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Determination of the Biodegradation Rate of Asphalt
for the Hanford Grout Vaults

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SUMMARY

Testing was initiated in March 1991 and completed in November 1992 to determine the rate at which asphalt is biodegraded by microorganisms native to the Hanford Site soils. The asphalt tested (AR-6000, U.S. Oil, Tacoma, Washington) is to be used in the construction of a diffusion barrier for the Hanford grout vaults. Experiments to determine asphalt biodegradation rates were conducted using three separate test sets. These test sets were initiated in March 1991, January 1992, and June 1992 and ran for periods of 6 months, 11 months, and 6 months, respectively. The experimental method used was one originally developed by Bartha and Pramer (1965), and further refined by Bowerman et al. (1985), that determined the asphalt biodegradation rate through the measurement of carbon dioxide evolved.

Using data from the January and June 1992 test sets, asphalt biodegradation rates were determined for test flasks containing soil and irradiated asphalt sample incubated at ambient and elevated ($\approx 35^{\circ}\text{C}$) temperature. The average asphalt biodegradation rate determined at ambient temperature for test flasks incubated for less than 160 days was 1.6×10^{-4} cm/yr, with a high endpoint at the 95% confidence interval of 1.9×10^{-4} cm/yr and a low endpoint at the 95% confidence interval of 1.4×10^{-4} cm/yr. For ambient temperature test flasks incubated up to 310 days, the average asphalt biodegradation rate was determined to be 1.0×10^{-4} cm/yr, indicating that the rate of biodegradation decreases as a function of time. For test flasks at elevated temperature, the average asphalt biodegradation rate was determined to be 1.1×10^{-3} cm/yr with a high endpoint of 1.3×10^{-3} cm/yr and a low endpoint of 9.9×10^{-4} cm/yr.

Conservatively, the asphalt diffusion barrier will degrade at 1.3×10^{-3} cm/yr during elevated temperatures with the rate decreasing to 1.9×10^{-4} cm/yr at lower, ambient temperature. This is a conservative estimate of the long-term degradation rates. Parameters such as available water, available oxygen, and available nutrients will affect the long-term biodegradation rate. Since the environment surrounding the asphalt diffusion barrier will likely be deficient in these parameters, the long-term biodegradation rate is predicted to be lower than determined by this study. However, additional work is needed to investigate the influence of these parameters, and others, on the asphalt biodegradation rate and refine the estimated long-term rate.

Extrapolating the biodegradation rate to 10,000 years indicates that approximately 3 cm of asphalt would degrade, assuming that the asphalt diffusion barrier remained at elevated (35°C) temperature for 1,000 years and then was at ambient temperature for 9,000 years. This amount of degradation is within the 10-cm allowance in the design criteria for the asphalt diffusion barrier.

This allowance would be approached only if the barrier remained at elevated temperature during the entire 10,000-year performance period (13 cm of asphalt would degrade based upon the conservative asphalt biodegradation rate).

To provide a check on the experimental method used, one of the test conditions studied contained nutrient agar, irradiated asphalt, and a bacterial culture (ATCC 13388). This bacterial culture is known to degrade asphalt and has been used in previous asphalt biodegradation studies as reported in the literature. Results for these test flasks indicated an asphalt biodegradation rate of 3.6×10^{-3} cm/yr with a high of 4.5×10^{-3} cm/yr and a low of 2.4×10^{-3} cm/yr, which is the same order of magnitude as the 2.44×10^{-3} cm/yr found by Barletta et al. (1987) for a similar test condition. The determination that the degradation rate calculated by this study and that reported in the literature are of the same order of magnitude provides confidence that the experimental method was implemented correctly.

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1.0 INTRODUCTION

The grout disposal system to be used on the Hanford Site requires a diffusion barrier to minimize both the migration of contaminants from the grout and the diffusion of water vapor toward the grout. The current vault design calls for a 102-cm- (40-in.-) thick asphalt pavement diffusion barrier. The grout performance assessment assumes that a 92-cm- (36-in.-) thick asphalt barrier functions as designed for a period of 10,000 years. The extra 10 cm (4 in.) of material provides an allowance for degradation of the barrier over the 10,000-year performance period.^(a) As discussed below, experiments conducted on asphalt and bitumens indicate that degradation rates from biological activity are less than 8×10^{-4} cm/yr (3.2×10^{-4} in./yr), which extrapolates to 8 cm (3.2 in.) in 10,000 years, well within the allowance specified in the design for the asphalt pavement diffusion barrier.

Numerous researchers have studied the rate at which microorganisms degrade asphalt and bitumen. Barletta et al. (1987) investigated the rate at which microorganisms (both native and foreign to a particular soil location) degraded blown bitumen samples. Using a technique developed by Bowerman et al. (1985) (which was a modification of a procedure originally used by Bartha and Pramer in 1965), Barletta determined the biodegradation rates for a bitumen material by various microorganisms. The rate of carbon dioxide (CO₂) evolution was measured to determine the rate of aerobic biodegradation. Soil from different locations with their native microorganisms, bacteria specified in ASTM G22 [American Society for Testing Materials (ASTM) 1981a], and fungi specified in ASTM G21 (ASTM 1981b) were used in the study. Table 1.1 illustrates the test conditions studied. The controls used consisted of growth medium (either soil or nutrient agar) and microbes only, without the addition of a bitumen sample. Degradation rates determined by Barletta are also given in Table 1.1. As can be seen from Table 1.1, considering the wide range of conditions studied, the variation in determined degradation rates is small.

Equipment similar to Bartha-Pramer flasks (named as such for they are patterned after the ones first used by Bartha and Pramer in 1965) were used by Abdellah and Pedersen (1984) in their investigation of the biodegradation of Mexphalate R 90/40 (a type of bitumen). Abdellah and

(a) The 10,000-year time period is consistent with that evaluated in the Hanford Defense Waste Environmental Impact Statement (HDW-EIS) (U.S. DOE 1987). This 10,000-year time period is more stringent than the 300-year extrapolation time frame recommended by Bowerman et al. (1985).

TABLE 1.1. Experimental Conditions Studied and Biodegradation Rates Determined by Barletta. Samples consisted of four nominal 1-cm- x 1-cm- (.39-in.- x .39-in.-) sized cylinders except where noted (adapted from Barletta et al. 1987).

<u>Medium</u>	<u>Microbe</u>	Surface <u>Area (cm²)</u>	Pre-test Moisture <u>Wt %</u>	<u>Length of</u> <u>Test (days)</u>	<u>Rate x 10⁻⁴</u> <u>(cm/yr)</u>
Barnwell soil	native	30 ± 3	2.6 ± -	177	5.78
Upton soil	native	30 ± 3	5.1 ± 2.6	197	3.65
Upton soil	Pseudomonas	30 ± 3	5.1 ± 2.6	35	2.11
Richland soil	native	30 ± 3	5.2 ± 0.3	197	5.96
Barnwell soil	native	30 ± 3	5.6 ± 0.2	196	10.5
Barnwell soil	native	30 ±	18.3 ± 0.4	181	4.58
Barnwell soil	native	20 ± 2 ^a	5.6 ± 0.2	192	6.37
Barnwell soil	native	134 ± 13 ^b	6.5 ± 0.2	206	5.43
Agar	Pseudomonas	30 ± 3	N/A	54	24.4
Agar	fungi	30 ± 3	N/A	59	55.6

^a Nominal 2-cm x 2-cm cylinder.

^b 1-mm-thick sheet.

Pedersen's approach was to measure the pressure drop in the flasks. The rate of the measured pressure drop was proportional to the consumption of oxygen, an indication of asphalt biodegradation. The microorganisms used were those indigenous to the soil used as an inoculum. Calculated degradation rates at various days in Abdellah's experiment are summarized in Table 1.2.

In addition to the quantitative work by Barletta and Abdellah for estimating the biodegradation rates for bitumen samples, work by Brown, Pabst and Marcev (1990), Colombo and Neilson (1979), and Roffey and Hjalmarsson (1983) qualitatively demonstrate the ability of microorganisms to degrade asphalt material. The microorganisms used by these researchers were isolated from the soil from various sites, suggesting that asphalt-degrading microorganisms are widespread.

The mechanism for the biodegradation of asphalt is a complex process influenced by asphalt composition, microorganism type and diversity, and environmental conditions. Asphalt is primarily composed of a complex mixture of alkyl and aromatic hydrocarbons that vary in their susceptibility to biodegradation. Likewise, microorganisms differ in their ability to degrade the

TABLE 1.2. Degradation Rates Determined by Abdellah. Degradation rate based on the consumption of oxygen as measured by a drop in container pressure (adapted from Abdellah and Pederson 1984).

<u>Day</u>	<u>Rate (cm/yr)</u>	<u>Rate (in./yr)</u>
16	3.47×10^{-4}	1.37×10^{-4}
32	3.10×10^{-4}	1.22×10^{-4}
41	7.30×10^{-4}	2.87×10^{-4}

different hydrocarbon components of asphalt. This complex mechanism suggests that the rate of asphalt biodegradation likely is not linear. However, since asphalt components that are easily degraded will likely be consumed first, it is expected that the rate of asphalt biodegradation should decrease with time.

Biodegradation of the asphalt will occur from the surfaces of the asphalt exposed to oxygen, where the primary nutrients (asphalt, O₂, and other trace minerals) are all present in adequate levels. Surface degradation should be limited by oxygen diffusion and diffusion or presence of needed minerals/ions (i.e., phosphorous and nitrogen). As time progresses, degradation should diminish as all of these become limited. In addition, the mechanism for biodegradation may be such that a residue which is resistant to biodegradation remains on the surface, protecting the underlying asphalt from further biodegradation (Pendrys 1989).

The objective for this task was to determine the rate at which microorganisms native to the Hanford Site degrade AR-6000 asphalt, the asphalt to be used as a diffusion barrier for the Hanford grout vaults. Experimental work was initiated in 1991 and continued through 1992. Surface soil from the grout vault area, soil from the bottom of the pit for the grout vaults, and excavation soil to be used as backfill are sources for different microorganisms and were used at various times for this task. The soils selected for these experiments were expected to provide a consortium of microorganisms which will potentially contact the asphalt diffusion barrier. The size and composition of the microbial populations indigenous to the Hanford Site soil have been previously characterized (Bentjen et al. 1989; Fredrickson et al. 1989, 1990; Bolton et al. 1989, 1990; Li et al. 1991). Aerobic test conditions were studied because they have been shown to result in higher biodegradation rates relative to anaerobic conditions (Abdellah and Pederson 1984, Bowerman et al. 1985). This report describes the experimental conditions and results for this asphalt biodegradation study.

2.0 BACKGROUND

Testing was initiated in March 1991 to determine the rate at which asphalt is biodegraded by microorganisms native to the Hanford soil. The asphalt (AR-6000, U.S. Oil, Tacoma, Washington) is to be used in the construction of a diffusion barrier for the Hanford grout vaults. Initial experiments to determine biodegradation rates were done over a period of 6 months (middle of March 1991 to the middle of September 1991) using a method originally developed by Bartha and Pramer (1965), and further refined by Bowerman et al. (1985), that determines the asphalt biodegradation rate through the measurement of carbon dioxide evolved as the asphalt is biodegraded. Appendix A contains detail on initial experimental work performed in 1991.

Results from testing in 1991 indicated that no significant biodegradation occurred except potentially for flasks containing asphalt and excavation soil incubated at 35°C. This determination was made through a comparison of the control flasks with surface soil only (to determine the background CO₂ level) to flasks with both asphalt and soil. Flasks incubated at 35°C did show an elevated rate of CO₂ evolution compared to the control flask; however, whether this elevated rate indicated a significant biodegradation rate is uncertain because of questions about the rate of CO₂ evolution from the control flasks. The CO₂ evolution rate from the control flasks was questioned because it was comparable to literature values for optimal conditions (i.e., nutrient agar with bacteria that degrade asphalt). In addition, flasks containing only asphalt samples demonstrated levels of CO₂ evolution that were of the same magnitude as the control flasks and other tested, ambient conditions.

Testing with flasks initiated in March 1991 was terminated in November 1991 because of a problem with the inlet carbon dioxide removal system. Ascarite used to scrub carbon dioxide from ambient air became saturated with CO₂ after the final sampling in September 1991, resulting in 1) the loss of CO₂ scrubbing capacity and 2) the release of aqueous hydroxide into the test flasks. The latter result compromised the integrity of the test flask system and made continued testing with the flasks impossible. In addition, measurements of the pH of the flask contents indicated values in excess of 13. Because of these high pH values, it was determined that collection of microbial smear for microbial identification would not provide accurate determinations of the microorganisms present. A possible mechanism for the high CO₂ evolution rates for flasks containing asphalt only was biodegradation. Microbial identification was to be used to verify this hypothesis.

Qualitative determination of the asphalt biodegradation rate was not possible with the 1991 test data. Prior to continued experimentation in 1992, alternatives to the Bartha-Pramer method for

following the rate of asphalt biodegradation were discussed with individuals knowledgeable with the behavior of asphalt in general. Initial discussions identified gravimetric analysis as a means for following the loss of asphalt material. However, following further calculations using estimated biodegradation rates, it was determined that this technique would not be sensitive enough to determine a biodegradation rate. Based on these discussions, it was determined that the Bartha-Pramer method was best suited for studying asphalt biodegradation and should be used for continued testing in 1992.

3.0 EXPERIMENTAL METHODS

This section describes the sample preparation methods used, the equipment used, the established testing matrix, and the testing procedures followed for determining the biodegradation rate for AR-6000 asphalt.

3.1 TEST MATERIALS AND MATERIAL PREPARATION

Soil was collected from the vicinity where grout vaults will be constructed, located on the 200 Area plateau of the Hanford Site in southeast Washington. Soil samples from the bottom of the grout vault pit were aseptically collected and immediately transported to the laboratory for storage at temperatures below 15°C prior to preparation of the test flasks. Soil was stored at a reduced temperature to slow metabolic activity of the microorganisms present and thus assure that the microorganisms remained viable prior to testing.

The water-holding capacity of the bottom soil in the grout vault pit was determined before preparing the test flasks by adding 15 ml of water to a 250-ml graduated cylinder packed with a known weight (and volume) of soil. After a 24-hour period, the "wet" mark was taken and the water-holding capacity determined as illustrated in equation (1).

$$\text{Water-Holding Capacity} = \frac{(\text{volume water added}) * (\text{density of water})}{[(\text{soil volume}) - (\text{wet mark volume})] * (\text{soil bulk density})} \quad (1)$$

A water-holding capacity of 0.121 was determined for the grout pit soil.

Soil moisture content was also determined by taking known weights of soil samples, drying the soil, and calculating the percent water on a dry weight basis as shown in equation (2).

$$\text{Wt\% Water} = \frac{(\text{wt wet soil}) - (\text{wt dry soil})}{(\text{wt dry soil})} \quad (2)$$

The water percent calculated (and the percentage of the water-holding capacity) determined for the grout pit soil was 5.69 wt% (47%). Because water-holding capacity is a better indicator for biological activity than is soil moisture content, a target water content of 70% of the water-holding

capacity for the grout pit soil was used. This adjustment to the soil moisture content was made prior to the preparation of the test flasks.

A target water-holding capacity of 70% was selected to provide an optimum level for biological activities. This water-holding capacity is within the range of 50% to 75% needed to promote activities of aerobic bacteria (Alexander 1977). Use of this water-holding capacity will result in a conservative estimate for asphalt biodegradation.

AR-6000 asphalt is made of a multitude of long-chain hydrocarbon units containing predominately hydrogen and carbon. Measurement of the evolution of carbon dioxide is proportional to the amount of carbon degraded; the percent carbon in asphalt must be known to determine the amount of asphalt degraded. Total organic carbon analyses were done in triplicate on asphalt samples. A mean value of 89.6% carbon was determined and used for degradation rate calculations.

Difco 0001 nutrient agar (Difco Laboratory, Detroit, Michigan) was selected for use in flasks containing nutrient agar, bacteria specified in ASTM G22, and asphalt. This nutrient agar lacks only a carbon source. The bacteria *Pseudomonas aeruginosa*, ATCC 13388, was purchased from the American Type Culture Collection (ATCC), Rockville, Maryland. The dehydrated culture was received on nutrient agar (Difco 0001) and preserved in 40% glycerol in an -85°C deep freezer. Prior to use for testing, the ATCC 13388 bacteria was grown overnight in nutrient broth (Difco 0003) and resuspended in phosphate buffer. Chemicals used in the preparation of the phosphate buffer solution were of reagent grade.

The cell density of the suspended bacterial culture was determined on a Beckman DU-70 spectrophotometer. Using phosphate buffer solution as a blank, the optical density of the bacterial solution was measured at 650 nm (OD_{650}) and found to be 0.8561. This density measurement corresponds to approximately 8.6×10^8 cells/ml.^(a) Preparation of the inoculated flasks resulted in approximately 2.6×10^9 cells per 50 ml of nutrient agar.

3.2 TEST EQUIPMENT

Testing for the biodegradation of asphalt was performed in standard Bartha-Pramer flasks (Bellco Glass Inc., Vineland, New Jersey) as shown in Figure 3.1. AR-6000 asphalt was heated then poured into the bottom of the test flask and allowed to flow until it covered the bottom of the

(a) OD_{650} of 1.00 $\approx 1 \times 10^9$ cells/ml [CRC Handbook "Toxicity Testing Using Microorganisms" (CRC 1986)].

FIGURE 3.1. Illustration of Experimental Setup with Bartha-Pramer Flask

flask; this weight was then recorded. This procedure is a different approach than that used in the March 1991 tests, as explained in Appendix A. The March 1991 tests used asphalt coupons formed into regular geometric shapes; however, the asphalt was found to be somewhat fluid at room temperature and did not retain its shape, thus affecting the surface area available for degradation. The procedure adopted for 1992 testing eliminated this variability.

The test flasks were then sealed with the rubber stoppers^(a) and the test apparatus sterilized by either gamma irradiation or autoclaving. Gamma irradiation was performed by positioning the test flasks around a GB650 ⁶⁰Co irradiator for a period of approximately 68 hours. The dose rate for the test flasks was 8.84×10^4 rad/hr, resulting in a total dose of ≈ 6 Mrads. Autoclaving of the test flasks was performed on two consecutive days at $\approx 120^\circ\text{C}$ and ≈ 140 kPa.

Also shown in Figure 3.1 is an ascarite-filled syringe and a KOH trap. The syringe was packed with ascarite and glass wool and used to remove carbon dioxide from air entering the flasks. The vials containing the 10 ml of 0.1 M KOH solution were autoclaved prior to being filled with KOH solution and subsequently placed in the Bartha-Pramer flasks. A CO_2 -free, 0.1 M KOH solution was prepared by dissolving KOH in millipore water from which the CO_2 had been removed by boiling the water for 15 minutes. The KOH acted as a CO_2 trap for any CO_2 evolved in the flasks during biodegradation of the asphalt, or from degradation of background organics found in each soil type.

Analysis of the KOH solutions for trapped CO_2 was performed on a Mettler DL70 autotitrator. The HCl used for determining the CO_2 levels in the KOH solutions was calibrated with a sodium hydroxide (NaOH) solution that had been previously calibrated on the titrator with a potassium hydrogen phthalate solution.

(a) Teflon-wrapped stoppers were used at the beginning of the test. However, as will be discussed in the next section, it was found that the Teflon did not seal sufficiently to the glass and led to a leakage of carbon dioxide into the test flasks.

3.3 EXPERIMENTAL SAMPLE SCHEME

Table 3.1 shows the ten different test conditions used for determining the biodegradation rate of asphalt.^(a) As will be discussed in the following section, nine test conditions (excluding the soil-only flask incubated at elevated temperature) were used for test flasks initiated in January 1992. Four additional sets of test flasks, including the soil-only flasks at elevated temperature, were initiated in June 1992. Three replicate flasks were prepared for each condition to provide additional data for a given test condition at a point in time. As shown in Table 3.1, test flasks contained 100 g of medium (either soil from the bottom of the vault pit or nutrient agar) with or without asphalt, just asphalt, or nothing.

TABLE 3.1. Experimental Sample Scheme. Some test conditions have more than three test flasks because additional sets of flasks were initiated in June 1992.

<u>Number of Flasks</u>	<u>Condition I.D.</u>	<u>Test Condition</u>
3	A	Nothing
3	B	Asphalt (Gamma Irradiated/Sterilized)
3	C	Asphalt (Autoclaved/Sterilized)
6	D	100-g Vault Pit Bottom Soil
3	E	100-g Vault Pit Bottom Soil Incubated Between 30°C and 35°C
3	F	100-g Vault Pit Bottom Soil (Autoclaved/Sterilized)
6	G	100-g Vault Pit Bottom Soil Asphalt (Gamma Irradiated/Sterilized)
6	H	100-g Vault Pit Bottom Soil Asphalt (Gamma Irradiated/Sterilized) Incubated between 30°C and 35°C
3	I	100-g Vault Pit Bottom Soil Asphalt (Autoclaved/Sterilized)
3	J	100-g Nutrient Salts Agar Asphalt (Gamma Irradiated/Sterilized) <i>Pseudomonas aeruginosa</i> bacteria (ATCC 13388)

(a) The asphalt samples were sterilized to minimize the potential to skew the test results by handling the samples. Also, the sterilized asphalt samples are representative of asphalt to be used in the diffusion barrier since the asphalt will be heated to high temperatures immediately before barrier installation.

3.4 EXPERIMENTAL PROCEDURE

After the test flasks were sterilized (either by gamma irradiation or autoclaving), 100 g of soil (water content adjusted to 70% of the water-holding capacity) was added to the appropriate flasks. For flasks containing asphalt, the soil was added so that the entire surface of the asphalt was covered with soil. The vial containing 10 ml of KOH solution was placed into the side arm and the flask sealed with two rubber stoppers, one for the reaction chamber fitted with a syringe filled with ascarite and glass wool and a second to seal the KOH trap chamber. All of these soil-containing flasks, except for flasks to be incubated at elevated temperatures, were stored in a foil-covered glove box. As will be discussed in the following section, this test configuration was implemented in April 1992 to prevent leakage of CO₂ into the test flasks. Before April 1992, the test flasks were stored in a dark cabinet. The flasks for the test condition of soil and asphalt at 35°C were placed into a forced air oven set to maintain a temperature of 32-40°C. Flasks containing nutrient agar were prepared in a similar manner to the soil filled flasks except that, instead of soil, 100 g of a nutrient agar containing a suspension of ATCC 13388 culture completely covered the asphalt. These test flasks were also stored in the glove box.

Incubations began on 7 January 1992 for an initial set of test flasks. A second set of test flasks began incubating on 8 June 1992. At approximately 30-day intervals from the incubation start time, the KOH vial was removed to determine the amount of CO₂ that evolved during the 30-day sampling period. A fresh KOH vial was inserted to collect CO₂ during the next sampling period.

Of the 10 ml of KOH solution in the sample vials, only 2.5 ml were used for the titration to determine the amount of CO₂ evolved during the collection period. For the titration, the autotitrator was set to measure the required amount of HCl solution (approximately 0.05 M) needed to bring the KOH solution first to pH 8.3 and then to pH 3.7. The amount of standard acid required to titrate between the two pH values was used to calculate the amount of CO₂ present. From Parkinson and Paul (1982), 1 ml of 0.05 M HCl is equivalent to 0.6 mg CO₂-C.

Because it is very difficult to keep CO₂ from entering the stock KOH solution, the background levels of CO₂ present in the KOH solution were also measured. This background level was then subtracted from the volume of HCl required to titrate between pH 8.3 and 3.7 for the individual flasks. The adjusted volume of HCl was converted to milligrams of CO₂-C using the Parkinson and Paul relationship corrected for the actual molarity of the titrating HCl solution.

As a final step, this value was adjusted to represent the full 10-ml KOH sample by multiplying by 4 (10 ml/2.5 ml).

To determine the biodegradation rate, the incremental rate of CO₂-C evolution (mg/day) was plotted as a function of time. This incremental rate was determined by dividing the amount of CO₂-C collected in a sampling period by the duration of the sampling period. An outlier test was performed on the resulting data to determine if data from one of the three replicate flasks was statistically different at the 95% confidence level from the other two (American Society for Testing and Materials 1969).

A linear regression using methods discussed by Jaech (1973) was then performed on the incremental rate CO₂-C data to obtain a best fit line for each test condition. Two assumptions that allow a linear regression to be performed are 1) that the relationship is linear and 2) that there is statistical independence between any two observations on the independent variable (for this experiment, time is the independent variable).

The linear regression lines were then tested to determine if the slope was significantly different from zero (Weisberg 1985). If the slope was found to not be significantly different from zero at the 95% confidence level, then an average CO₂-C evolution rate was used to calculate the asphalt biodegradation rate. To determine the equation for the net CO₂-C evolution rate for asphalt, the resulting lines (equations) for each test condition have the line for the control flasks (soil only) subtracted from it and a new line determined. This resulting line allows an estimation to be made for the net CO₂-C evolution rate from the biodegradation of asphalt at a given point in time. The asphalt biodegradation rate may be determined at this point in time using:

$$\text{Biodegradation Rate} = (\text{Net CO}_2\text{-C Evolution Rate}) / (\text{Bulk Density} \times \text{Surface Area} \times \% \text{Carbon}).$$

An estimation of the error associated with this biodegradation rate is determined using methods described by Weisberg (1973).

It is anticipated that the asphalt biodegradation rate will decrease as a function of time. The highest predicted asphalt biodegradation rate determined from the above method would then be expected to occur at shorter incubation times.

4.0 RESULTS AND DISCUSSION

Table 4.1 summarizes the calculated amounts of CO₂-C evolved for each of the different test flasks begun on 7 January 1992. Samples were collected for CO₂ analyses at approximately 30-day intervals. A data set at day 60 is missing from this table because of an error in preparation of the KOH trap solution. The trap solution was found to be 0.01 N instead of 0.1 N for this sample period. As shown in Table 4.1, there is very little difference in evolved CO₂-C for days 30 and 90 among any of the test conditions. This slight difference in CO₂-C evolution was attributed to a leakage of CO₂ into the test flasks. The cause of the CO₂ leakage was found to be an inadequate seal from the Teflon-wrapped rubber stoppers. A corrective measure was taken after the sampling at day 90 (April 1992) whereupon all of the Teflon-wrapped rubber stoppers were replaced with unwrapped rubber stoppers. In addition, the January test flask set was placed into glove boxes that contained ascarite traps to further minimize the potential for CO₂ to enter the Bartha-Pramer test flasks. As Table 4.1 illustrates, this corrective measure was very successful in eliminating the leakage problem.

Once the new test configuration was found to successfully eliminate the CO₂ leakage problem, a second set of test flasks was initiated in June 1992 to collect data during the incubation period from time zero on. Table 4.2 summarizes the calculated amounts of CO₂-C for each of the different test flasks begun in June 1992. This second set of test flasks was limited to test conditions with soil only (both at ambient and elevated temperature) and soil and irradiated asphalt (both at ambient and elevated temperature).

This section discusses results for the test flask sets initiated in January 1992 and June 1992. The data are also analyzed to determine an asphalt biodegradation rate by microorganisms native to the Hanford soil.

4.1 RESULTS FROM JANUARY 1992 TEST SET

As previously discussed, the lack of a difference in evolved CO₂-C between the different test conditions at days 30 and 90 indicated a problem with the experimental setup. This problem was attributed to a leakage caused by the Teflon wrapping around the rubber stoppers. Once this wrapping was removed and the test flasks were placed into a glove box, the evolved CO₂-C for many of the test conditions decreased from the day 30 and day 90 levels and a discernible difference between the test conditions was observed. Figure 4.1 illustrates the decrease in evolved CO₂-C levels for test flasks containing nothing, irradiated asphalt only, and autoclaved asphalt

TABLE 4.1. Evolved mg CO₂-C for Test Flasks Initiated January 1992. The mg CO₂-C in the table has been corrected for the background levels of CO₂ in the KOH solution. No data collected for Day 60, KOH trap solution found to be 0.01N instead of 0.1N. Sampling day is measured from start of incubation.

Condition	Day 30	Day 90	Day 120	Day 153	Day 183	Day 219	Day 252	Day 279	309 Day
I.D.1									
A	5.21	5.36	0.06	0.07	0.00	0.06	0.00	0.01	0.00
	5.84	6.04	0.03	0.01	0.08	0.03	0.01	0.04	0.00
	6.12	5.91	0.02	0.02	0.03	0.03	0.03	0.00	0.00
B	3.73	4.80	0.06	0.00	0.00	0.05	0.04	0.00	0.00
	4.10	5.08	---	0.06	0.02	0.43	0.00	0.00	0.00
	3.72	4.36	0.03	0.13	0.00	0.06	0.01	0.02	0.00
C	4.34	5.50	0.06	0.03	0.07	0.07	0.01	0.00	0.02
	7.52	7.33	0.05	0.09	0.03	0.08	0.00	0.01	0.03
	6.33	6.11	0.04	0.06	0.01	0.05	0.06	0.04	0.02
D	6.88	6.54	2.10	0.87	1.16	1.29	0.91	0.58	0.53
	5.93	6.05	1.50	1.01	0.96	1.17	0.92	0.57	0.58
	7.14	6.22	1.61	0.95	0.98	1.20	0.76	0.54	0.58
F	6.65	6.25	0.59	0.13	0.14	0.18	0.42	0.10	0.07
	6.40	6.06	0.26	0.10	0.11	0.14	0.16	0.04	0.03
	5.57	4.67	0.25	0.11	0.11	0.19	0.36	0.10	0.08
G	5.57	5.61	2.18	1.37	1.30	1.60	1.19	0.77	0.81
	5.23	4.98	1.58	1.36	1.26	1.51	1.15	0.74	0.93
	5.54	5.05	1.63	1.34	1.28	1.48	1.02	0.62	0.82
H	7.19	6.69	2.84	2.15	7.19	7.74	7.08	3.87	3.07
	6.03	5.49	4.91	3.19	4.08	6.3	4.19	0.95	0.24
	6.09	6.48	4.48	3.55	2.88	1.91	4.3	1.73	1.83
I	6.25	6.58	1.63	1.16	1.07	1.21	0.99	0.62	0.65
	6.67	6.45	1.81	1.17	1.21	1.39	1.03	0.70	0.76
	6.82	6.11	1.73	1.32	1.27	1.54	1.23	0.75	0.92
J	9.34	8.56	11.30	7.31	6.19	5.04	3.08	1.90	1.83
	9.33	8.46	10.93	7.53	6.55	5.42	3.12	1.81	2.11
	9.38	8.02	10.83	7.24	6.10	5.26	3.23	1.88	1.82

A = Nothing

B = Irradiated Asphalt

C = Autoclaved Asphalt

D = Soil

E = Soil (35°C)

F = Autoclaved Soil

G = Soil, Irradiated Asphalt

H = Soil, Irradiated Asphalt (35°C)

I = Soil, Autoclaved Asphalt

J = Agar, Irradiated Asphalt, ATCC 13388

TABLE 4.2. Evolved mg CO₂-C for Test Flasks Initiated in June 1992. The mg CO₂-C in the table has been corrected for the background levels of CO₂ in the KOH solution. Sampling day is measured from start of incubation.

Condition I.D. ¹	Day 30	Day 66	Day 99	Day 126	Day 156	Cumulative
D	2.11	1.35	1.97	0.47	0.53	6.43
	3.09	2.87	3.44	1.39	2.23	13.02
	2.08	1.39	0.89	0.51	0.65	5.52
E	4.70	7.92	0.19	0.15	0.25	13.21
	4.07	2.39	2.59	1.35	0.14	10.54
	4.06	5.22	2.56	1.21	0.08	13.13
G	2.64	1.90	1.23	0.80	0.77	7.34
	2.52	1.58	1.02	0.71	0.84	6.67
	2.66	1.75	1.12	0.71	0.80	7.04
H	4.66	3.80	5.08	4.39	3.92	21.85
	4.54	3.16	3.29	0.99	0.19	12.17
	4.63	7.92	3.95	2.80	3.25	22.55

¹ D = Soil Only E = Soil Only (35°C)
G = Soil, Irradiated Asphalt H = Soil, Irradiated Asphalt (35°C)

FIGURE 4.1. Evolved CO₂-C Results After New Test Configuration Implemented - Data for Flasks Containing Nothing, Irradiated Asphalt Only, and Autoclaved Asphalt Only

only after the change in the experimental test configuration. The levels of CO₂-C observed after implementation of the new test configuration are consistent with the absence of a CO₂ source in the flasks.

To some extent, all of the test conditions showed a decrease in the levels of evolved CO₂-C except for test flasks containing nutrient agar, irradiated asphalt, and the bacterial culture (ATCC 13388). Figure 4.2 illustrates the evolved CO₂-C for the agar test condition. Results from the first data set after the change in test flask configuration show an evolved CO₂-C level greater than that observed for either the day 30 or day 90 data sets. Since the results from each of the three flasks for this test condition were similar, the elevated levels are considered to be real. There are two explanations for this observation: 1) since the leak in the test flasks is a two-way path in which CO₂ may either enter or escape, the actual amount of evolved CO₂ for days 30 and 90 was not entirely collected by the KOH trap, and 2) the evolved CO₂-C was trending upward during the first 90 days of incubation with the peak occurring during the sample collection period for day 120.

FIGURE 4.2. Evolved CO₂-C Results After New Test Configuration Implemented - Data for Flasks Containing Nutrient Agar, Asphalt, and ATCC 13388 Culture

Since the ATCC culture is known to degrade many components of asphalt, and the results for days 30 and 90 indicate a CO₂ evolution rate higher than the other test flasks, the former explanation for the observations in Figure 4.2 is more likely.

Also shown in Figure 4.2 is a significant decreasing trend in the amount of evolved CO₂-C in a given sampling period. Figure 4.3 better shows the overall effect of this trend by illustrating the cumulative CO₂-C evolved as a function of time for the new test configuration data. As shown by Figure 4.3, the rate at which CO₂-C is evolved, which is proportional to the biodegradation rate of the asphalt, is decreasing as a function of time. The data suggest that the biodegradation rate will continue to decrease, which would correspond to a plateau in the cumulative CO₂-C plot in Figure 4.3. The mechanism for this plateau of the CO₂-C evolution rate may be either an oxygen limitation because of diffusion through the biomass or a limited supply of trace nutrients.

The test condition containing nutrient agar, asphalt, and ATCC 13388 culture is considered to be optimal for asphalt biodegradation. The asphalt is in direct contact with bacteria known to

FIGURE 4.3. Cumulative CO₂-C for Agar-Containing Test Flasks. Data from day 120 and on.

degrade asphalt, and the nutrient agar provides additional substrates to promote bacterial growth. The observed decrease in the CO₂-C evolution rate is also expected for test conditions containing just soil and test conditions containing both soil and asphalt; however, the underlying mechanism for this decrease in the CO₂-C evolution rate is expected to be different.

Figure 4.4 illustrates the cumulative CO₂-C evolved for the control flasks with only soil and test flasks containing soil that had been autoclaved (sterilized). For the control flasks, the decreasing evolution rate trend is similar to that observed for the test flasks with the nutrient agar. Comparison between Figures 4.3 and 4.4 illustrates the difference between the optimal conditions of the nutrient agar flasks (optimal growth conditions, accessible carbon source) and the baseline conditions of the control flasks (less optimal growth conditions, limited access to a carbon source). The overall CO₂-C evolution rate for the test flasks containing nutrient agar is ≈ 0.16 mg/d while the overall evolution rate for the control flasks is ≈ 0.03 mg/d.

Figure 4.4 also illustrates the cumulative CO₂-C for test flasks containing only soil that had been autoclaved. As Figure 4.4 shows, the autoclaving process was successful in killing many of

FIGURE 4.4. Cumulative CO₂-C for Test Flasks Containing Only Soil and Test Flasks Containing Only Autoclaved Soil. Data from day 120 and on.

the microorganisms present in the Hanford soil. The source of the evolved CO₂-C from these test flasks may be naturally occurring microorganisms in the soil that were not killed or another microorganism that was introduced. The latter scenario is more likely since the test flasks were not maintained in a sterile environment during the entire incubation test period.

Figure 4.5 illustrates the cumulative CO₂-C for test flasks containing both asphalt and soil. One test set contained asphalt sterilized by gamma irradiation, while the other test set contained asphalt sterilized by autoclaving. The two differently prepared asphalt samples were used to determine the effect on asphalt biodegradation. As expected, a decreasing CO₂-C evolution rate is observed in Figure 4.5. Unlike the case with the nutrient agar, the mechanism for this decrease is most likely due to a loss of the primary organic substrate. Some nutrient limitation may also be a contributing mechanism.

Figure 4.5 illustrates a potential slight difference in the biodegradation rate for the differently prepared asphalt samples. The irradiated asphalt tends to show a slightly higher level of

FIGURE 4.5. Cumulative CO₂-C for Test Flasks Containing Soil and Irradiated Asphalt and Test Flasks Containing Soil and Autoclaved Asphalt. Data from day 120 and on.

evolved CO₂-C than the asphalt that was autoclaved. This difference may be due to the gamma irradiation degrading some of the larger asphalt compounds into smaller ones. The smaller organic compounds are generally easier to degrade, thus leading to a higher level of evolved CO₂-C. However, the difference may also be due to the volatilization of some compounds during the autoclaving process. Since smaller compounds are usually more volatile than larger ones, the smaller organic compounds would be driven out of the asphalt, leaving a proportionally greater number of larger, and less degradable, compounds. What is common to both proposed mechanisms is the prediction of a lower level of evolved CO₂-C for asphalt containing a greater proportion of larger organic compounds.

To determine if the observed differences in Figure 4.5 were significant, a set of statistical analyses were performed. For each time period, the mean and standard deviation of the three observations was calculated. The standard deviations were squared and the variances tested under the hypothesis that they were equal using an F-test (Steel and Torrie 1980). The variances tested to be equal for all time periods except day 183. Also, the means for each treatment were compared under the hypothesis that they were equal using a t-test (Steel and Torrie 1980). A different t-test must be used depending on the equality of the variances as tested in the previous step. The results showed that the means tested to be equal except for Day 153. For Day 153, the mean for test flasks with soil and irradiated asphalt tested to be significantly higher than the mean for test flasks with soil and autoclaved asphalt. This difference perpetuated itself for the remaining sample periods, as shown in Figure 4.5.

Figure 4.6 depicts the cumulative CO₂-C for the test flasks containing soil and asphalt that were incubated at elevated temperature. The results for each of the three replicate flasks are shown to illustrate the observed differences between the flasks. As illustrated in Figure 4.6, the spread of the data is greater than that observed for any of the other test conditions. The cause for this spread could be a combination of the differing acclimation periods for the microorganisms to the elevated temperature and/or a difference in the population of microorganisms that adapt well in each test flask. Comparing Figure 4.6 with Figure 4.3 (cumulative CO₂-C for test flasks with the nutrient agar), it is apparent that the overall rate of CO₂-C evolution is comparable between the two test conditions (≈ 0.12 mg/day for the elevated temperature test flasks vs. ≈ 0.16 mg/day for the agar-containing test flasks). However, whereas the rate for the agar-containing test flasks is decreasing, the rate for the test flasks at elevated temperature started out low and then increased. Due to the scatter in the data, it is difficult to determine if the observed trend for the cumulative CO₂-C is going to continue upward or level off as seen previously for the other data sets.

FIGURE 4.6. Cumulative CO₂-C for Test Flasks Containing Soil and Irradiated Asphalt Incubated at Elevated Temperature. Data from day 120 and on.

4.2 RESULTS FROM JUNE 1992 TEST SET

Once the new test configuration was found to be successful at eliminating the CO₂ leakage problem, a second set of test flasks was initiated in June 1992 to collect data during the incubation period from time zero on. This second set of test flasks was limited to test conditions with only soil (both at ambient and elevated temperature) and soil and irradiated asphalt (both at ambient and elevated temperature). These test conditions were identified as conditions for which data was necessary to allow a combined interpretation of the results for both the January and June test sets. The data from the test flasks with only soil incubated at elevated temperature was especially important because the January test set did not have a soil control at an elevated temperature.

Figure 4.7 illustrates the cumulative CO₂-C evolved for test flasks containing only soil and flasks containing soil and irradiated asphalt, each set of flasks being stored at ambient temperature. Except for the data for one of the three flasks with only soil, the cumulative CO₂-C curves for the

FIGURE 4.7. Cumulative CO₂-C for Test Flasks Containing Only Soil and Test Flasks Containing Soil and Irradiated Asphalt - June Test Set

two different test conditions are identical. This suggests that the native microorganisms prefer the naturally occurring organic material in the soil (a material they are used to) and/or require time to adapt to the different carbon source (i.e., the asphalt). Figure 4.8 shows the cumulative CO₂-C curves from the January test set for test flasks containing only soil and test flasks containing soil and irradiated asphalt. As shown in Figure 4.8, there is little difference between the two test conditions in the amount of CO₂-C evolved at day 120, which is consistent with observations for the June test set. However, as the incubation period increases, the test flasks containing soil and irradiated asphalt begin to evolve greater amounts of CO₂-C in a given sampling period compared to the control flasks with only soil.

The higher cumulative CO₂-C curve observed in Figure 4.7 for one of the test flasks containing only soil was due to elevated levels of CO₂-C produced during the sampling period between day 30 and day 97. Microorganisms in this test flask either had an easily accessible source of organic carbon or acclimated to the test flasks better than the microorganisms in the other two control flasks with only soil. The overall decreasing trend is the same as for the other two

FIGURE 4.8. Cumulative CO₂-C for Test Flasks Containing Only Soil and Test Flasks Containing Soil and Irradiated Asphalt - January Test Set. Day 120 on.

control test flasks, as seen by a comparison of the slopes for each of the control flasks in Figure 4.7. To a lesser degree than observed for the June test set, one of the control flasks for the January test set also has a higher cumulative level of CO₂-C compared to the other two.

Figure 4.9 illustrates the cumulative CO₂-C evolved for test flasks containing only soil and flasks containing soil and irradiated asphalt, each set of flasks being incubated at an elevated temperature close to 35°C. Unlike the results illustrated in Figure 4.7 for test flasks incubated at ambient temperature, the results for test flasks incubated at elevated temperature show a greater degree of variability than flasks incubated at ambient temperature. Also shown in Figure 4.9 is a distinct difference between the control flasks with only soil and flasks with both soil and irradiated asphalt. At day 30, the amount of CO₂-C evolved is the same for both test conditions; however, beginning at day 64, the flasks containing asphalt begin to show an increased rate of CO₂-C evolution relative to the control flasks.

FIGURE 4.9. Cumulative CO₂-C for Test Flasks Containing Only Soil and Test Flasks Containing Soil and Irradiated Asphalt Incubated at 35°C - June Test Set

Figure 4.10 compares the cumulative CO₂-C evolved curves for test flasks containing only soil at both ambient and elevated temperature. As expected, results for test flasks incubated at elevated temperatures are generally higher than for test flasks incubated at ambient temperature. The difference, however, does not appear to be very great, as illustrated by the fact there is some overlap between the highs and lows of one test condition with the other. For both test conditions, the overall trend is again a decrease in the CO₂-C evolution rate as a function of time.

4.3 DETERMINATION OF ASPHALT BIODEGRADATION RATE

As discussed previously, results for the June test set indicate a trend in which the rate of CO₂-C evolution decreases as a function of time. Results from the January test set indicate that this decreasing trend for the rate of CO₂-C evolution continues at longer incubation time (January test set incubated for greater than 270 days). However, the degree to which the CO₂-C evolution

FIGURE 4.10. Cumulative CO₂-C for Test Flasks Containing Only Soil and Test Flasks Containing Only Soil Incubated at 35°C - June Test Set

rate decreases is less at a longer incubation time than at a shorter incubation time. Figure 4.11 better illustrates this point by showing the CO₂-C evolution rate for different sampling points from the January and June test sets for test flasks containing only soil.

Figure 4.12 illustrates the CO₂-C evolution rate for different sampling points from the January and June test sets for test flasks containing both soil and irradiated asphalt. As shown in Figure 4.11 for the control flasks with only soil, results for test flasks with soil and asphalt also show that the degree to which the CO₂-C evolution rate decreases is less at longer incubation times than at a shorter incubation time. The analysis of the January and June test sets to determine the asphalt biodegradation rate will be performed separately so that potential differences in the handling of the test flasks does not affect the calculated results.

As discussed in Section 3.0, the determination of the asphalt biodegradation rate requires five steps. First, the CO₂-C evolution rate is plotted as a function of time for each of the test conditions. A "least-squares" linear regression is then performed on the data to obtain a best fit

FIGURE 4.11. CO₂-C Evolution Rate for Soil-Only Test Flasks - January and June Test Sets

FIGURE 4.12. CO₂-C Evolution Rate for Test Flasks Containing Soil and Irradiated Asphalt - January and June Test Sets

line. The resulting line (equation) for the control flasks (soil only) is then subtracted from the line determined for each of the other test conditions. This process results in a new line that predicts the net CO₂-C evolution rate as a function of time. This CO₂-C evolution rate is inserted into Equation (1) to determine the asphalt biodegradation rate:

$$\text{Biodegradation Rate} = \frac{\text{Net CO}_2\text{-C Evolution Rate}}{\text{Bulk Density} \times \text{Surface Area} \times \% \text{Carbon}} \quad (1)$$

Figures 4.13 and 4.14 illustrate the linearly regressed lines for the control flasks with only soil and test flasks with soil and irradiated asphalt, respectively. As discussed previously, the data from the January and June test sets were analyzed separately, as shown by the two different linear regression curves in Figure 4.13 and 4.14. The high data set for a single test flask with only soil was removed from the June test set because it was determined to be statistically different from the other two test flasks. This determination was done using an outlier test (American Society for Testing and Materials 1969).

From Figure 4.13, the equation for the linearly regressed line for the January test flasks with only soil was determined to be

$$\text{CO}_2\text{-C evolution rate} = -1.6 \times 10^{-4}(\text{day}) + 6.6 \times 10^{-2} \quad (r^2 = 0.64).$$

For the June test flasks with only soil, the determined linear regression line was

$$\text{CO}_2\text{-C evolution rate} = -3.9 \times 10^{-4}(\text{day}) + 7.5 \times 10^{-2} \quad (r^2 = 0.72).$$

From Figure 4.14, the linear regression line determined for the January test flasks with soil and asphalt was

$$\text{CO}_2\text{-C evolution rate} = -1.5 \times 10^{-4}(\text{day}) + 7.2 \times 10^{-2} \quad (r^2 = 0.73),$$

and for the June test flasks

$$\text{CO}_2\text{-C evolution rate} = -4.7 \times 10^{-4}(\text{day}) + 8.9 \times 10^{-2} \quad (r^2 = 0.82).$$

For each of the test conditions shown in Figures 4.13 and 4.14, the slope of the linear regression line is negative. This indicates that the overall evolution rate of CO₂-C evolution is decreasing as a function of time. A determination of the asphalt biodegradation rate requires the net CO₂-C evolution rate. This net rate is determined by subtracting the equation for the control flasks (containing only soil) from the equation for test flasks containing soil and irradiated asphalt. The

FIGURE 4.13. Linear Regression for Test Flasks Containing Only Soil

FIGURE 4.14. Linear Regression for Test Flasks Containing Soil and Irradiated Asphalt

resulting lines for the net CO₂-C evolution rate are illustrated in Figure 4.15. The equations for these lines were:

$$\text{Net CO}_2\text{-C evolution rate} = -7.5 \times 10^{-5}(\text{day}) + 1.4 \times 10^{-2} \quad (r^2 = .99)$$

for the June data set and

$$\text{Net CO}_2\text{-C evolution rate} = 9.6 \times 10^{-6}(\text{day}) + 5.7 \times 10^{-3} \quad (r^2 > 0.99)$$

for the January data set.

The negative slope for the net CO₂-C evolution rate equation for the June data set indicates that the net rate of CO₂-C evolution is decreasing with time. Consequently, the highest net CO₂-C evolution rate, and corresponding asphalt biodegradation rate, should occur early. The incubation time for the June data set was from day zero to day 156. Therefore, the highest net CO₂-C evolution rate for the June data set would be at time zero. This net rate is $1.4 \pm 0.2 \times 10^{-2}$ mg/day. The asphalt biodegradation rate for the June data set is determined by inserting this net CO₂-C evolution rate into Eq. 1 with values of 1037 mg/cm³, 38.48 cm², and 78.8% for the bulk density, average surface area, and percent carbon, respectively. For the June test set, the average asphalt

FIGURE 4.15. Net Evolved CO₂-C for Soil and Irradiated Asphalt Test Condition

biodegradation rate for the test condition with soil and irradiated asphalt is 1.6×10^{-4} cm/yr with a high endpoint at the 95% confidence interval equal to 1.9×10^{-4} cm/yr and a low endpoint at the 95% confidence interval equal to 1.4×10^{-4} cm/yr. This range of degradation rates is on the same order of magnitude as those found by Barletta et al. (1987) for Richland, Washington soils. Extrapolating the highest estimated asphalt biodegradation rate to 10,000 years^(a) indicates that approximately 2 cm would be degraded in this time period. This is well within the 10-cm allowance currently in the design criteria for the asphalt diffusion barrier.

The positive slope for the net CO₂-C evolution rate equation for the January data set indicates that the net rate of CO₂-C evolution is increasing with time. Since the incubation time period for the January data set was from day zero to day 309, a conservative estimation of the asphalt biodegradation rate for the January data set would be obtained by inserting 330 days into the net CO₂-C evolution rate equation and then inserting this calculated net CO₂-C evolution rate into Equation 1.^(b) The net CO₂-C evolution rate at day 330 is 8.9×10^{-3} mg/day. This net CO₂-C evolution rates results in an asphalt biodegradation rate of 1.0×10^{-4} cm/yr. An estimation of the error for the January data set linear regression equation shows the error to not be statistically significant for two significant figures. Therefore, a range of asphalt biodegradation rates cannot be calculated.

The asphalt biodegradation rate for the test condition with soil and irradiated asphalt incubated at ambient temperature was found to be the same order of magnitude as that reported by Barletta et al. (1987). To increase confidence that the Bartha-Pramer technique was being correctly implemented, a set of test flasks containing nutrient agar, irradiated asphalt, and a known inoculum, ATCC 13388, was included in the test matrix. Comparison of the results from this test condition to the literature values provides a check on the experimental techniques used in this study.

Figure 4.16 illustrates the least-squares linear regression fit for the test flask containing the nutrient agar, irradiated asphalt, and ATCC 13388 culture. The equation for the linear regression line was found to be:

$$\text{CO}_2\text{-C Evolution Rate} = -0.002(\text{day}) + 0.494 \quad (r^2 = 0.904).$$

(a) Ten thousand years is the evaluation time period used in the performance assessment of the asphalt diffusion barrier for the Hanford grout vaults.

(b) Extrapolating a net CO₂-C evolution rate for a time longer than day 330 would result in a questionable value because it would be significantly outside the constraints of the test data.

FIGURE 4.16. Linear Regression for Test Flasks Containing Nutrient Agar, Irradiated Asphalt, and ATCC 13388

The negative slope indicates that the CO₂-C evolution rate decreases as a function of time. Consequently, the highest biodegradation rate (which is proportional to the CO₂-C evolution rate) occurs at an early incubation time. Inserting 120 days into the equation for CO₂-C evolution rate gives an average value of 3.1×10^{-1} mg/day with a low endpoint at the 95% confidence interval of 2.4×10^{-1} mg/day and a high endpoint at the 95% confidence interval of 3.9×10^{-1} mg/day. Using Equation 1 and this range of calculated CO₂-C evolution rates leads to a predicted biodegradation rate of 3.6×10^{-3} cm/yr with a low of 2.8×10^{-3} cm/yr and a high of 4.5×10^{-3} cm/yr. This biodegradation rate is of the same order of magnitude as that found by Barletta et al. (1987), which was 2.44×10^{-3} cm/yr.

Figures 4.6 and 4.9 indicated that the amount of CO₂-C evolved for test conditions incubated at elevated temperature ($\approx 35^\circ\text{C}$) was generally higher than that for corresponding test conditions incubated at ambient temperature ($\approx 25^\circ\text{C}$). This difference suggests that the degradation rate for both naturally occurring organic material and asphalt is higher at elevated temperature. To determine if the biodegradation rate is higher at elevated temperature, the method previously described is applied to the June test set for test flasks incubated at elevated temperatures. This method is not applicable to the January data set because a soil-only control at elevated temperature was not included in the January data set.

Figures 4.17 and 4.18 illustrate the least-squares linear regression lines for the control flasks with only soil and flasks with soil and asphalt, both sets incubated at $\approx 35^{\circ}\text{C}$, respectively. The resulting equation for the soil-only control flask was

$$\text{CO}_2\text{-C evolution rate} = -1.2 \times 10^{-3}(\text{day}) + 1.9 \times 10^{-1} \quad (r^2 = 0.66),$$

and the resulting equation for the test flasks with both soil and irradiated asphalt was

$$\text{CO}_2\text{-C evolution rate} = -5.7 \times 10^{-4}(\text{day}) + 1.7 \times 10^{-1} \quad (r^2 = 0.25).$$

Figure 4.19 illustrates the line resulting from subtracting the results for the control flasks with soil from the results for the test flasks with soil and irradiated asphalt. The resulting equation for the net $\text{CO}_2\text{-C}$ evolution rate is:

$$\text{Net CO}_2\text{-C Evolution Rate} = 6.5 \times 10^{-4}(\text{day}) - 2.0 \times 10^{-2} \quad (r^2 = .99).$$

The positive slope of this resulting line indicates that the net $\text{CO}_2\text{-C}$ evolution rate is increasing as a function of time. Since the incubation period for this data set was from time zero to day 156, inserting 180 days into the net $\text{CO}_2\text{-C}$ evolution rate equation results in a conservative estimation of the net $\text{CO}_2\text{-C}$. The calculated net $\text{CO}_2\text{-C}$ evolution rate is $9.7 \times 10^{-2} \text{ mg/day}$ with a high endpoint

FIGURE 4.17. Linear Regression for Test Flasks Containing Only Soil Incubated at 35°C

FIGURE 4.18. Linear Regression for Test Flasks Containing Soil and Irradiated Asphalt Incubated at 35°

FIGURE 4.19. Net Evolved CO₂-C for Soil and Irradiated Asphalt Test Condition at 35°C

at the 95% confidence interval equal to 1.1×10^{-1} mg/day and a low endpoint at the 95% confidence interval equal to 8.5×10^{-2} mg/day. Inserting these values into Equation 1 results in an average asphalt biodegradation rate of 1.1×10^{-3} cm/yr with a high of 1.3×10^{-3} cm/yr and a low of 9.9×10^{-4} cm/yr.

Extrapolating the highest estimated asphalt biodegradation rate to 10,000 years indicates that approximately 13 cm of the asphalt diffusion barrier would be degraded in this period. This is higher than the 10-cm allowance currently in the design criteria for the asphalt diffusion barrier; however, it should be noted that this estimated asphalt biodegradation rate is for incubation at elevated temperature ($\approx 35^{\circ}\text{C}$). The average asphalt biodegradation rate during the course of the 10,000-year performance period would be expected to lie between that estimated for ambient incubation temperatures and that estimated for elevated temperatures.

5.0 CONCLUSIONS

The results from testing initiated in March 1991 and completed in November 1992 indicate that asphalt is degraded by microorganisms native to the Hanford Site. Experiments to determine asphalt biodegradation rates were conducted using three separate test sets. These test sets were initiated in March 1991, January 1992, and June 1992 and ran for periods of 6 months, 11 months, and 6 months, respectively. Results from the March 1991 tests were inconclusive because of unanticipated results for control flasks. Biodegradation results from the test sets initiated in 1992 were as follows:

- For test flasks containing soil and irradiated asphalt incubated up to 160 days, the average asphalt biodegradation rate at ambient temperature was determined to be 1.6×10^{-4} cm/yr. The high endpoint at the 95% confidence interval was 1.9×10^{-4} cm/yr and the low endpoint at the 95% confidence interval was 1.4×10^{-4} cm/yr.
- For test flasks containing soil and irradiated asphalt incubated up to 310 days, the average asphalt biodegradation rate at ambient temperature was determined to be 1.0×10^{-4} cm/yr.
- For test flasks containing soil and irradiated asphalt incubated up to 160 days, the average asphalt biodegradation rate at elevated temperature ($\approx 35^\circ\text{C}$) was determined to be 1.1×10^{-3} cm/yr. The high endpoint was 1.3×10^{-3} cm/yr and the low endpoint was 9.9×10^{-4} cm/yr.

Observations from the test sets initiated in 1992 follow:

- The asphalt biodegradation rate at ambient temperature decreases as a function of time.
- Results for test flasks containing nutrient agar, irradiated asphalt, and an ATCC bacterial culture known to degrade asphalt were comparable to results reported in the literature.
- Results for test flasks containing nutrient agar were included in the test matrix as both a check on the experimental method and to provide optimal conditions for asphalt biodegradation. The biodegradation rate for these test flasks was also found to decrease as a function of time.
- A comparison between test flasks containing soil and irradiated asphalt and test flasks containing soil and autoclaved asphalt showed little difference in the amount of $\text{CO}_2\text{-C}$ evolution ($\text{CO}_2\text{-C}$ evolution is proportional to asphalt biodegradation).

Results and observations for the test sets initiated in 1992 indicate that microorganisms native to the Hanford Site will degrade the asphalt to be used as a diffusion barrier for the Hanford grout vaults. The rate at which these microorganisms will degrade the asphalt was found to be dependent on the incubation temperature (higher degradation rates seen at elevated temperatures) and incubation time (degradation rate observed to decrease as a function of time). However, the rate for asphalt biodegradation seemed to be independent of the preparation method for the asphalt. Asphalt subjected to gamma irradiation was not observed to biodegrade at a rate higher or lower than asphalt subjected to an autoclaving process.

6.0 RECOMMENDATIONS

This initial study evaluated the capability of microorganisms native to the Hanford Site to degrade asphalt to be used as a diffusion barrier for the Hanford grout vaults. The asphalt biodegradation rates determined from this study are conservative values that may be used to estimate the amount of degradation over time. Results from this study indicate that the temperature in which biodegradation occurs affects the biodegradation rate and that the biodegradation rate decreases as a function of time. Results from this study also indicate that the biodegradation rate is approximately the same for asphalt that is exposed to sterilization doses of gamma irradiation and asphalt exposed to autoclaving temperatures and pressure.

Based on the results from this initial study, we recommend the following work so that a complete evaluation of the effects of biodegradation on the asphalt diffusion barrier can be made.

1) Long-term testing with the Bartha-Pramer flasks - The asphalt biodegradation rate was determined to decrease with time. This determination was made based on data obtained from test flasks incubated up to 310 days. Estimation of a biodegradation rate at longer time periods would result in a questionable value because of the extrapolation outside of the constraints of the test system. Long-term testing would confirm, or contradict, the decreasing trend for the biodegradation rate.

2) Parametric study with Bartha-Pramer flasks - This initial study showed that temperature was an important parameter affecting the asphalt biodegradation rate and that physical treatment of the asphalt (gamma irradiation and autoclaving) was not. Additional studies are needed to identify other parameters that may affect the asphalt biodegradation rate. The identification of these parameters, in combination with predictions of the environment surrounding the asphalt diffusion barrier, would allow a better estimation of the effect biodegradation has on the asphalt barrier. For example, if moisture level is found to be an important parameter for the asphalt biodegradation rate, then the estimation of the asphalt biodegradation rate based on predicted moisture levels in contact with the asphalt barrier would be more accurate than an estimated rate without this consideration.

3) Mechanistic study for asphalt biodegradation - Determining the mechanism by which asphalt is biodegraded by microorganisms native to the Hanford Site is necessary to evaluate scenarios that are not amenable to testing in the laboratory. For example, testing with the Bartha-

Pramer flasks estimates an overall asphalt biodegradation rate for an asphalt sample. The method does not provide information on whether the biodegradation occurs in cracks in the asphalt sample or strictly on the surface. If biodegradation does occur in the cracks, to what extent does diffusion limit the rate of biodegradation? To answer these questions requires an understanding of the asphalt biodegradation mechanism.

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APPENDIX A

**SUMMARY OF 1991 ASPHALT
BIODEGRADATION TESTING**

APPENDIX A

SUMMARY OF 1991 ASPHALT BIODEGRADATION TESTING

The results from 6 months of testing (March 1991 to September 1991) suggest that asphalt is degraded at a minimal rate by microorganisms native to the Hanford Site. However, further work is needed to quantify the rate of biodegradation for use in evaluating the performance requirements of the asphalt diffusion barrier. Thirteen different test conditions were evaluated using combinations of asphalt (with and without lime) and support medium (nutrient agar or soil from various locations around the grout vaults). Only flasks containing excavation soil with coupons incubated at elevated temperatures (35°C) showed CO₂ evolution levels (an indication of asphalt biodegradation) that were statistically different (95% confidence interval) from that observed for the control flasks containing surface soil only. Estimation of the asphalt biodegradation rate for this test condition was found to be 6.84×10^{-4} cm/yr (2.69×10^{-4} in./yr). Unusual results for the control flasks make the determined biodegradation rate for the flasks incubated at elevated temperatures suspect. Further testing is needed to verify this result. Degradation rates were not evaluated for the remaining test conditions because results were not found to be statistically different from those of the control flasks.

Results for flasks with asphalt coupons only showed a CO₂ evolution level that was significantly higher than the KOH solution blanks and was comparable to that seen in the surface soil-only control flasks. This result is unexpected because of the lack of an identified mechanism to explain this high rate of CO₂ evolution. In addition, the results for test conditions with both soil and asphalt coupons do not show a CO₂ evolution rate that is higher than that for soil only or asphalt coupons only, as would be expected.

EXPERIMENTAL METHODS

This section describes the sample preparation method used, the equipment use, the established testing matrix, and the testing procedures followed for determining the biodegradation rate for AR-6000 asphalt.

Test Materials and Material Preparation

Soils were collected from the vicinity where grout vaults will be constructed, located on the 200 Area plateau of the Hanford Site in southeast Washington. Soil samples from the surface,

vault pit bottom, and excavation activities were aseptically collected and immediately transported to the laboratory for storage below 15°C prior to the preparation of the test flasks. Soil was stored at a reduced temperature to slow the metabolic activity of the microorganisms present and thus assure that the microorganisms remained viable prior to testing.

AR-6000 asphalt with hydrated lime was prepared by heating 213.0 g of asphalt to 142°C and mixing in 6.582 g of lime for 5 minutes (asphalt with lime is believed to be less susceptible to biodegradation). This process produced a batch of asphalt with 3.1 wt% hydrated lime. AR-6000 asphalt, with and without lime, was formed into cylindrical shapes by pouring asphalt in the liquid phase (achieved by heating the asphalt above 140°C) into standard stainless steel Swagelok 3/4 in. front ferrules resting on an aluminum plate (both the plate and the ferrules were stored in a freezer until just before the ferrules were filled). Prior to being filled with asphalt, each ferrule was weighed, numbered, and coated with a silicon spray.^(a) After each ferrule was filled with asphalt, the weight of each asphalt/ferrule unit was measured and the net weight of each asphalt "coupon" determined. Immersed weight in water was also measured for some of the asphalt/ferrule units so that the average density of the asphalt coupons could be determined.

To ensure that the volume and the surface area of each asphalt coupon was approximately the same, each of the ferrules was filled to the "top" with asphalt. The dimensions of the asphalt coupons are those of the inside of the ferrule. Measurement of three ferrules determined that the average volume of each asphalt coupon was 2115 mm³ with a surface area of 1019 mm².

Asphalt is made of a multitude of long-chain hydrocarbon units containing predominately hydrogen and carbon. Measurement of the evolution of carbon dioxide is proportional to the amount of carbon degraded; the percent carbon in asphalt must be known to determine the amount of asphalt degraded. Total organic carbon analyses were done in triplicate on an asphalt coupon without lime and a coupon with lime.^(b) Results from the analysis showed very little difference between the two coupon types; therefore, the data was combined, with a resulting total organic carbon content of 78.8%.

Difco 0001 nutrient agar was selected for use in flasks containing nutrient agar, bacteria specified in ASTM G22, and asphalt coupons (with and without lime). This nutrient agar lacks only a carbon source. The bacteria *Pseudomonas aeruginosa*, ATCC 13388, was purchased from

(a) The silicon spray assists in removing the asphalt coupon from the ferrule mold. Silicon spray is not used on the aluminum plate that forms the "bottom" of the mold.

(b) Total organic carbon determined by PNML Procedure 7-40.37.

the American Type Culture Collection (ATCC). The dehydrated culture was received on nutrient agar (Difco 0001) and preserved in 40% glycerol in an -85°C deep freezer. Prior to use for testing, the ATCC 13388 bacteria was grown overnight in nutrient broth (Difco 0003) and resuspended in phosphate buffer.

The cell density of the suspended bacterial culture was determined on a Beckman DU-70 spectrophotometer. Using phosphate buffer solution as a blank, the optical density of the bacterial solution was measured at 650 nm (OD_{650}) and found to be 0.8561. This density measurement corresponds to approximately 8.6×10^8 cells/ml.^(a) Preparation of the inoculated flasks resulted in approximately 2.6×10^9 cells per 50 ml of nutrient agar.

Test Equipment

Testing for the biodegradation of asphalt was performed in standard Bartha-Pramer flasks as shown in Figure 1. The flasks and fittings were autoclaved prior to use and then filled with test material in a laminar flow hood. Rubber stoppers for the flasks were wrapped with Teflon so that a better seal with the glass could be attained. A syringe that penetrated the rubber stopper was packed with ascarite and glass wool and used to remove carbon dioxide from air entering the flasks. The vials containing the 10 ml of 0.1 M KOH solution were also autoclaved prior to being filled with KOH solution and subsequently placed in the Bartha-Pramer flasks. A CO_2 -free, 0.1 M KOH solution was prepared by dissolving KOH (Sigma Catalog #17-1767 88% KOH, 0.8% K_2CO_3) in millipore water from which the CO_2 had been removed by boiling the water for 15 minutes. The KOH solution acted as a CO_2 trap for any CO_2 evolved in the flask as a result of biodegradation of the asphalt coupons, or of degradation of background organics found in each soil type.

Analysis of the KOH solutions for trapped CO_2 was performed on a Mettler DL70 autotitrator. The HCl used for determining the CO_2 levels in the KOH solutions was calibrated with a sodium hydroxide (NaOH) solution that had been previously calibrated on the titrator with a potassium hydrogen phthalate [KHP] ($KHC_2H_4O_4$). This method determined that the concentration of HCl used for the titration of the KOH solutions containing the trapped CO_2 was 0.052 M.

(a) OD_{650} of 1.00 $\approx 1 \times 10^9$ cells/ml (CRC Handbook "Toxicity Testing Using Microorganisms" 1986).

FIGURE 1. Illustration of Experimental Setup with the Bartha-Pramer Flask.

Experimental Sample Scheme

Thirteen different test conditions were used for determining the biodegradation rate of asphalt. Three replicate flasks for each condition were prepared as shown in Table 1. Each flask contained 50 g of the medium type (either soil or nutrient agar) except for flasks 37, 38, and 39, which contained two asphalt coupons only. Each flask also contained two asphalt coupons with or without lime, except for the control flasks with soil only (flasks 34, 35, and 36).

TABLE 1. Experimental Sample Scheme. 50 g of the medium type (soil or nutrient agar) are used for each flask except for flasks with coupons only.

<u>Flask I.D.</u>	<u>Contents</u>
1, 2, 3	Surface Soil + 2 Asphalt Coupons
4, 5, 6	Surface Soil + 2 Asphalt Coupons with Lime
7, 8, 9	Vault Pit Bottom Soil + 2 Asphalt Coupons
10, 11, 12	Vault Pit Bottom Soil + 2 Asphalt Coupons with Lime
13, 14, 15	Excavation Soil + 2 Asphalt Coupons
16, 17, 18	Excavation Soil + 2 Asphalt Coupons with Lime
19, 20, 21	Sterilized Surface Soil + 2 Asphalt Coupons
22, 23, 24	Nutrient Agar + ATCC 13388 + 2 Asphalt Coupons
25, 26, 27	Nutrient Agar + ATCC 13388 + 2 Asphalt Coupons with Lime
28, 29, 30	Excavation Soil Amended to 5 wt% water (41.7% holding capacity) + 2 Asphalt Coupons
31, 32, 33	Excavation Soil + 2 Asphalt Coupons (Flasks Stored at 32 - 40°C)
34, 35, 36	Surface Soil Only
37, 38, 39	2 Asphalt Coupons Only

RESULTS AND DISCUSSIONS

Table 2 summarizes the calculated amounts of CO₂-C evolved for each of the different test flasks over the six-month test period. The table is missing points because not every flask was sampled every 30 days. As indicated by Table 2, within a sample set (i.e., 30 day, 60 day, etc.) there does not appear to be a significant difference between the various conditions being studied. Figure 2 further illustrates this lack of a discernible difference by showing the cumulative mg of CO₂-C evolved for the flasks containing surface soil only, surface soil with two asphalt coupons, and vault pit bottom soil with two asphalt coupons. To verify this observation, a statistical analysis was conducted on the raw data--i.e., the volume difference required to titrate between pH 8.3 and 3.7.

Figure 3 illustrates this difference as a function of sample time for each of the 13 test conditions. A statistical test for differences among the different test conditions for each sample collection point was performed, with the results shown in Table 3. Because results from day 90 are inconsistent with the other data, no statistics were computed for day 90. When the temperature was elevated (flasks 31, 32, and 33), the titration difference was higher than when the temperature

TABLE 2. Evolved mg CO₂-C for Each Sample Set. The mg CO₂-C given below have been corrected for the background levels of CO₂ in the KOH solution.

<u>Flask</u>	<u>30 Day</u>	<u>60 Day</u>	<u>90 Day</u>	<u>120 Day</u>	<u>150 Day</u>	<u>180 Day</u>	<u>Cumulative</u>
1	7.03		11.90		7.44	1.22	26.37
2		8.17		8.88		7.68	24.73
3		8.27		8.98		6.27	23.52
4	7.42		13.89		7.90	5.40	34.61
5		7.97		8.79		8.48	25.24
6		8.14		8.78		4.79	21.71
7	7.41		12.55		8.60	5.35	33.91
8		7.98		9.06		8.28	25.32
9		7.95		8.55		5.77	22.27
10	6.66		4.79		2.80	0.75	15.00
11		8.39		9.36		6.99	24.74
12		8.04		8.76		8.61	25.41
13	6.78		5.75		7.16	1.07	20.76
14		8.03		9.13		6.32	23.48
15		8.09		9.33		6.47	23.89
16	6.76		6.06		8.19	4.06	25.07
17		8.33		10.15		7.01	25.49
18		7.85		8.50		6.25	22.60
19	7.48		5.36		8.30	4.62	25.76
20		7.84		8.81		8.05	24.70
21		8.03		8.60		4.76	21.39
22	8.73		6.54		8.70	6.27	30.24
23		9.11		9.25		8.48	26.84
24		8.80		8.11		4.13	21.04
25	8.86		6.51		8.72	6.17	30.26
26		8.75		9.21		8.69	24.62
27		8.96		8.97		6.90	24.83
28	7.80		6.80		8.51	4.24	27.35
29		9.75		10.34		7.46	27.55
30		8.32		8.87		6.41	23.60
31	7.63		8.86		9.16	5.10	30.75
32		10.49		11.45		6.69	28.63
33		10.88		11.71		7.39	29.98
34	7.65		12.94		6.50	0.88	27.97
35		8.42		8.85		6.77	24.04
36		7.58		8.39		6.66	22.63
37	7.90		6.89		8.92	1.57	25.28
38		9.12		9.51		8.06	26.69
39		9.02		10.28		5.31	24.61

FIGURE 2. Cumulative mg of CO₂-C Evolved for Selected Conditions

FIGURE 3. Plot of Titration Difference for Each Test Condition

TABLE 3. Test for Differences Among the Different Test Conditions. The statistics were computed using a reduced set of data. Data not included in this test were that from day 90, that for flasks at elevated temperature, and the data from day 150 for flasks 10,11,12.

<u>Day</u>	<u>F-Value</u>	<u>Level</u>
30	3.69	0.02
60	3.04	0.03
120	0.96	0.52
150	1.78	0.19
180	0.18	0.99

was not elevated, so this data was not used to calculate the statistics shown in Table 3. In addition, the titration observed for flasks 10, 11, and 12 on day 150 was extremely low and not used.

One very striking feature of Figure 3 is the results for day 90, which has four conditions at a significantly higher value than those values for the rest of the conditions. The four conditions are surface soil with coupons (1, 2, 3), surface soil with limed coupons (4, 5, 6), vault pit bottom soil with coupons (7, 8, 9), and surface soil only (34, 35, 36). This high spike is not believed to be real because 1) the control flask is contained within this group of "outliers," and 2) the results for day 120 for this group are comparable to the other flasks. Parafilm was added around the base of each stopper after the 90-day sample set was collected to assure an airtight seal; therefore, it is possible that the abnormally high values resulted from a leak. The low outlier for day 150 (vault pit bottom soil with limed coupons) probably did not result from a leak, but there is no explanation for this anomalous result.

Also illustrated by Figure 3 is the consistency of higher values for flasks 31, 32, and 33 (excavation soil and coupons incubated at 35°C) than for the other flasks between days 30 and 150. This result is not unexpected because biological activity, with subsequent evolution of carbon dioxide, is higher for many microorganisms at elevated temperatures. The fact that values at day 30 are not higher probably is due to a lag phase in which the native microorganisms become acclimated to the warmer environment. An apparent decrease in evolved CO₂ at day 180 may be caused by a decrease in the amount of available organic carbon, a trend which appears to begin between day 120 and 150.

Figure 4 illustrates the data for the surface soil-only control flask and the resulting linear least squares fitted line. The slope for the linear least square line was found to be 0.128 mg CO₂-C/day. This slope value can be converted to an average CO₂ evolution rate of 0.45 mg CO₂/day. This rate is five times higher than the rate determined by Barletta et al. (1987) for flasks with

FIGURE 4. Cumulative CO₂ Evolved for Surface Soil-Only Controls

Hanford soils only and comparable to rates determined for optimal (pure culture in nutrient agar) conditions studied by Barletta et al. (1987). This is further illustrated in Figure 5, which compares the cumulative CO₂ evolved for the control flasks containing surface soil only with the flasks containing nutrient agar, bacteria, and asphalt coupons.

The bacteria in the flasks added to nutrient agar are those known to degrade asphalt. Carbon dioxide evolution from the control flasks can be attributed only to biodegradation of the natural organic carbon in the soil. Since the amount of organic material in the surface soil is limited (estimated to be less than 0.5 wt% at the surface and decreasing to less than 0.05 wt% at greater depths), the rate of CO₂ evolution should decrease with time as the accessible organic carbon is depleted. Further testing at longer times may help to evaluate the level of CO₂ evolution resulting from the biodegradation of asphalt.

Overall, except for the differences noted above (specifically those at the elevated temperatures), there is not a discernible difference between the varying test conditions between days 30 and 180. The data for 180 days suggests a potential downward trend for some of the conditions, although this trend needs to be verified with data beyond 180 days. Because the

FIGURE 5. Comparison Between Surface Soil-Only Flasks and Flasks with Nutrient Agar, ATCC 13388 and Coupons

control flasks (surface soil only) are not statistically different from the other test conditions, with the exception of the flasks at elevated temperatures, there does not appear to be significant biodegradation of the asphalt. However, a quantitative degradation rate cannot be calculated because there is not a statistical difference between the conditions and controls at ambient temperatures. In addition, because of the perplexing results from flasks that contain asphalt coupons only, the validity of the degradation rate at elevated temperatures is questionable.

Figure 6 illustrates the cumulative results obtained for the flasks containing asphalt coupons only as compared to results for flasks with surface soil only, vault pit bottom soil with coupons, and excavation soil with coupons. As shown, the evolution rate for the flasks with coupons only is steady and comparable in value to flasks known to have a carbon source. Proposed mechanisms and hypotheses to explain the unexpected results for flasks containing the asphalt coupons only are discussed below.

FIGURE 6. Comparison of Results Between Coupon-Only Flasks and Selected Flasks with Soil. Some type of microorganisms present.^(a)

Abiotic degradation, such as air oxidation and photo decomposition, is a possible mechanism for the observed degradation seen in the flasks with asphalt coupons only. Photo decomposition is unlikely because the flasks were stored in a dark cabinet. Degradation of the asphalt because of thermal effects is also improbable because the coupon-only flasks were stored in a dark cabinet at room temperature. Air oxidation is a potential mechanism that may occur in the cabinet in which the flasks were stored; however, existing literature does not identify such a mechanism as being prevalent for the degradation of asphalt.

Biodegradation of the asphalt from natural airborne microorganisms, and other contamination routes, is also a possible mechanism to explain the observed degradation in flasks containing asphalt only. This mechanism can be supported by taking a smear from an asphalt coupon and placing it on a nutrient agar plate to determine if microorganisms are present.

(a) Soils naturally contain some organic matter; therefore, flasks with only surface soil do have a limited carbon source. The asphalt coupons provide an additional source of carbon for flasks containing soil and coupons.

However, if biodegradation is occurring, the flasks with both soil and coupons should show a CO₂ evolution rate that is higher than that seen for flasks with soil only or asphalt coupons only. One explanation is that either the microorganisms native to the soil, or those that are airborne, dominate in the flasks containing both soil and asphalt coupons, so the effect from only one is observed. In addition, a search of the literature for other abiotic mechanisms for the degradation of asphalt would allow a better determination for the cause of the unexpected result with coupons only.

A final explanation for the observed test results is the possibility that the test flasks had leakage of CO₂. This would account for the comparable levels of CO₂ measured in the different test flasks. Additional material was wrapped around the rubber stoppers during testing to minimize potential leakage; however, independent testing of the technique was not performed to determine if this was successful. Future testing with the Bartha-Pramer flasks may need to address leakage; especially if other mechanisms for CO₂ evolution are eliminated.

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