24 h. In general all isolates had a peak response to all three volatile compounds within 12 h, followed by a decrease (Fig. 2). The data are reported as a change in absorbance from the initial reading at time 0 to 6, 12 or 24 h. Each data point represents the mean of eight wells. For isolate IO163 (Fig. 2), the greatest absorbance increase was with toluene, while *o*-xylene showed an intermediate result, and carbon tetrachloride slightly increased but eventually decreased. There was only a slight increase in the control treatment. This increase in the control is likely to be a result of endogenous nutrient metabolism. Starving the cultures overnight in saline was designed to minimize these effects. For most cultures that were starved longer (e.g. 48–72 h) the control peak was not significantly reduced although some isolates did benefit from longer holding times. Furthermore, growing the cultures in a variety of minimal media (1% PTYG, 5% PTYG, nobel agar and glycerol) did not have an affect on these control values. Gorden et al. [10] used washed cells and reported no color change for controls. In the great majority of cases, control values did not exceed treatment values and were treated as suspect if they did. Elimination of the cell washing step for these preliminary screening experiments saved time and money.

Extension of the incubation time to 48 and 72 h rarely showed further increase in absorbance values. Thus, the incubation time was limited to 48 h (read at 0, 12, 24 and 48 h) not only to increase screening efficiency but also because of a noticeable deterioration in the 96-well plate beginning at approximately 72 h.

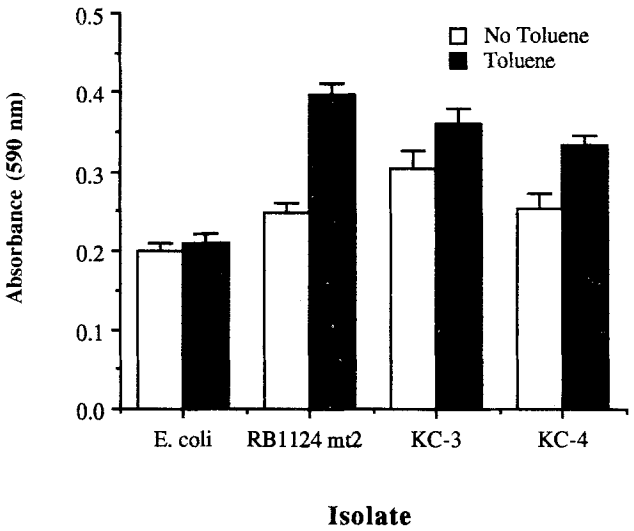

Fig. 1. Mean absorbance value ($n = 8$) and standard deviations of isolates incubated in air or toluene vapors for 24 h. Negative control of *E. coli*, and known toluene degraders *Pseudomonas putida* mt-2 and known xylene degraders *P. putida* KC-3 and KC-4.

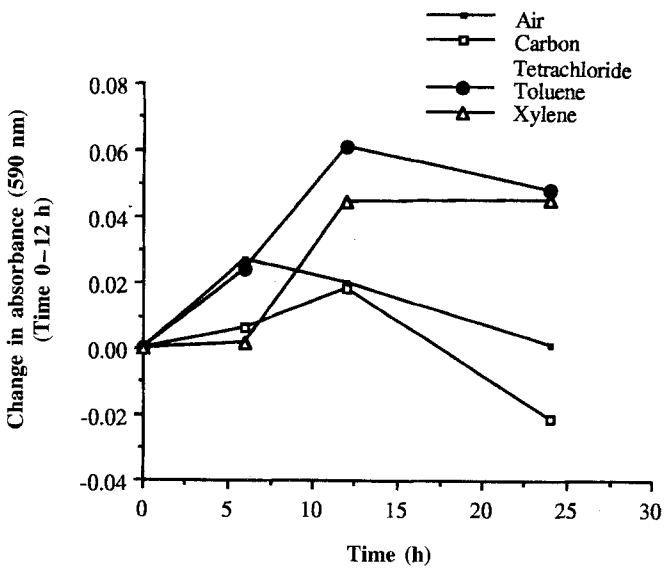

Fig. 2. Absorbance values for isolate IO163 recorded at 0, 6, 12 and 24 h. Each data point represents the mean of eight wells. The standard deviations have been omitted for clarity; however they ranged from 0.01 to 0.04.

Cellular respiration could be due to metabolism of plate constituents (e.g. plasticizers) as the plates deteriorated. This assay was designed to identify potential degraders, thereby reducing the more time-consuming traditional assays. Those isolates which underwent a color change were further tested in quantitative batch experiments for their ability to degrade any of the volatile compounds. Out of 150 isolates, 37 (25%) were identified as potential degraders and 35% of these were quantitatively identified as toluene and *o*-xylene degraders.

At an initial inoculum which had an OD of 0.6, an increased frequency of positives was observed over that seen with an inoculum which had an OD of 0.3. Numerous isolates were evaluated and exhibited similar behavior at the two absorbance levels; representative data are shown in Fig. 3. The isolate, S25 (from an enrichment experiment), had very different results for the two initial inoculation concentrations. A treatment with an inoculum optical density of 0.3 did show a slight absorbance increase for toluene (solid square), and *o*-xylene (solid triangle), while the control and carbon tetrachloride treatments showed a decrease in absorbance. However, when inoculum optical density was increased to 0.6 the isolate was positive for all three carbon compounds. Increasing the initial concentration increases the control response (open circle) but the response was greater in the presence of the contaminants. Some of this increase in absorbance is a consequence of increased microbial biomass and possible carryover of stored nutrients. However, isolate S25 (Fig. 3, top) grows on toluene and *o*-xylene in more conventional experiments (unpublished data). A second enrichment isolate S17 (Fig. 3, bottom) did not show an increase in absorbance for either an optical density of 0.6 or 0.3 and subsequent experiments indicate that degradation of these contaminants did not occur. Furthermore, visual inspection of these plates showed a deep purple color for S25 and little color for S17. Therefore, in order to avoid missing some positive isolates and accepting higher background values, we standardized all the initial inoculum concentrations to an optical density of 0.6 absorbance.

Some of the isolates obtained from the subsurface at INEL exhibited potential oxidation of the volatile carbon sources (Fig. 4) despite the nonselective procedure (i.e. nonenrichment) used to isolate them. There was a positive response to toluene for four of the isolates (solid bars) from this site. In addition, isolates IO116, IO097 and IO093 were positive for *o*-xylene (hatched bars) and isolates IO097 and IO093 were positive for carbon tetrachloride (striped bars). Isolate IO116 had a slight response in air whereas isolates IO277, IO097 and IO093 showed decreasing absorbance. Thus, this method has been useful in efficiently identifying bacteria from various sites and isolation regimes that have the ability to utilize volatile carbon sources. These isolates are being quantitatively analyzed to verify degradation of toluene, *o*-xylene and carbon tetrachloride.

In some cases the absorbance values decreased (Fig. 4). This absorbance reduction may be a result of cell lysis due to stress or the lack of utilizable carbon. Another explanation for those exposed to toluene, *o*-xylene and carbon tetra-

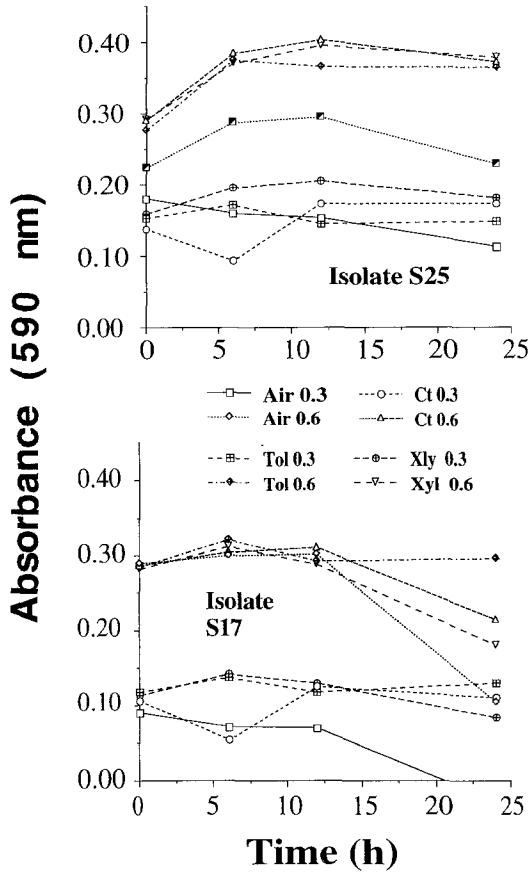

Fig. 3. Mean absorbance values (n = 8) over time for two isolates from Yakima Barricade, Hanford, WA. Each data point is the mean of eight OD values. The standard deviations have been omitted for clarity; however they ranged from 0.01 to 0.07.

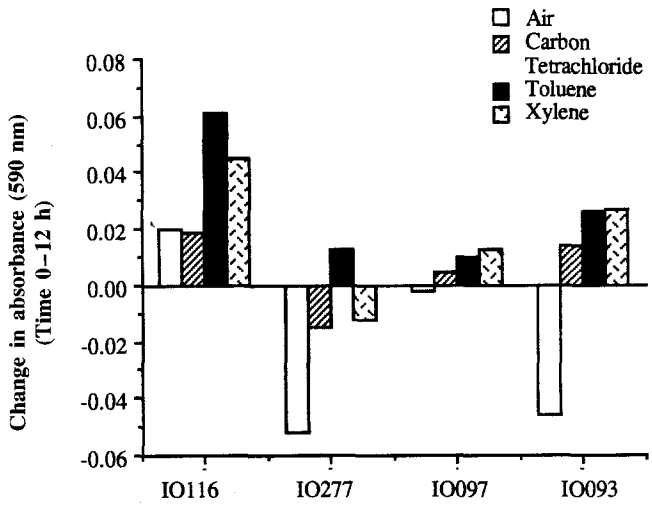

Fig. 4. Absorbance change during a 12-h incubation of four INEL isolates with toluene, xylene, and carbon tetrachloride. Initial inoculum absorbance = 0.6. Bars indicate averages of eight determinations.

chloride vapors is toxicity. The concentrations of these compounds in saturated solutions are relatively high (175–800 p.p.m.) due to the delivery method. Thus, they may cause cell death and lysis resulting in lower absorbance. We are investigating whether this modified procedure can be used to evaluate and identify a toxic reponse (by a decrease in absorbance) to a particular carbon compound. It may have use in conjunction with conventional respirometric measurement and inhibitors of degradation [18]. We are also investigating numerous methods of lowering the contaminant concentration in the 96-well plates.

We have found this screening method to be particularly useful in combination with enrichment for contaminant degradation. Enrichments are a very common and powerful method of obtaining degradative organisms [16]. Bacteria originating from our enrichments were isolated into pure cultures and assayed as described above. In general, the magnitude of the positive oxidative response to the volatile compounds was greater for enrichment isolates than for those previously maintained on nutrient plates. Isolate S25 (Fig. 5) showed the greatest change in absorbance from 0 to 12 h not only in air but also in carbon tetrachloride, toluene and *o*-xylene. This increased oxidation of the volatile compounds may be a result of cellular enzyme 'priming' due to the enrichment regime. In contrast to isolate S25, isolate S17 exhibited only a slight response for carbon tetrachloride over control incubation and a decrease in response to toluene and *o*-xylene vapors. We are currently evaluating the positive response to the carbon tetrachloride treatment.

Not all bacterial isolates obtained from the volatile enrichments yielded positive results when tested in pure culture. In batch studies isolate S18a originally displayed visible growth in a mixed (toluene, *o*-xylene, carbon tetrachloride) enrichment experiment; however, it was not positive for any single carbon compound (compared to the background). It may be that the incubation regime in the 96-well plates was not carried out for an adequate period of time. We have also examined the oxidative response of reconstructed consortia. Preliminary results suggest that oxidative metabolism for the reconstructed consortia is greater than for individual isolates.

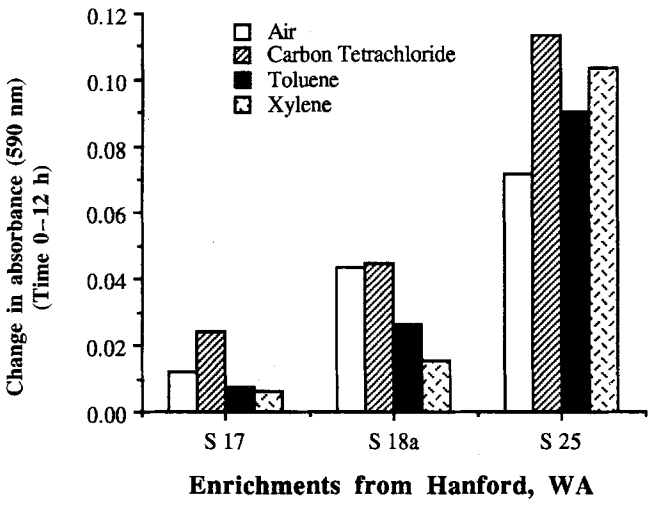

Fig. 5. Isolates from soil enrichments, Yakima Barricade, Hanford, WA with air, toluene, *o*-xylene and carbon tetrachloride. The inoculum concentration had an OD of 0.6.

Experiments reported here have examined soil and bacteria from uncontaminated sites. We have recently expanded the use of this system to include enriched soil samples from contaminated sites and have obtained consortia that can grow in the presence of the volatile compounds. Further analysis of these results is ongoing. Once microbial cultures have been identified as having significant degradative abilities they can be transferred to environmental remediation activities in the DOE and to users outside of the DOE.

This specific experimental design was developed in response to a need to efficiently screen a large number of bacterial isolates for their potential to degrade contaminant compounds. In our initial screening experiments, we reduced the number of isolates to be examined from the original 150 to 37 within 4 days as well as reducing the supplies, space and time. These 37 isolates have produced 13 isolates that, based on GC analysis, degraded toluene and *o*-xylene. Thus, the screening procedure greatly saved time and resources.

There are numerous situations where the experimental design presented here may not be optimal. Some subsurface isolates are very slow growers and it is likely that they would require incubation times greater than 48 h. However, as the incubation time increases, the liquid within the wells may evaporate, causing an increase in absorbance. Incubation times may also be limited by plate integrity to the contaminant compound.

In addition to incubation times, the composition of volatile compound(s) as well as the contaminant concentration may affect the results. Based on the solubility values, the concentration of volatile compounds in these experiments was high (175–800 p.p.m.). There may be toxic effects as the decrease in absorbance values suggests. These high contaminant concentrations may inhibit degraders that would function efficiently at lower contaminant levels. We are continuing to develop methods for reducing the concentration of volatiles within the microtiter wells and examining the potential of this system to determine contaminant toxicity levels. Application of this design modification can eliminate the need for many of the costly and tedious methods traditionally used in screening procedures. Using the Biolog system, investigators should be able to identify bacteria quickly and efficiently that show the greatest potential to utilize a particular carbon source. The bacteria we have identified as being contaminant degraders will not only add additional species capable of biodegradation to our remediation arsenal but because of their original source (deep core material containing recalcitrant carbon sources) they may be better adapted to survive within contaminated sites where nutrients are low. These contaminant-degrading isolates can be further characterized regarding degradation kinetics, nutrient requirements for optimum degradation activity, and tolerance to numerous contaminants in a variety of forms and concentrations. Isolation of the gene(s) involved

should provide a unique source of genetic material for application in other bioremediation programs. Subsequent reports will address the application of this system to specific microbial isolates from the subsurface at Savannah River Laboratory (SRL), Idaho National Engineering Laboratory (INEL), Pacific Northwest Laboratory (PNL) and Oak Ridge National Laboratory (ORNL) as well as its application to enrichment experiments and reconstructed consortia.

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