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Methodological Modifications for Accurate and Efficient Determination of Contaminant Biodegradation in Unsaturated Calcareous Soils

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Many techniques for quantifying microbial biodegradation of ^{14}C-labeled compounds use soil-water slurries and trap mineralization-derived $^{14}CO_2$ in solution wells suspended within the incubation flasks. These methods are not satisfactory for studies of arid-region soils that are highly calcareous and unsaturated because (i) slurries do not simulate unsaturated conditions and (ii) the amount of CO_2 released from calcareous soils exceeds the capacity of the suspended well. This report describes simple, inexpensive methodological modifications for quantifying microbial degradation of [^{14}C]benzene and 1,2-dichloro[U-^{14}C]ethane in calcareous soils under unsaturated conditions. Soils at 50% water holding capacity were incubated with labeled contaminants for periods up to 10 weeks, followed by acidification of the soil and trapping of the evolved CO_2 in a separate container of 2 N NaOH. The CO_2 was transferred from the incubation flask to the trap solution by a gas transfer shunt containing activated charcoal to remove any volatilized labeled organics. The amount of $^{14}CO_2$ in the trap solution was measured by scintillation counting (disintegrations per minute). The method was tested by using two regional unamended surface soils, a sandy aridisol and a clay-rich riparian soil. The results demonstrated that both [^{14}C]benzene and 1,2-dichloro[U-^{14}C]ethane were mineralized to release substantial amounts of $^{14}CO_2$ within 10 weeks. Levels of mineralization varied with contaminant type, soil type, and aeration status (anaerobic vs. aerobic); no significant degradation was observed in abiotic control samples. Methodological refinements of this technique resulted in total $^{14}CO_2$ recovery efficiency of approximately 90%.

Contamination of soil and groundwater systems is an urgent problem demanding rigorous interdisciplinary research to develop appropriate remedial technologies (7, 10). Indigenous subsurface microbial populations possess the ability to degrade a wide variety of organic compounds (2, 4, 6, 15). In situ bioremediation, the enhancement of the subsurface environment to maximize microbial degradation of contaminants, has been shown to be a cost-effective and noninvasive treatment when incorporated into a comprehensive remediation program (7, 14, 16). As a result, there is great interest in quantifying the ability of microorganisms to degrade organic contaminants, and numerous laboratory studies have measured the rate of decomposition of organic contaminants in soil–water slurry experiments (1, 3, 9, 11, 13, 15).

However, this general experimental approach has several potential drawbacks with respect to quantifying contaminant degradation in calcareous unsaturated soils, like many found in the southwestern United States. The design and implementation of remediation strategies require that the ability of indigenous microbial populations to degrade organic contaminants under natural field conditions be thoroughly characterized (7). Aqueous slurry incubations do not simulate natural conditions found in unsaturated (vadose zone) soils, many of which are contaminated with organic pollutants. The design of a bioremediation strategy for an unsaturated soil on the basis of laboratory slurry experiments may be incorrect because of an inaccurate assessment of the ability of the indigenous microorganisms to degrade the targeted contaminant. Preliminary laboratory data generated from experiments conducted under natural moisture and aeration conditions are required to produce accurate estimates of biodegradation potentials for a particular site.

The basic methodological steps involved in traditional ^{14}C-contaminant mineralization techniques must be modified to quantify biodegradation in highly calcareous unsaturated soils. Many published techniques measure the mineralization-derived $^{14}CO_2$ by driving all CO_2 into the flask headspace via sample acidification after slurry-contaminant incubation (1, 9, 11, 13, 15). The $^{14}CO_2$ is then trapped in an NaOH, ethanolamine, or KOH solution held in a small well suspended from the flask stoppers. In some studies, a fluted filter paper is inserted into the well to maximize CO_2 recovery (5). These designs are not suitable for calcareous soils because the production of unlabeled CO_2 after acidification exceeds the absorptive capacity of the trap, even with the additional capacity of a filter paper. In many cases, pressure from the CO_2 produced upon acidification of these soils is sufficient to force the stoppers from the flasks. Obviously, this situation prohibits quantification of the $^{14}CO_2$ fraction.

The current study was conducted to develop a reliable, simple, and inexpensive method for quantifying biodegradation of volatile organic pollutants by indigenous microbial communities under unsaturated conditions. The method described, derived from the modification and combination of existing techniques, enables the containment of relatively large amounts of total CO_2 so that mineralization-derived $^{14}CO_2$ can be quantitatively recovered and measured. Two U.S. Environmental Protection Agency priority pollutants associated with petroleum contamination, benzene and 1,2-dichloroethane, were chosen for use in this study. The compounds represent two different structural classes of priority pollutants, aromatic and chlorinated aliphatic, which appear to require different aeration conditions for complete

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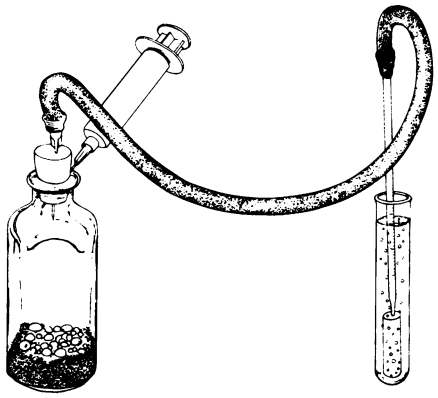

FIG. 1. Design and application of experimental gas transfer shunt.

mineralization (6, 10). Biodegradation of each pollutant was quantified under both aerobic and anaerobic conditions in each of the two surface soils examined.

MATERIALS AND METHODS

Soil sample collection and handling. Two regional surface soils, a sandy aridisol from the grounds of Sandia National Laboratories (Sandia soil) and a riparian soil from the banks of the Rio Grande (Bosque soil), were collected in Albuquerque, N.M., in Fall 1987. The Bosque soil had more organic matter and was finer textured than the Sandia soil. Large root material and rocks were removed by hand, and soils were passed through a 2.0-mm-pore-size sieve. All soil samples were stored at 5°C prior to analyses, and analyses were initiated within 1 week of sample collection.

Determination of water holding capacity. Soil subsamples were placed into stoppered funnels fitted with a glass-wool plug, covered completely with water, and allowed to remain in contact with the water for 30 min. The subsamples were then drained by gravity for 30 min, and water content was determined by loss upon heating at 105°C to constant weight. The water holding capacity was calculated as the amount of water retained per gram of soil.

Gas transfer shunt. Shunts were designed to transfer mineralization-derived $^{14}CO_2$ from incubation flasks to trap solution containers (Fig. 1). Each shunt consisted of a 30-cm length of latex tubing connected to the incubation flasks via a hypodermic needle. The tubing was filled with 6- to 14-mesh granular activated carbon (Fisher Scientific Co.) to retain any ^{14}C-labeled volatile organics and thus prevent artificially high $^{14}CO_2$ determinations. The tubing was attached to a sterile 1-ml pipette, and a standard 5-cm aquarium bubbler was fitted to the end of the pipette. The bubbler was immersed in 5 ml of 2 N NaOH. The bubbler increased the surface-to-volume ratio of gas (CO_2) bubbles to maximize the amount of physical contact between $^{14}CO_2$ molecules and the trap solution, thus enhancing the $^{14}CO_2$ trapping efficiency of the NaOH.

The $^{14}CO_2$ recovery efficiency of the gas transfer shunt at various stages of development was tested by acidifying soil samples amended with NaH[^{14}C]CO_3 (23.7 GBq mmol^{-1}; Amersham), which released $^{14}CO_2$ upon acidification. The $^{14}CO_2$ recovery associated with the final shunt design was used to correct biodegradation data. Ethanolamine has been cited in the literature as an effective CO_2 trap solution and was tested for its $^{14}CO_2$ recovery efficiency, but 2 N NaOH proved to be the more efficient trap and was used for the biodegradation experiments.

Biodegradation experiments. The amount of water necessary to adjust a known amount of each soil to one-half of its water holding capacity was placed in the bottom of a 2-liter Erlenmeyer flask. A volume of [^{14}C]benzene or 1,2-dichloro[U-^{14}C]ethane (4.48 GBq mmol^{-1} and 1.41 GBq mmol^{-1}, respectively; Amersham) was added beneath the water level in the flask, and bulk soil samples were then added to the radiolabeled solutions. The soils were allowed to absorb the radiolabeled solutions through capillary action for 12 h. After shaking the labeled material, 50-g portions were placed in 160-ml incubation flasks. The resulting concentration of each organic contaminant in the soil subsamples was approximately 100 ppb (100 ng/g).

Three flasks of each soil-contaminant combination were immediately extracted with ether, which effectively solubilizes and removes organic compounds. Scintillation counting of the ether extracts confirmed that the ^{14}C label was present at approximately 30,000 dpm g^{-1} and was evenly dispersed in all subsamples (standard error, approximately 3.7%). Flasks were sealed with silicon stoppers, and subsequent experiments revealed that less than 4.0% of the ^{14}C label was absorbed by these stoppers.

Half of the flasks containing soil and radiolabeled contaminants were incubated aerobically in an environmental chamber, and half were purged with inert gas and incubated in an anaerobic chamber. Abiotic soil samples for each soil type were provided by irradiation with a cobalt source at Sandia National Laboratories, and all incubations were conducted at 25°C.

After incubation periods of up to 10 weeks, triplicate samples of each combination of soil type, contaminant type, and aeration status, as well as corresponding abiotic control samples, were used to quantify contaminant biodegradation. Gas transfer shunts (Fig. 1) and trap solution containers were placed in line as described above.

After connection of the shunt and trap solution, soil samples were acidified by adding an amount of 4 N H_2SO_4 (with swirling) sufficient to completely acidify the sample (pH, <2.0). Acidification of the samples caused noticeable bubbling within the flasks and consequently within the trap solutions, where a stream of fine bubbles emerged through the aquarium bubblers at the bottom of the solution. After the visible bubbling in the flasks and trap solution containers had subsided (with shunts and traps still in line), nitrogen (N_2) was used to purge the atmosphere of the flasks and ensure complete transfer of CO_2 into the trap solution. The flow of N_2 gas was maintained until the volume of the flask was replaced at least three times. At this point, with the N_2 gas still flowing through the system. the bubble filters were removed from the trap solutions, rinsed into the solutions thoroughly with deionized, distilled water, and properly disposed.

Experiments were conducted to determine the optimal amount of trap solution to use for scintillation counting. On the basis of these results, additional distilled water was added to each trap solution to achieve a final volume of 25 ml. A 1-ml aliquot of each solution was mixed with 20 ml of Scintiverse II scintillation fluid and subjected to scintillation counting (disintegrations per minute) on a Hewlett-Packard model 1500 scintillation counter. Counts were quench corrected by automatic external standardization. Data generated as disintegrations per minute of ^{14}C-labeled CO_2 were

TABLE 1. Recovery efficiencies of $^{14}CO_2$ achieved by use of ethanolamine or NaOH as CO_2 trapping solution

Expt	Soil type	Trap solution	Technical modification[a]	$^{14}CO_2$ recovered[b] (%)
1	Sandia	Ethanolamine	0.5 ml counted	20.4
2	Bosque	Ethanolamine	0.5 ml counted	25.4
3	Sandia	2 N NaOH	0.5 ml counted	63.7
4	Bosque	2 N NaOH	0.5 ml counted	58.3
5	Sandia	2 N NaOH	Diluted 1:5; 1.0 ml counted	68.5
6	Bosque	2 N NaOH	Diluted 1:5; 1.0 ml counted	78.3
7	Sandia	2 N NaOH	Air bubblers added and rinsed into trap	89.8
8	Bosque	2 N NaOH	Air bubblers added and rinsed into trap	86.6

[a] Modifications are listed in sequential order.
[b] Expressed as percentage of total added disintegrations per minute.

expressed as the percentage of contaminant that had been mineralized to CO_2 during each time period.

RESULTS

Relative $^{14}CO_2$ recovery efficiencies achieved with each major step in the technical refinement of the gas transfer shunt methodology are shown in Table 1. Initial experiments were conducted without bubblers, which were later added to increase trapping efficiency. Ethanolamine was withdrawn from consideration as a CO_2 trap solution because of the extremely low efficiencies observed in this study. Recoveries associated with 2 N NaOH were substantially higher, and this solution was used throughout the study to trap CO_2. It was necessary to dilute the NaOH solution 1:5 (vol/vol) with deionized water prior to scintillation counting to remove the quenching effect of the milkiness resulting from the mixing of undiluted 2 N NaOH with Scintiverse II scintillation cocktail. Separate experiments confirmed that a 1:20 (vol/vol) NaOH:Scintiverse II ratio resulted in the highest scintillation counting efficiency (Table 2).

The attachment of aquarium bubblers to the ends of the pipettes submerged in the trap solution further enhanced the $^{14}CO_2$ recovery efficiencies by increasing the surface-to-volume ratio of the CO_2 gas bubbles and thereby maximizing the physical contact between the CO_2 and the trap solution. Thorough rinsing of the bubblers into the trap solution while the N_2 gas continued to flow proved to be a critical factor. Without the rinsing step, up to 20% of the total counts remained on the bubbler itself. However, thorough rinsing reduced the bubbler counts to negligible levels.

To guard against contamination by nondegraded ^{14}C-labeled organics, gas transfer shunts contained granular activated carbon to remove benzene or 1,2-dichloroethane before it reached the NaOH trap solution. Scintillation counting of random samples of both suspended granular activated carbon and of ether extracts of the material removed from the shunts demonstrated that negligible amounts (less than 2.1% of the total counts for 20 random samples) of nonmineralized volatile organics were being purged from the incubation flasks.

Table 3 shows the actual application of this method for quantifying degradation of ^{14}C-labeled compounds. Within 4 weeks, indigenous microbial populations in the Sandia and Bosque soils degraded [^{14}C]benzene and 1,2-dichloro[U-^{14}C]ethane under aerobic and anaerobic conditions. Neither compound appeared to undergo significant degradation in the irradiated soil samples, confirming that mineralization was due to the activity of viable microbial populations existing within the soils.

TABLE 2. Recovery of NaH[^{14}C]CO_3 in 20 ml of Scintiverse II scintillation cocktail with increasing amounts of NaOH solution added

Dilute NaOH[a] added (ml)	Count (dpm)	Recovery (%)
0	106,704	97
1	103,427	94
2	96,969	89
3	101,487	92
4	100,156	91
5	94,461	86
6	94,605	86
7	88,670	81
8	77,622	71

[a] Ratio of 2 N NaOH to dH_2O, 1:5.

DISCUSSION

The primary objective of this study was to modify existing techniques to facilitate reliable, simplified, inexpensive quantification of contaminant biodegradation in highly calcareous, unsaturated, unamended soils. Many aspects of conventional ^{14}C-compound mineralization studies remain appropriate for use with this type of soil and were incorporated into this study. The measurement of $^{14}CO_2$ derived from ^{14}C-labeled contaminant mineralization by scintillation counting provides researchers with a sensitive, convenient, and flexible method in a laboratory setting. However, the use of a suspended trap solution well is not appropriate when the soils being examined release substantial quantities of CO_2 upon acidification. The use of a gas transfer shunt eliminates this problem, because any conceivable amount of CO_2 generated could be contained and measured simply by

TABLE 3. Biodegradation of benzene and 1,2-dichloroethane in Sandia and Bosque soils over a period of 4 weeks[a]

Soil	Aeration status	Benzene (%)		1,2-Dichloroethane (%)	
		Live	Killed	Live	Killed
Sandia	Aerobic	14.85 (1.1)	1.23 (0.6)	23.85 (1.4)	0.81 (0.4)
Sandia	Anaerobic	2.43 (1.9)	0.64 (0.1)	3.36 (2.7)	0.65 (0.1)
Bosque	Aerobic	47.08 (20.6)	1.62 (0.2)	15.12 (5.8)	5.13 (2.5)
Bosque	Anaerobic	16.49 (5.6)	0.79 (0.1)	3.33 (1.8)	0.48 (0.2)

[a] Values are percentages of 100-ppb (100-ng/g) starting concentrations of each compound expressed as means of triplicate determinations with the standard deviation in parentheses.

increasing the volume of the trap solution in the external vessel.

With respect to actual results obtained with this technique, substantial amounts of microbially mediated mineralization of ^{14}C-labeled contaminants occurred in both soils within 4 weeks. Differences in biodegradation levels between the two soil types were not unexpected and likely reflect natural variation with respect to microbial population and activity levels (15) as well as contaminant-specific enzyme production (6). In both soils, maximum levels of benzene mineralization occurred under aerobic conditions. Substantial, albeit lower, levels of benzene degradation were also observed during anaerobic degradation with either soil type. The latter result suggests that an alternative mechanism (13), such as denitrification, methanogenesis, or sulfate reduction (8, 12), is a viable biodegradation pathway in these soils under anoxic conditions. A black coloration, commonly associated with sulfate reduction and metal precipitation, was noted in anaerobic flasks containing both of the soil-contaminant combinations after 4 weeks. Mixed consortia of sulfate reducers, methanogens, and denitrifiers may thus be involved in the anaerobic degradation of this contaminant. With respect to 1,2-dichloroethane, more degradation occurred aerobically, which contrasts with literature citations classifying the compound as one which only undergoes significant degradation anaerobically (6). This discrepancy could be due to the presence of anaerobic microsites present in the unsaturated soils utilized here. Several researchers have noted elevated chlorinated aliphatic degradation during incubations where the aeration status was changed from anaerobic to aerobic. The presence of anaerobic microsites in a generally aerobic soil could provide the interface environments that favor this type of degradation. This example points out the importance of examining soil contaminant biodegradation under natural conditions; the results can be quite different from those obtained under artificial slurry conditions.

In addition to the obvious benefits of this technique with respect to measuring biodegradation in calcareous unsaturated soils, there are broader advantages associated with the use of gas transfer shunts. The shunts developed in this study resulted in very acceptable $^{14}CO_2$ recoveries, and all of the materials required to construct the shunts are readily available and quite inexpensive. During this study, multiple samples were processed at the same time by placing up to 18 shunts in line and acidifying corresponding samples. A single technician can easily process 36 samples during a typical workday. Study-specific variables, such as amount and type of trap solution, amount of soil used, soil moisture and aeration status, and length of carbon-filled tubing, can easily be manipulated. These factors enhance the applicability of the technique; it is a convenient and time-efficient means of collecting accurate measurements of biodegradation rates, even under conditions of limited experimental funding.

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