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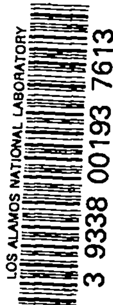
# Potential Microbial Impact on Transuranic Wastes Under Conditions Expected in the Waste Isolation Pilot Plant (WIPP)

October 1—December 15, 1978

University of California



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# Potential Microbial Impact on Transuranic Wastes Under Conditions Expected in the Waste Isolation Pilot Plant (WIPP)

## October 1—December 15, 1978

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**CONTENTS**

ABSTRACT . . . 1

I. INTRODUCTION . . . 2

II. ASSESSMENT OF MICROFLORA IN LASL TRU BURIAL SITE SOIL (LASL) . . . 2

III. ALKYLATION OF RADIONUCLIDES (LASL) . . . 5

IV. CHELATE DEGRADATION (UNM) . . . 7

V. GAS PRODUCTION (UNM) . . . 11

VI. SPECIFIC MILESTONES FOR SECOND QUARTER, FY 1979 (LASL) . . . 13

VII. MILESTONES FOR THE MICROBIOLOGICAL STUDIES (WIPP R & D PROGRAM BY QUARTERS (LASL, UNM) . . . 15

REFERENCES . . . 16

# POTENTIAL MICROBIAL IMPACT ON TRANSURANIC WASTES UNDER CONDITIONS EXPECTED IN THE WASTE ISOLATION PILOT PLANT (WIPP)

October 1—December 15, 1978

by

Benjamin J. Barnhart, Evelyn W. Campbell, Julia M. Hardin, Eleuterio Martinez, Douglas E. Caldwell, and Richard Hallett

## ABSTRACT

This is the second quarterly progress report for this project designed to assess potential microbial interactions with plutonium and other transuranics (TRU) in organic matrix radioactive wastes as may possibly occur in the Waste Isolation Pilot Plant (WIPP). At this writing, only 2 months have elapsed in the quarter, making a total of 4 months work on the project for which funding began August 1, 1978. Since October 1, we have successfully enumerated microorganisms in soil from an area adjacent to a shallow burial site (Area C) at the Los Alamos Scientific Laboratory. From 1.33 to $2.98 \times 10^6$ aerobic colony forming units (CFU) were found per gram of soil as compared with 1.99 to $2.97 \times 10^5$ anaerobic CFU per gram. The fungus population ranged from $3.5 \times 10^3$ to $3.2 \times 10^4$ CFU per gram. The most probable number (MPN) demonstrated in nutrient broth by the extinction dilution method was similar for aerobic and anaerobic bacteria. Direct counting of microflora in fluorescent-stained soil samples has been initiated using the epifluorescent microscopy capability at the University of New Mexico. We have demonstrated the almost quantitative methylation of mercury and have attempted to alkylate europium in an abiotic reaction with methylcobalamine, a metabolic intermediate in bacterial metabolism, but methylation was not detected over a range of pH (4.0 to 7.0) and at two temperatures (25°C and 37°C). The alkylation of thorium is being pursued as a prerequisite to working with plutonium.

Studies of europium citrate and europium EDTA chelate degradation have begun. Both degrade, but EDTA degrades at a much slower rate than citrate. Methodology has been improved in preparation for analogous studies of plutonium chelate degradation.

The microbial production of gases under conditions expected for the WIPP has been studied. Carbon dioxide is the only significant gas detected. Hydrogen sulfide is also produced if a high concentration of proteinaceous substrate is present.

## I. INTRODUCTION

The WIPP is primarily intended for the terminal isolation of defense-related transuranic radioactive waste. This waste material may include celluloses, rubbers, plastics, etc.; radionuclides; actinides; and, quite possibly, residual chelating chemicals from decontamination operations and microorganisms. The bacteria and fungi in the wastes are derived from humans who work with the materials, laboratory animals, air, and soil. Microorganisms can metabolize organic materials in radioactive waste and microbial metabolites may react with radionuclides to enhance their mobilities if leaching occurs.

Although microbial activity in radioactive wastes has not been a prominent area of research, there are some relevant reports. Colombo et al,[1] have enumerated and classified as aerobic or anaerobic bacteria from trench water in low-level radioactive waste disposal sites (near surface) at Maxey Flats, Kentucky, and West Valley, New York. Au[2] and Au and Beckert[3] have analyzed the microbial population in soil of the Nevada Test Site. However, there have been no reports enumerating or classifying microorganisms extant in radioactive waste itself or describing microbial alteration of the chemical or physical states of transuranic elements. Due to the lack of information in this area, a round-table discussion has been organized to bring workers in the field together to identify problems and to discuss methods for their solution. Both principal investigators for the current Los Alamos Scientific Laboratory-University of New Mexico (LASL-UNM) project have been invited to participate. The round-table discussion will be held in May 1979, during the American Society for Microbiology meetings in Los Angeles.

Microbial interaction with radionuclides may cause enhanced volatilization via alkylation reactions, or in solubilization and concentration by chelation and degradation of chelates, respectively.

There is an obvious need to determine the potential for microbial interaction with radioactive waste materials. The scope of this project includes investigations in several important areas of potential microbial influence on radionuclides. Following an initial study to enumerate, and at least partially classify, the microflora in shallow burial low-level transuranic waste, we are investigating microbial production and degradation of chelated radionuclides, changes in radionuclide oxidation states, and alkylation reactions.

Microorganisms will cause production and transformation of gases within the WIPP disposal site. Carbon dioxide is the major gas expected as a result of bacterial decomposition of organic waste. The rates of $CO_2$ production under WIPP conditions are under study. In addition, other gases that may be produced are being identified and rates of production determined.

## II. ASSESSMENT OF MICROFLORA IN LASL TRU BURIAL SITE SOIL (LASL)

### A. Background

To date, we have developed standard operating procedures for enumerating microflora from LASL TRU shallow burial site TA-54, Area C, using the dilution agar-plate technique[4] to estimate colony forming units (CFU)/g of soil and the most probable number (MPN) method,[5] which permits estimation of population density without an actual count of single cells or colonies.

It was necessary to develop these procedures using soil not contaminated with radioactivity. The characterized soil was collected from just outside the fenced TRU burial site along the southern perimeter. The microbiological enumerations are reproducible and will serve as a reference to compare with TRU-contaminated soil samples collected in the TA-54 area. From these experiments, we have found that it is possible to collect samples in plastic bags, freeze them in dry ice and ethanol at the collection site, and store them in a laboratory freezer without a loss in microorganisms until it is convenient to set up the cultures.

### B. Estimation of CFU by Dilution Agar-Plate Technique

All manipulations were performed in a Bioquest Biological laminar flow cabinet. A 10-g aliquot of moist soil sample was transferred to an Erlenmeyer flask containing 95 mℓ of sterile 3X distilled water or 1% peptone broth and a sterile stirring rod. The flask was placed on a magnetic stirrer for 15 min to

disperse soil particles. Immediately, 10 mℓ from the center of the suspension was transferred to a 90-mℓ blank establishing the $10^{-2}$ dilution. From this second dilution, 10-mℓ quantities were transferred to other 90-mℓ blanks to provide a dilution series through $10^{9}$. A 1-mℓ portion of each dilution was transferred to each of three Petri dishes. About 12 mℓ of molten agar, cooled to 42°C, was poured into each inoculated Petri dish, and the plate was rotated by hand to swirl the agar and ensure thorough mixing with the inoculant. The agar was allowed to solidify without being disturbed, then the dishes were incubated at 28°C for 7 days. Colonies were counted at 7 days, except where noted. Dishes from the dilution at which 30 to 300 colonies had developed were considered satisfactory to count.

Table I shows the results of three experiments with 10-g aliquots of the same soil sample and growth medium used in each case.

These experiments resulted in an average of $1.94 \times 10^{6}$ CFU/g of soil (incubated at 28°C for 7 days under aerobic conditions). The excellent agreement between experiments shows that our technique, described in Sec. II. A, is reproducible. It does not make much difference if 3X distilled $H_2O$ or 1% peptone is used for diluting microorganisms. There is essentially no difference between numbers and types of colonies appearing on dishes using trypticase soy agar (TSA) 1:10 or TSA 1:100.

The anaerobic cultures resulted in an average of $2.66 \times 10^{5}$ CFU/g of soil with good agreement between experiments. Anaerobic agar (DIFCO) will be the medium of choice in later experiments. Except for *Clostridium*, colonies appearing were facultative anaerobes.

Mycosel (fungus selection agar) in the first two experiments had to be counted in 3 days before overgrowth made them impossible to count. In the

**TABLE I**

**CFU PER GRAM OF SOIL**

| Sample | Bacteria | | Fungi |
|---|---|---|---|
| | Aerobic | Anaerobic | |
| Soil Collected outside fenced area TA-54, Area C 2940 on 10/20/78 plated immediately on trypticase soy agar (TSA) 1:10 | $1.33 \times 10^{6}$ | $1.99 \times 10^{5}$ | Mycosel $3.6 \times 10^{5}$ @ 3 days |
| Aliquot of same sample stored 11 days in freezer, plated on 10/31/78 on TSA 1:10 | $1.61 \times 10^{6}$ | $3.03 \times 10^{5}$ | |
| TSA 1:100 | $1.59 \times 10^{6}$ | Anaerobic Agar (DIFCO) | Mycosel $3.5 \times 10^{5}$ @ 3 days |
| Aliquot of same sample using 1% peptone as diluent, plated on 12/4/78 on TSA 1:10 | $2.19 \times 10^{6}$ | $2.97 \times 10^{5}$ | Mycosel $3.2 \times 10^{4}$ |
| TSA 1:100 | $2.98 \times 10^{6}$ | Anaerobic Agar (DIFCO) | |

third experiment, there were no colonies at 3 days, indicating that perhaps the agar was too hot when the plates were poured, so the result (counted at 7 days) cannot be compared with the other two experiments.

### C. Estimation of Microbial Population Density by the MPN

Prepared as described above, 1-mℓ portions of each soil dilution were used to inoculate a series of five tubes, each containing trypticase soy broth (TSB) or thioglycollate medium. The tubes were examined microscopically for evidence of microbial growth after 7 days at 28°C and recorded as positive or negative. The negative dilution tubes were further incubated to 14 days. The first experiment (October 20, 1978) had growth in all tubes, indicating that further dilutions must be made to find the highest dilution at which growth can be obtained. Results of the October 31, 1978 experiment, in which tubes were incubated for 14 days at 28°C, are shown in Table II.

The MPN of organisms in the original sample was calculated using a factor from the table of MPN for use with 10-fold dilutions and 5 tubes per dilution.[6] The factor was multiplied by the appropriate dilution factor to obtain the MPN of the original sample.

Either TSB 1:10 or 1:100 can be used, as they give the same result of $2.3 \times 10^6$ MPN microorganisms/g of soil under aerobic conditions. The growth in the tubes containing Sabouraud with antibiotics may not all be due to fungi since the antibiotics (added to prevent bacteria from growing) are unstable with heat and on standing.

### D. Summary of Microbial Results

The agar-plate method gives total counts of the species of microorganisms that can grow on the substrate under the incubation conditions used. Therefore, the total count is only a fraction of the total count actually present. However, if the same conditions are rigidly applied, it is possible to get reproducible numbers for any given group of soil samples and, in a relative way, to compare soil samples collected in the shallow TRU burial site (TA-54 area) with soil samples collected outside of the area as a control.

Estimates of the MPN have a low order of precision. Large numbers of tubes must be inoculated at each dilution. The use of a twofold dilution series would give far greater precision than 10-fold dilution. However, when the population density is unknown, the mechanics of counting usually necessitate a 10-fold dilution ratio with a minimum of 5. As in the case of the plate-count method, rigid attention must be given to details to get reproducible results. It was necessary to work with soil samples not contaminated with radioactivity to work out these details and to establish a set of base-line data.

Direct enumeration of microorganisms on soil has not been possible. That is, however, an important capability since only 1-10% of the microorganisms on any given soil sample can be cultivated. Our Zeiss epifluorescent microscope is arriving, and when all parts have been assembled, we will quantitate the soil microflora using fluorescent stains and epifluorescent microscopy.[7,8]

We are initiating work on soil taken from core samples of shallow burial sites (LASL) containing low-level TRU waste. The microorganisms will be

**TABLE II**

**MPN MICROORGANISMS PER GRAM OF SOIL**

| Medium | Bacteria | | Fungi |
|---|---|---|---|
| | Aerobic | Anaerobic | |
| TSB | | | |
| 1:10 | $2.3 \times 10^6$ | | |
| 1:100 | $2.3 \times 10^6$ | | |
| Thioglycollate medium | | $3.3 \times 10^6$ | |
| Sabouraud broth with antibiotics | | | $4.9 \times 10^4$ |

enumerated using fluorescent stain and epifluorescent microscopy and by the MPN method. CFU will be estimated using the dilution agar-plate technique. Descriptions of microorganisms will be limited to aerobic/anaerobic, cell morphology, gram stain reaction, motility, gas production, and sensitivity and resistance to ionizing radiation. More information on these tasks will be available in the next quarterly report.

## III. ALKYLATION OF RADIONUCLIDES (LASL)

### A. Background

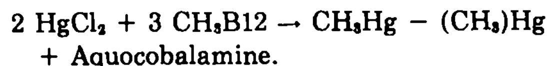
Microbial methylation of mercury[9,10] has refocused attention on the possibility that heavy elements in the lanthanide and actinide series may be similarly modified. Although the methylation of mercury, arsenic, selenium, lead, and tellurium[11] has been known for some time, nothing is known about the methylation of the transuranic elements.

Because factors affecting the reaction sequences are not adequately understood, the possibility that such alkylation may take place in nature has been largely ignored. Schroeder and coworkers[12] found organic derivatives of transition metals had longer retention time in rat tissues than did their inorganic salts. Because of the toxicity of radionuclides, particularly plutonium isotopes, and because of the increased retention time of organic derivatives, it is of considerable importance to assess the possible methylation of transuranic elements by microorganisms.

This investigation will establish some of the factors affecting the alkylation of lanthanide and actinide elements by methylcobalamine *in vitro*. Methylcobalamine is a major metabolite of microorganisms found in various soils and sediments.[13,14]

### B. Methylation of Mercury

The methylation of mercury by microorganisms in the environment has been documented and well summarized.[15] In this report, we present data on experiments with $HgCl_2$, $EuCl_3$, and $Th(NO_3)_4 \cdot 4H_2O$ using methyl-CO-5-6-dimethylbenzimidazalcobamide[14] ($CH_3B12$) as a methyl donor. When mercury was treated with $CH_3B12$, the reaction confirmed what other investigators had found.[16,9]



$$2\ HgCl_2 + 3\ CH_3B12 \rightarrow CH_3Hg - (CH_3)Hg + \text{Aquocobalamine.}$$

Methylcobalamine (0.5 $\mu$mol) was reacted with a 2-$\mu$mol mercuric chloride phosphate buffer at 37°C in 1 m$\ell$ of 0.2 M $KH_2PO_4$, pH 7.0. Care was taken that most operations were carried out in the dark.[17] The reaction proceeded at a high rate, and inorganic mercury was methylated almost quantitatively.

The amount of methylcobalamine that decomposed into aquocobalamine after release of the methyl group was monitored spectrophotometrically by an increase in absorbance at $A_{351}$ nm (Fig. 1) and a concomitant decrease at $A_{380}$ nm (Fig. 2). After a reaction time of about 4.5 h, the methyl group was released almost quantitatively. At 24 h, the reaction mixture was extracted with 1 m$\ell$ of benzene, and the upper layer was removed and allowed to evaporate to 500 $\mu\ell$.

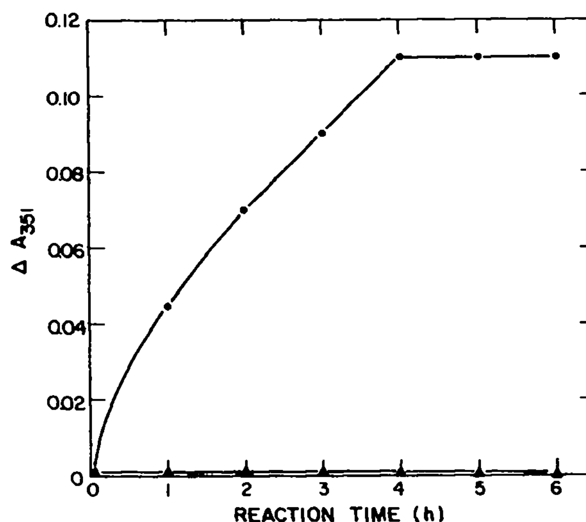



*Fig. 1.*

*Abiotic methylation of mercury by methylcobalamine ($CH_3B12$). The reaction mixture contained $HgCl_2$:$CH_3B12$ in a molar ratio of 2:1 in 0.2 M potassium phosphate buffer at pH 7.0 and at 37°C. All readings were taken in a Beckman DU-2 Spectrophotometer. Symbols: ● = complete reaction mixture containing $HgCl_2$; ▲ = control mixture lacking mercury.*

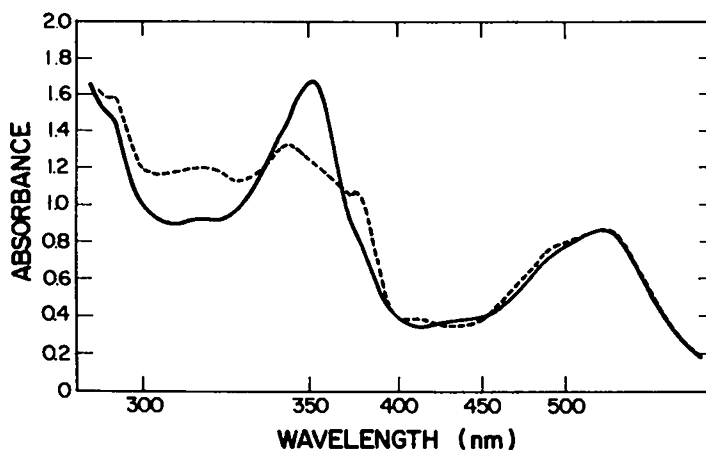



*Fig. 2.*
*Spectral analysis of the abiotic methylation of mercury by methylcobalamine ($CH_3B12$). Reaction conditions as described in Fig. 1. These spectra were obtained using a Unicam Continuous Recording Spectrophotometer. Symbols: Broken line = $CH_3B12$ at 0 h of reaction time; solid line = $CH_3B12$ after 6 h of reaction time.*

The 50 $\mu\ell$ of the benzene extract were spotted on a silica gel thin-layer chromatography (TLC) plate. The solvent used to develop the chromatogram was hexane:acetone, 90:10 (vol%). The 20 $\mu\ell$ of standard mercuric chloride (7 $\mu g/\mu\ell$) were spotted to check the unknown sample, and the chromatographs were developed for 1.5 h. The RFs were 0.30 for monomethyl mercury and about 0.75 for the dimethyl mercury. The $(CH_3)_2Hg$, because of its extreme volatility, was lost from the TLC plate within 24 h. Mercury was detected by spraying with a 0.05% dithizon-chloroform solution. The mercury spot turned canary yellow.

### C. Methylation of Europium

After methylation of mercury, we attempted to methylate europium[18,19] chloride ($EuCl_3$) with $CH_3B12$, using conditions similar to those used with mercury. The concentration of $CH_3B12$ was held constant at 1.5 $\mu$mol in all reactions. The 10 $\mu$mol of $EuCl_3$ was used in one set of reactions, which were done at pH 4.0, 4.5, and 5.0, using 0.1 M acetic acid as the solvent mixture, and at room temperature for 24 h. Another set of reaction mixtures contained 20 $\mu$mol of $EuCl_3$. Spectrophotometric readings were taken at zero time and every hour thereafter for the next 5 h. The reactions did not appear to proceed under these conditions. After 24 h at room temperature, the samples were placed in a constant temperature bath at 37°C overnight. $A_{351}$ and $A_{380}$ readings were taken after 18 h, but no changes in absorption could be detected.

Europium-3+ (Refs. 19 and 20) is a strong water seeker and in an aqueous medium is completely enveloped with molecules of $H_2O$. This situation renders the europium ion inaccessible to the methyl group donated by $CH_3B12$.

There appears to be a degree of conversion of the methylcobalamine to aquocobalamine with time in the absence of an acceptor atom. The control sample of $CH_3B12$ also shows some degradation with time.

Ultraviolet absorption for the control varied slightly while the reaction was in progress. The $A_{351}$ = 0.565, and the $A_{380}$ = 0.960 as initial readings. After four days, the $A_{351}$ = 0.550 and $A_{380}$ = 0.410; there was some conversion of the methylcobalamine to the aquocobalamine form. Likewise, for six reactions at different molar ratios, the $A_{351}$ = 0.586 and $A_{380}$ = 0.481, as average initial absorbances. The final readings for the reactions after 4 days were $A_{351}$ = 0.559 and $A_{380}$ = 0.448. These slight shifts were attributed to chemical degradation of the vitamin B12 analog by interactions with solvent components and to light degradation exposure at sampling times.

After 4 days reaction time, all samples were extracted with 500 $\mu\ell$ of benzene. About 25 $\mu\ell$ of each

sample were spotted on a thin-layer chromatography plate with a control of $EuCl_3$. Europium can be detected by spraying with a 15% solution of sodium tungstate.[21] TLC proved positive; however, the benzene extract for all reactions was negative.

Under experimental conditions approximating a bacterial environment,[22] i.e., pH 4.0, 4.5, and 5.0 in acetic acid, the reaction does not proceed.

The benzene extract layer was also checked with Arsenazo I,[23] which gives a strong purple color when in contact with europium or the actinide elements. The reaction filtrate that contains $CH_3B12$ + acetic acid + $EuCl_3$ shows a strong positive purple when spotted on Whatman #1 paper or silica gel TLC. This indicates the europium is still unreacted.

### D. Methylation of Thorium

Even though europium methylation could not be detected, the question remained whether the heavy elements of the transuranic series might be alkylated by microorganisms. For our investigation, we chose thorium[24,25] as our model compound. Naturally occurring thorium is weakly radioactive, yet its chemical properties are similar to $^{239}$Pu IV. Some thorium isotopes and daughter nuclides are strongly radioactive and may be found in nuclear wastes. The highly charged positive ion $Th^{+4}$ has a strong tendency to hydrolyze and form complexes with anions, which may be present in solution. Thorium extraction can be achieved in the same manner as $^{239}$Pu by changing concentrations of complexing agents.

Using $Th(NO_3)_4 \cdot 4H_2O$, spectrophotometric data show that the methyl group of $CH_3B12$ is lost as in the methylation of mercury (Fig. 2). However, the reaction products have not been characterized. The shift in the spectra of $CH_3B12$ at $A_{351}$ nm and $A_{380}$ nm occurs only when the alkylation of mercury occurs. As a result, this same spectral shift in the thorium $CH_3B12$ mixture strongly suggests that thorium may also be alkylated.

## IV. CHELATE DEGRADATION (UNM)

### A. Background

The microbiology of chelate degradation and the methods proposed for studies of chelate degradation under WIPP conditions were discussed in the June 1-30, 1978, quarterly report, "Microbial Biogeochemistry of WIPP Wastes," by D. E. Caldwell. These studies are preliminary and europium is used as an analog of plutonium. Procedures for studying TRU waste simulations have been modified. Degradation rates and their implications are discussed.

### B. Materials and Methods

The experimental procedure for studying the degradation of plutonium chelates was developed using europium, a nonradioactive lanthanide, as an analog of plutonium. Two procedures were used to study degradation rates of the europium chelates. The simpler procedure used 30-mℓ glass screw-cap scintillation vials as the reaction vessels with a $CO_2$ gas trap placed inside the vial. The second procedure used a soil perfusion column with gas washing bottles containing NaOH attached at the outlet to trap the evolved $CO_2$. Only those chelating agents available with $^{14}$C labels were tested with these methods. A different procedure will be used for Desferol.

Two stock solutions were prepared for each of the chelating agents. One stock solution of each chelating agent was prepared to contain approximately 1 mg of the chelating agent per mℓ of solution. The other stock solution was prepared with the $^{14}$C-labeled agent to yield a scintillation count of 100 000 cpm/mℓ. Simulated organic matrix TRU waste (LASL composite waste), as described in Table III, was cut into small pieces for addition to the substrate in the degradation vials.

The 11 conditions used for the degradation studies were aerobic, anaerobic, 25°C, 70°C, 1% water, 91% water, salt brine, nutrient solution, a control with no radionuclide or metal ion analog, a control with no soil inoculant, and a sterile control.

**1. Simulation of Chelate Decomposition by TRU Waste.** The aerobic degradation vials were prepared by adding 1 g of TRU waste (Table III) to the bottom of each vial followed by 1 mℓ of the $EuCl_3$-$6H_2O$ solution. This provided a 10:1 molar excess of metal ion to chelating agent. In those experiments requiring only 1% water, the contents of the vial were allowed to dry overnight in a 70°C oven,

TABLE III

COMPOSITION OF SIMULATED TRU WASTE PER VIAL
(For Gas Evolution and Chelate Degradation Studies)

| Waste Material | mg |
|---|---|
| Kimwipe (cellulose) | 170 |
| Rags (cellulose) | 170 |
| Polyethylene | 240 |
| Polyvinyl chloride | 120 |
| Neoprene rubber | 75 |
| Hypalon rubber | 75 |
| Butyl rubber | 75 |
| Latex rubber | 75 |
| TOTAL | 1 000 |

and 10 mg of water was added to the vial the following day. In the first four vials that were prepared for sodium citrate, the soil inoculant used was 20 mg of a soil mixture. In all subsequent vials, 20 mg of aspen forest soil was used for uniformity and reproducibility. A 6-mm by 50-mm Durham test tube containing 0.7 mℓ of 0.1 N NaOH was placed inside each reaction vial to act as a gas trap for the $CO_2$.

To test the effectiveness of the gas trap, some initial experiments were conducted using $^{14}C$-labeled glucose. It was found that the transfer of the labeled carbon from the substrate to the NaOH trap was effective, and there was no loss of $CO_2$ from the vial.

The anaerobic vials were prepared as were the aerobic vials with these exceptions. In place of the hard plastic screw caps, a cap with a silicon-Teflon septum was used to allow sampling of the NaOH gas trap with a syringe and needle so as not to disturb the anaerobic conditions inside the vial. A second Durham tube containing 0.7 mℓ of TSB was put into the vial for the purpose of eliminating any residual $O_2$ from the vial after it was closed and flushed with argon. Standard methods for the removal of $O_2$ were not applicable for TRU waste simulators.

Sampling of the aerobic gas traps was accomplished simply by opening the vial and extracting the entire contents of the Durham tube with a syringe. The NaOH was then transferred to a Nalgene 10-mℓ plastic film envelope in preparation for scintillation counting. The syringe was rinsed three times before proceeding to the next sample. A needle was inserted through the septa of the anaerobic vials to the bottom of the Durham tube to extract the NaOH. After the NaOH was withdrawn, the syringe was separated from the needle, and a second syringe containing fresh NaOH was attached. The Durham tube was refilled with $O_2$-free NaOH before the long needle was withdrawn from the vial.

Liquid scintillation counting was used to detect $^{14}C$-$CO_2$. Biofluor scintillation cocktail and a Beckman Model LS230 counter were used.

**2. Chelate Degradation in Soil Perfusion Columns.** The perfusion column consisted of a 300-mm by 37-mm i.d. glass cylinder plugged at the top with a 2-hole rubber stopper and at the bottom with a 1-hole stopper. About 2 cm of 8-mm-mesh gravel was placed in the bottom of the column followed by 1 cm of glass wool and 9 cm of soil. To this was added 10 mℓ of a solution containing $^{14}C$-sodium citrate and a 10-fold molar excess of europium chloride. The $^{14}C$-sodium citrate was measured to yield 4 800 cpm/mℓ in the 500-mℓ gas washing bottle when the degradation reached completion.

The europium-citrate solution was percolated and recycled through the coil in the column by connecting the apparatus to a vacuum line. The incoming air was first passed through a 500-mℓ gas washing bottle containing 0.1 N NaOH to remove atmospheric $CO_2$ so as not to overload the main collection bottle and to humidify the air so as not to dry the perfusion column. Two traps were used between the soil column and the vacuum line to ensure that all the $CO_2$ was captured. Each gas washing bottle was determined to be about 85% efficient in removing the $CO_2$ from the transporting air. The pair of bottles gave a trapping efficiency of greater than 96% when connected in series.

## C. Results and Discussion

These data are tentative and preliminary; confirmatory experiments are in progress.

**1. Degradation of Europium Chelate.** Europium is reported to be toxic to microbial growth in concentrations of 0.011 M.[26] The maximum concentration of europium used was 0.005 M. Citrate was degraded most rapidly in the absence of europium (Table IV). Inhibition of degradation may be due to both chelated and unchelated europium. The europium-citrate chelate degraded at a rapid rate in solution at 25°C. Increasing the temperature to 70°C (Table V) reduced the degradation rate except for treatments where no europium was present. Anaerobic conditions did not slow the rate of degradation of the europium-citrate chelate at 25 or 70°C, and in fact, the data indicate that the rate of degradation may be slightly accelerated (see Tables VI and VII). Of the four uninoculated controls, those with TRU waste all showed chelate degradation, which indicates that the TRU waste contains sufficient microbial flora for degradation. No degradation has been detected in samples containing the brine solutions.

**2. Degradation of Europium-EDTA Chelate.** The ethylenediaminetetraacetate (EDTA) chelate degraded at 1/10 the rate of citrate degradation. Table VIII shows a maximum of 2.6% after 19 days compared to 26.8% degradation for citrate in 18 days (Table IV). Otherwise, the degradation of EDTA chelate appears to be following a similar pattern to that of the citrate. However, the inhibitory effects of 70°C and anaerobic conditions seem to be much more pronounced. Anaerobic degradation was only 0.9% after 15 days (25°C) (Table VIII) compared to 2.6% in 19 days for aerobic conditions. At 70°C, the rate of degradation was even slower.

**3. Evaluation of TRU Waste Simulation Systems.** The aerobic waste simulators were opened at regular intervals to remove the $^{14}C$-$CO_2$ collected in the alkaline trap, and some $^{14}C$-$CO_2$ was lost. The system used for anaerobic simulators did not involve opening the vials and did not result in loss of $^{14}C$-$CO_2$. As a result, the methodology developed for the anaerobic simulators (to maintain anaerobic conditions) will be used for both aerobic and anaerobic vials.

**TABLE IV**

**PERCENT AEROBIC DEGRADATION OF EUROPIUM CITRATE CHELATE AT 25°C IN 91% $H_2O$**

| | Molar Ratio Citrate/$EuCl_3:6H_2O$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation Time (Days) | 10.0 | 5.0 | 1.0 | 1.0 Control No Inoculum No TRU Waste | 0.2 | 0.1 | 0.1 | 0 Control No $EuCl_3$ | 0.1 Soil Soil Perfusion Column |
| 3 | --- | 0.84 | --- | 0.4 | --- | --- | --- | --- | --- |
| 9 | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 10 | --- | --- | --- | --- | 17.2 | 6.6 | --- | --- | 57.0 |
| 11 | --- | --- | --- | --- | --- | --- | 23.0 | --- | --- |
| 15 | --- | --- | --- | --- | --- | --- | --- | 34.0 | --- |
| 17 | 34.8 | --- | --- | --- | --- | --- | --- | --- | --- |
| 18 | --- | --- | --- | --- | --- | --- | 26.8 | --- | --- |
| 21 | --- | 13.3 | --- | 0.4 | --- | --- | --- | --- | --- |
| 26 | --- | --- | 3.4 | --- | --- | --- | --- | --- | 81.0 |
| 31 | --- | --- | --- | --- | 28.8 | 12.3 | --- | --- | --- |
| 56 | --- | 26.9 | 4.0 | --- | --- | --- | --- | --- | --- |
| 57 | --- | --- | --- | --- | --- | --- | --- | 34.3 | --- |
| 62 | 41.5 | 34.3 | 5.7 | 0.4 | --- | --- | --- | --- | --- |

($5 \times 10^{-7}$) moles sodium citrate per vial.)

## TABLE V

## PERCENT AEROBIC DEGRADATION OF EUROPIUM CITRATE CHELATE AT 70°C IN 90% $H_2O$

| Incubation Time (Days) | Inoculated | Inoculated (Replicate) | No Inoculum | No Europium |
|---|---|---|---|---|
| 11 | 5.6 | 4.8 | 9.4 | 43.5 |
| 18 | 7.6 | 6.8 | 11.1 | 46.0 |

(5 × $10^{-7}$ moles sodium citrate per vial.
Molar ratio citrate/$EuCl_3$ = 1/10.)

## TABLE VI

## PERCENT ANAEROBIC DEGRADATION OF EUROPIUM CITRATE CHELATE AT 25°C IN 90% $H_2O$

| Incubation Time (Days) | Inoculated 75% $H_2O$ | Inoculated | No Inoculum | No Europium |
|---|---|---|---|---|
| 11 | 5.2 | 25.4 | 8.0 | 44.5 |
| 18 | 6.7 | 31.4 | 13.7 | 55.8 |

(5 × $10^{-7}$ moles sodium citrate per vial.
Molar ratio of citrate/$EuCl_3$ = 1/10.)

## TABLE VII

## PERCENT ANAEROBIC DEGRADATION OF EUROPIUM CITRATE CHELATE AT 70°C IN 90% $H_2O$

| Incubation Time (Days) | Inoculated 75% $H_2O$ | Inoculated | No Inoculum | No Europium |
|---|---|---|---|---|
| 11 | 2.5 | 14.5 | 1.5 | 16.7 |
| 18 | 4.5 | 23.0 | 5.0 | 23.3 |

(5 × $10^{-7}$ moles sodium citrate per vial.
Molar ratio of citrate/$EuCl_3$ = 1/10.)

TABLE VIII

PERCENT DEGRADATION OF EUROPIUM EDTA CHELATE IN 91% $H_2O$ AT 25°C

| | Aerobic Inoculation (19 Days) | | | Anaerobic Inoculation (15 Days) | | |
|---|---|---|---|---|---|---|
| | Inoculated | No Inoculum | No Europium | Inoculated | No Inoculum | No Europium |
| 25°C | 2.6 | 2.3 | 11.0 | 0.9 | 0.8 | 0.5 |
| 70°C | 0.55 | --- | --- | 0.3 | 0.2 | 0.2 |

($5 \times 10^{-7}$ moles sodium citrate per vial. Molar ratio of citrate/$EuCl_3$ = 1/10.)

## V. GAS PRODUCTION (UNM)

### A. Background

The microbiology of gas production and methods proposed for studies of gas production by LASL wastes under projected WIPP conditions were discussed in the June 1-30, 1978, quarterly report. Modifications of the proposed work and preliminary results are discussed here.

### B. Materials and Methods

The substrates for gas production studies included LASL waste (34% cellulose, 24% polyethylene, 12% polyvinyl chloride, 7.5% each neoprene, latex, hypalon, and butyl rubber), carboxymethyl cellulose (cm-cellulose), asphalt, and bovine serum albumin (Tables IX and X). Substrates were incubated in 25- by 70-mm glass vials sealed with a Teflon silicon septum and one gram of substrate was added to each vial. The vials were then inoculated with 20 g of a soil mixture. Subsequently, either 10 or 100 $\mu \ell$ of phosphate buffer (0.01 M, pH 7.0) was added, and the vials were incubated at 25, 40, 50, 60, or 70°C. One series of prepared vials was made anaerobic by flushing them with argon gas. Control vials were sterilized using steam and were incubated.

The atmosphere of each vial was sampled by withdrawing 10 to 100 $\mu \ell$ through the septum and injecting it directly into the gas chromatograph (GC). The Varian 3700 automatic GC with thermal conductivity detector (TCD) was used. The detection sensitivity for the gases of interest ($H_2$, $N_2$, $O_2$, CO, $CO_2$, $CH_4$, NO, $N_2O$, $NH_3$, $H_2S$, $SO_2$) is $6 \times 10^{-10}$ g/s, and the minimum detectable level is $2 \times 10^{-8}$ mol/100 $\mu \ell$ for $CO_2$. We used a 16-ft by 1/8-in. stainless steel column packed in series with 8 ft of Porapak O (80/100 mesh) and 8 ft of Porapak R (80/100 mesh). The sampling schedule was daily, weekly, or monthly, depending on the refractory nature of the substrate and the harshness of the environmental conditions.

### C. Results and Discussion

Table IX shows that only those vials that contained 1000 $\mu \ell$ of buffer showed a significant rate of gas evolution. Although $H_2S$, $CH_4$, $CO_2$, $O_2$, NO, $N_2O$, and $SO_2$ could be detected in the nanomolar range, $CO_2$ (other than those normally present at atmospheric concentrations) was the only major gas detected as a product of LASL waste degradation. A small amount of $H_2S$ was produced from bovine serum albumin at low temperatures (25°C), see Table X. It appears from this series of preliminary experiments that the rate of $CO_2$ evolution is temperature-dependent, reaching a maximum at 50°C. At temperatures greater than 50°C, production decreases to a low but measurable rate at 70°C, the highest temperature tested. A comparison of the rates of $CO_2$ evolution seen with LASL waste and

TABLE IX

RATE OF $CO_2$ EVOLUTION
(nanomoles/g/day)

| | | Aerobic | | Anaerobic | | Sterile |
|---|---|---|---|---|---|---|
| | | With 10 μℓ Buffer | With 1000 μℓ Buffer | With 10 μℓ Buffer | With 1000 μℓ Buffer | With 1000 μℓ Buffer |
| 25°C | LASL waste | 8 | 48 | 1.2 | 65.5 | ND[a] |
| | Cm-cellulose | 73.6 | 494 | 14.4 | 9.6 | ND |
| | Bovine serum albumin | 7.0 | 2332 | 8.0 | 3480.0 | ND |
| | Asphalt | ND | 23 | ND | 6.0 | ND |
| 40°C | LASL waste | ND | 408 | ND | 92 | ND |
| | Cm-cellulose | ND | 368 | ND | 446 | ND |
| | Bovine serum albumin | ND | 2771 | ND | 1204 | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 50°C | LASL waste | ND | 658 | ND | 1714 | 0.30[b] |
| | Cm-cellulose | ND | 980 | ND | 134 | ND |
| | Bovine serum albumin | ND | 3776 | 54 | 54 | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 60°C | LASL waste | 8.0 | 656 | 17 | 204 | ND |
| | Cm-cellulose | ND | 208 | ND | 208 | ND |
| | Bovine serum albumin | 8.6 | 1721 | 16 | 2301 | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 70°C | LASL waste | 0.6 | 7.0 | ND | 7 | ND |
| | Cm-cellulose | ND | 32 | ND | 28 | ND |
| | Bovine serum albumin | ND | 44 | ND | 32 | ND |
| | Asphalt | ND | ND | ND | ND | ND |

[a]None detected above atmospheric $CO_2$ level.

[b]Sterility of this vial has not been tested.

cm-cellulose indicates that a correlation may exist, suggesting that the cellulose component of the LASL waste is the substrate utilized in $CO_2$ evolution. No evolution of methane was observed. This was probably due to the lack of an adequate inoculum and lack of strict anaerobic conditions. However, methane could be a major product if the WIPP site should become anaerobic.

As a result of these preliminary studies, several modifications to the existing experiment protocols will be made. Because gases other than $CO_2$ were not produced in significant amounts, gas chromatographic analysis will be replaced by a more precise anlysis of $CO_2$ alone. Titration of sodium hydroxide trapped $CO_2$ will be used for routine analysis, supplemented at 3-month intervals with gas chromatographic analysis for the production of other gases.

The screw-cap sample vial will be replaced with a serum vial sealed with a septum secured with an aluminum cap to ensure the integrity of the gas-tight seal at temperatures above 50°C.

**TABLE X**

**RATE OF $H_2S$ EVOLUTION (nanomoles/g/day)**

| | | Aerobic | | Anaerobic | | Sterile |
|---|---|---|---|---|---|---|
| | | With 10 $\mu\ell$ HOH | With 1000 $\mu\ell$ HOH | With 10 $\mu\ell$ HOH | With 1000 $\mu\ell$ HOH | With 1000 $\mu\ell$ HOH |
| 25°C | LASL waste | ND[a] | ND | ND | ND | ND[a] |
| | Cm-cellulose | ND | ND | ND | ND | ND |
| | Bovine serum albumin | ND | ND | ND | ND | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 40°C | LASL waste | ND | ND | ND | ND | ND |
| | Cm-cellulose | ND | ND | ND | ND | ND |
| | Bovine serum albumin | ND | 20 | ND | 22 | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 50°C | LASL waste | ND | ND | ND | ND | |
| | Cm-cellulose | ND | ND | ND | ND | ND |
| | Bovine serum albumin | ND | 180 | ND | ND | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 60°C | LASL waste | ND | ND | ND | ND | ND |
| | Cm-cellulose | ND | ND | ND | ND | ND |
| | Bovine serum albumin | --- | 4200 | ND | 4100 | ND |
| | Asphalt | ND | ND | ND | ND | ND |
| 70°C | LASL waste | ND | ND | ND | ND | ND |
| | Cm-cellulose | ND | ND | ND | ND | ND |
| | Bovine serum albumin | ND | ND | ND | ND | ND |
| | Asphalt | ND | ND | ND | ND | ND |

[a]None detected above atmospheric $CO_2$ level.

During a 90-day incubation, low rates of gas evolution were observed in samples containing 10 $\mu\ell$ of buffer and the sterile control, so sampling frequency for these vials will be reduced.

A series of vials containing LASL waste but lacking the cellulose component will be added to determine whether the other waste components contribute to the evolution of $CO_2$.

These alterations in experimental protocols should allow expansion of the treatment matrix to include variations in pH and salt concentration, and data will be obtained that reflect long-term microbial activity in the stored waste.

## VI. SPECIFIC MILESTONES FOR SECOND QUARTER, FY 1979 (LASL)

### A. Assessment of Microflora in LASL TRU Burial Site Soil

**1. Nature and Identification of Core Samples.**

a. Sampling was begun at TA-54, Area C, October 10, 1978. The cone spoon was 50 cm long by 32 cm diam. The sample totaling 120 cm was divided into 10-cm lengths, placed in sterile plastic bags, and frozen in dry ice and ethanol at the collection site. The samples are being stored in a laboratory freezer.

b. Overall radioactivity levels of and the presence of specific radionuclides in each core sample will be determined by LASL Group H-12. The core sampling is being pursued as a joint project with investigators in H-12, who are monitoring the burial site for radionuclide migration. The H-12 survey is supported under separate Department of Energy (DOE) funding.

**2. MPN Evaluation in Broth.** The techniques worked out in the previous quarter with soil samples collected outside TA-54, free of radioactivity, will be used to enumerate the MPN microorganisms/g of TRU burial site soil, and in LASL simulated waste. *Mycophil* broth with low pH (BBL) will be used instead of Sabouraud's, which contains antibiotics as a fungus selection medium.

**3. Dilution Agar-Plate Counts for CFU.** TRU burial site soil samples and LASL simulated waste will be diluted, plated, and counted according to the established technique used in reference soil samples collected outside TA-54. The *mycophil* agar with low pH (BBL) will be used as a fungus selection medium instead of *Mycosel*, which contains antibiotics.

**4. Direct Counts in Soil Samples by Epifluorescent Microscopy to Estimate Total Numbers of Microflora.** Different methods of enumerating bacteria directly from soil samples using the epifluorescent microscope and a variety of fluorochromes, including acridine orange, mithramycin, and propidium iodide are under investigation. It is desirable to find the stain that will give the least background staining of the soil particles, and that will give optimum staining to the microorganisms. Various techniques of bright field microscopy of stained soil samples will also be evaluated.

**5. Identification of Microorganisms to Genus.** Ten bacteria have been picked from isolated colonies on the TSA plates from nonradioactive soil near TA-54. They were grown in pure culture, frozen, and stored at −70°C for future identification and use in mock-up experiments. A similar collection will be made of microorganisms grown in isolated colonies on agar plates from the TRU burial site soil samples and LASL simulated waste. A continual effort will be made to determine specialized media best suited to the soil microorganisms and to identify them morphologically and biochemically using API identification systems, ancillary biochemical tests, and Berger's Manual of Determinative Bacteriology. These microorganisms will be compared with those isolated from nonradioactive soil to determine qualitative differences in microflora.

## B. Alkylation of Radionuclides

For the next quarter, we plan to conclude the thorium experiments and go on to experiments with weapon grade $^{239}$Pu. Many questions remain unanswered concerning modification of $^{239}$Pu or its fission by-products by alkylation. Where would they go? What would be a toxic level in man in the modified forms? What may be done to remove or neutralize their concentrations? Close examination of the chemistry of the $^{239}$Pu reveals how complex the problem is.

## VII. MILESTONES FOR THE MICROBIOLOGICAL STUDIES (WIPP R & D PROGRAM) BY QUARTERS (LASL, UNM)

| | FY 78 | FY 79 | | | | FY 80 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ---4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Quarterly reports | ● | ● | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annual reports | ● | | | | 0 | | | | 0 |
| Literature search and update | ● | ● | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Capital equipment ordered | ● | ● | | | | 0 | | | |
| Laboratory expendables ordered | ● | ● | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Enumeration and identification of microflora in LASL TRU burial site soil (LASL) | ● | ● | 0 | 0 | 0 | | | | |
| Enumeration and identification of microflora in metallic and wood LASL TRU waste containers (LASL) | | | 0 | 0 | 0 | | | | |
| Identification of saltbed microflora (UNM) | | | | 0 | 0 | | | | |
| Microbial gas generation (UNM) | | ● | 0 | 0 | 0 | | | | |
| Abiotic reactions (LASL) | | | | | | | | | |
| Alkylation of heavy metals and actinides | ● | ● | 0 | 0 | 0 | | | | |
| Chelation of heavy metals and actinides | | | 0 | 0 | 0 | | | | |
| Microbial degradation of chelates (UNM) | | ● | 0 | 0 | 0 | 0 | 0 | | |
| Microbial interaction with radionuclides (LASL): | | | | | | | | | |
| Alkylation/Volatilization | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chelation/Solubilization | | | | 0 | 0 | 0 | 0 | 0 | 0 |
| Chelate degradation | | | | 0 | 0 | 0 | 0 | 0 | 0 |
| Analysis and interpretation of data[a] (LASL, UNM) | | ● | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Final conclusions and recommendations | | | | | | | | | 0 |

● - On Schedule
0 - Planned

[a]The ongoing work may identify additional topics of research that may be included; other experiments may be deleted by mutual consent.

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