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Microbiology of a Pesticide Disposal Pit

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In 1977, 1.2 billion pounds of pesticides were used in the United States (USDA 1977). Pesticide-application operations create significant quantities of chemical waste which require disposal (MUNNECKE *et al.* 1976). Methods for pesticide disposal have been described (DAY 1976 and MUNNECKE *et al.* 1976) and may include photo-decomposition, chemical degradation and biodegradation. Advantages of biological disposal systems have been presented (DAY 1976 and MUNNECKE *et al.* 1976), but no data have appeared in the literature that describe the microbiology of such systems.

The microbiological activity in a concrete-lined pesticide disposal pit constructed at the Iowa State University Horticulture Research Station in 1969 was monitored. Resident microbial populations were determined after 8 years of use and our data are summarized here.

MATERIALS AND METHODS

Pit construction and sampling procedures. The pesticide pit used in this study was 0.9 m deep at one end and 1.2 m deep at the other end, and was 9.1 m long, and 3.7 m wide. The pit contents were stratified: The top 30 cm and the bottom layer consisted of 4-cm diam $Ca(CO_3)_2$ river gravel; the middle 30 cm was topsoil. All three strata usually were submerged because most disposals were large-volume rinsates from sprayer tanks. Detailed pit specifications are available (L. M. Johnson, M.S. Thesis, Iowa State University, Ames, Iowa, 1978). To prevent overflow, the pit was automatically covered during rainfall and uncovered at other times to facilitate evaporation. A wide variety of pesticides, including chlorinated hydrocarbons, triazines, organophosphates and carbamates, had been placed in the pit for a period of 8 years.

Four sample pipes were constructed by drilling holes in 7.08-cm diam plastic pipes. These were equipped with 3-mm (inside diam) plastic tubing to allow sampling at various levels. The pipes were placed 2 m apart along the central axis of the pit and were designated A, B, C, and D. Pipe A was placed in the shallow

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end of the pit and pipe D was placed in the deep end of the pit. Pipes B and C were spaced between pipes A and D. Male luer-slips (Nuclepore Corp., Pleasanton, CA) were placed on the ends of the plastic tubing to accommodate Luer-Lok syringes.

Aqueous samples that were processed aerobically were collected from the plastic sample tubing with 30-cc Luer-Lok syringes (Beckton, Dickinson and Co., Rutherford, NJ). Twenty-five-ml samples were withdrawn from each tube and placed in sterile prescription bottles.

Aqueous samples that were processed anaerobically were collected with 50-cc Luer-Lok syringes containing O_2-free CO_2. The gas was rendered O_2-free by passage over a heated copper catalyst (HOLDEMAN *et al.* 1973). All anaerobic samples were collected 15.0 cm from the bottom of the pit, placed in 2-liter, round-bottom flasks containing O_2-free CO_2 and processed within 30 min after collection.

Soil samples were collected near each sample pipe after the top layer of rocks had been removed.

Submerged rocks were collected randomly from the surface of the disposal pit and placed in sterile collection jars. Each rock was scrubbed aseptically in sterile, deionized water containing 0.1% (w/v) peptone. Appropriate dilutions of peptone water were plated to determine viable cell numbers. The average rock surface area was about 100 cm^2.

Plating and identification procedures. The types and numbers of organisms from rocks, soil and aqueous samples processed aerobically were determined after samples were plated on Trypticase Soy Agar (TSA, Baltimore Biological Laboratory, Beckton, Dickinson and Co., Cockeysville, MD) and peptonized milk agar (PMA), (LARKIN 1972). Deionized water containing 0.1% (w/v) peptone was used for all dilutions. Plates were inoculated by the spread plate technique with 0.1 ml of each sample or diluted sample. Colonies were counted after the plates were incubated at 30°C for 24 h.

To determine types and numbers of organisms in the anaerobically processed samples, pre-reduced media and dilution blanks were prepared according to methods described by HOLDEMAN and MOORE (1973). Three pre-reduced media were employed: TSA, TSA supplemented with 40% rumen fluid, and TSA prepared with pesticide pit liquid rather than deionized H_2O. Roll tubes (25 X 142 mm, Bellco Glass Inc., Vineland, NJ), containing 10.0 ml of molten (45°C) medium were inoculated, rolled in cool (10-15°C) H_2O, and incubated at 30°C. Colonies were counted after 48 h of incubation.

After counting, colonies were either picked at random to determine predominant types of bacteria or were selected on the basis of morphological differences to determine minority populations. Pure cultures from aerobically collected samples were grown at 30°C on TSA slants in screw-capped tubes and stored at

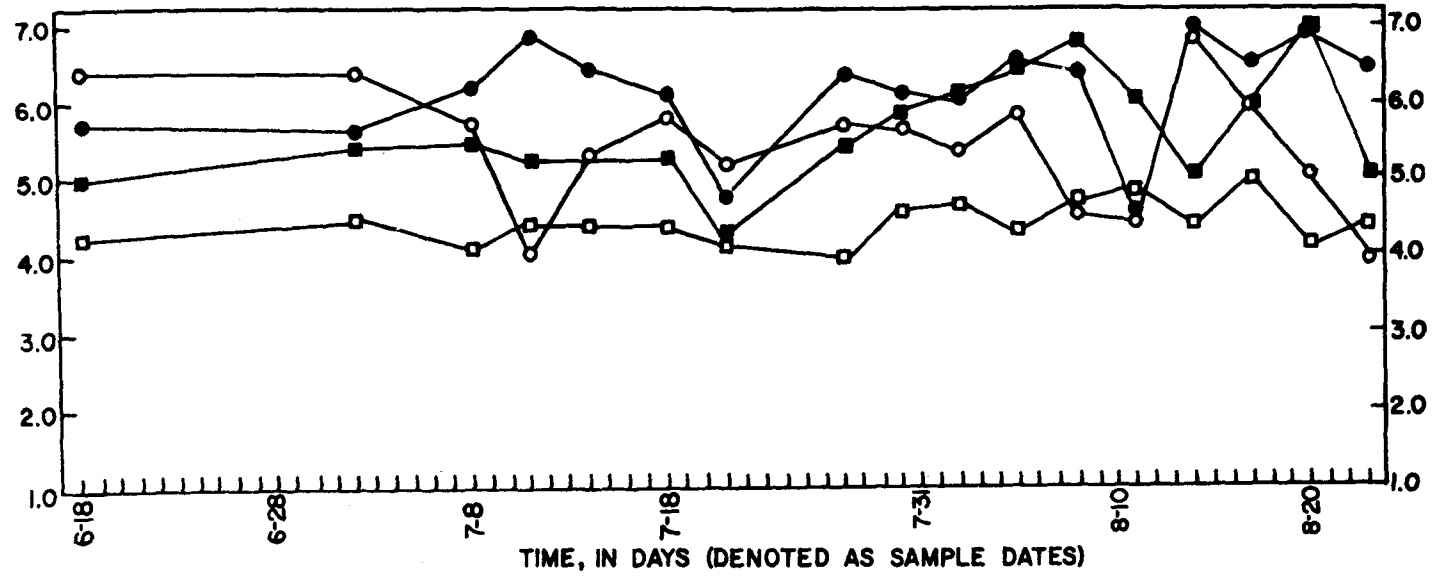

Figure 1. Log_{10} viable cells per ml of liquid samples collected 46 cm from the top of the pesticide disposal pit. Samples from four pipes, including pipes A (open squares), B (closed squares), C (closed circles) and D (open circles), were plated on TSA and incubated at 30°C for 24 h. Only data obtained in samples collected from June 18 through August 24 are presented.

4°C. Cell morphology and motility were determined on cultures grown in Trypticase Soy Broth (Difco) for 24 h at 30°C. All Gram-positive organisms were rod-shaped and formed endospores. These were identified according to GORDON *et al.* (1973). All Gram-negative bacteria obtained from aerobic samples were identified according to GRANT and HOLT (1977) and *Bergey's Manual of Determinative Bacteriology* (1974). Bacteria from anaerobic samples were initially streaked on TSA to verify oxygen tolerance. Since all of these organisms were oxygen-tolerant and Gram-negative, they were identified according to *Bergey's Manual* (1974), EWING (1973a and 1973b) and EWING and FIFE (1972).

RESULTS AND DISCUSSION

Differences in viable cell numbers existed between samples collected from different pipes (Table 1). The numbers of viable cells in samples collected at different depths from each sample pipe, however, were much the same.

TABLE 1

A comparison of viable counts from liquid samples collected at different depths from the top of each sample pipe. All counts were made on TSA plates incubated at 30°C for 24 h.

Samples source, depth	Average viable cells per ml
Pipe A, 15 cm	2.7×10^4
Pipe A, 46 cm	1.7×10^4
Pipe A, 76 cm	1.7×10^4
Pipe B, 15 cm	1.6×10^5
Pipe B, 46 cm	7.0×10^4
Pipe B, 76 cm	1.2×10^5
Pipe C, 15 cm	2.4×10^6
Pipe C, 46 cm	1.3×10^6
Pipe C, 76 cm	2.6×10^6
Pipe D, 15 cm	4.4×10^6
Pipe D, 46 cm	1.7×10^6
Pipe D, 76 cm	1.0×10^6
Pipe D, 106 cm	2.4×10^6

Log_{10} viable cells per ml of aerobic, liquid samples are presented in Figure 1. Representative data are presented on counts made only by using TSA because bacterial numbers recovered on TSA and PMA were similar. The mean viable counts per ml collected 46.0 cm from the top of sample pipes A, B, C and D over the entire sampling period were 1.6×10^5, 2.2×10^6, 2.5×10^6 and 4.2×10^5, respectively. The highest numbers of viable organisms collected per ml from pipes A, B, C and D over the sample period were 2.6×10^6, 2.2×10^7, 1.4×10^7 and 3.1×10^6, respectively. The lowest numbers of viable organisms collected per ml from these same sample pipes were, respectively, 1.4×10^3, 2.3×10^4, 1.5×10^4 and 1.2×10^4. The pit was used routinely and the sporadic introduction of pesticides most likely contributed to the fluctuation of viable cell numbers.

Numbers of bacteria recovered from pipes B anc C (near the middle of the pit) were similar. Numbers recovered from pipes A and D generally were lower than numbers observed at pipes B and C, probably because most pesticide disposals were made at central portions of the pit between pipes B and C and several pesticides and carriers can serve as growth substrates for bacteria (DAGLEY 1975 and MATSUMARA 1974).

Molds (*Fungi imperfecti*) and yeasts were occasionally encountered during the enumeration of plates. These organisms were present, however, at less than 1.0×10^3 colony forming units per ml.

Of 119 randomly selected isolates from pit liquid, 89% were Gram-negative rods; 65% of these organisms were pseudomonads. Several pseudomonads did not produce fluorescent pigments and did not accumulate poly-β-hydroxybutyrate; these bacteria resembled *Pseudomonas stutzeri*. One pseudomonad was identified as *Pseudomonas putida*. Twenty-nine percent of the isolates were unidentifiable Gram-negative, motile rods; most were oxidase-negative, facultative anaerobes and probably belonged to the family *Enterobacteriaceae*. Gram-positive bacteria were represented by the genera *Arthrobacter* (7%), *Micrococcus* (3%), and *Streptomyces* (2%). In another experiment, 20 isolates were selected on the basis of colonial morphology. The results were similar to those reported above, except that representatives of the genera *Alcaligenes* (2 isolates), *Arthrobacter*, *Mycobacterium* and *Xanthomonas* (1 isolate each) were also identified. The metabolic capabilities of these 20 isolates were determined by examining growth in filter-sterilized pesticide pit liquid. After one loopful of actively growing cells was placed in a tube of sterile pit liquid, the cultures were shaken at 30°C for two weeks. During this period, 6 of the 20 organisms grew well, 13 grew sparsely in sterile pesticide pit liquid and only 1 culture, the *Streptomyces* sp., was incapable of growing in sterile pesticide liquid.

Viable cells per gram of soil recovered on TSA ranged from 4.0×10^5 to 6.3×10^5. The types of bacteria recovered from the soil layer are presented in Table 2. *Bacillus cereus var. mycoides*, *B. circulans*, *B. coagulans*, *B. globisporus*, *B. larvae*, *B. licheniformis*, *B. macerans*, *B. polymyxa*, and 3 *Bacillus* spp. that we could not identify were observed. Of the pseudmonads isolated from soil, 4 were *Pseudomonas stutzeri*. Two *Citrobacter* species were encountered: *C. freundii* and *C. diversus*. A larger proportion of facultative anaerobes inhabited the soil than the liquid in the pit.

Types of bacteria isolated from rock surfaces (Table 2) were similar to those isolated from soil, but were different from those isolated from pit liquid. Several members of the genus *Bacillus* were observed. Four of the 8 *Bacillus* sp. could not be identified, but 4 were *B. coagulans*, *B. macerans*, *B. megaterium* and *B. polymyxa*.

TABLE 2

Distribution of 34 bacteria isolated from soil samples and 40 bacteria isolated from rock surfaces. Colonies were randomly picked from samples plated on TSA and PMA and incubated at 30° C for 24 h.

Bacterial group	Number (percent) of isolates from soil	Number (percent) of isolates from rock surfaces	Percent of all soil and rock isolates
Bacillus spp.	17 (50)	8 (20)	33.7
Pseudomonas spp.	7 (21)	17 (43)	32.4
Gram-negative rods	7 (21)	12 (30)	25.6
Citrobacter spp.	2 (4)	0 (0)	2.7
Enterobacter agglomerans	0 (0)	2 (5)	2.7
Arthrobacter spp.	1 (1)	1 (2)	2.7

Although 1.0×10^4 bacteria were recovered per ml of anaerobically processed pit liquid samples, no strict anaerobes were observed. Colonies were selected from roll tubes on the basis of morphology, and all organisms belonged to the family *Enterobacteriaceae*. Fourteen of 16 isolates were identified as either *Escherichia coli* that did not produce gas from glucose at 45°C or *Enterobacter agglomerans*. The 2 remaining cultures were *Citrobacter diversus*. Although the genera *Vibrio* and *Aeromonas* commonly are isolated from soil or water, none of the bacteria that we isolated from anaerobically processed samples were oxidase positive. Small wildlife typically find shelter under the cover of the disposal pit during winter months. Fecal materials from these animals could have served as a source of bacteria belonging to the family *Enterobacteriaceae*.

The average pH values of samples obtained from each pipe were 7.4 (pipe A), 7.7 (pipes B and D), and 7.8 (pipe C). The highest pH value recorded during the entire sampling period was 8.2, whereas the lowest pH value observed was 6.6.

Minor, day-to-day fluctuations were observed in the temperature of the pit liquid. When the rainfall-activated cover responded to precipitation, the pit was covered and usually cooled 1-2°C if the pit remained covered for 24 h or more. A difference of 1°C occurred between the shallow and deep ends of the disposal pit. The range of temperatures observed from June to September was 17°C to 24°C; the mean temperature during this period was 20.1°C. During the winter, the liquid in the pit was frozen solid.

The quantitative importance of our data would be more significant if data from a pesticide-free control pit were presented. No control pit was available for study, but data from soil and lake water have been presented by other authors (EDWARDS 1975 and JONES 1971). EDWARDS (1975) reported that 5.4×10^4 aerobic, heterotrophic bacteria were collected per ml lake water. JONES (1971) obtained counts of 1.9×10^3 viable bacteria per ml of low-

nutrient lake water and 8.2 X 10^3 per ml of high-nutrient lake water during a period of May to November. Bacterial numbers were consistently three orders of magnitude higher in mud (4.2 X 10^6 per ml) than in water samples (JONES 1971). Gram-negative bacteria predominated in lake water samples, and the diversity of genera isolated were significantly greater than the diversity of organisms that we isolated from the pesticide disposal pit. It is difficult to estimate numbers of viable bacteria in a heterogenous ecosystem such as the soil, but 10^8 to 10^{10} viable bacteria per gm of dry soil are commonly reported (ALEXANDER 1977). Although the numbers of bacteria that we isolated from the disposal pit may be lower than numbers isolated from dry soil, the numbers of bacteria isolated from the liquid in the disposal pit were significantly higher than those typically found in lake water. High numbers of bacteria in the liquid portion of the pit may be a result of the wide variety of pesticidal compounds that were introduced into the pit. This influx of pesticides, as well as variations in temperature and pH, could affect microbial populations.

The mere presence of bacteria in the disposal pit does not prove that the bacteria degraded pesticides. Some forms, such as the *Bacillus* spp. may be dormant, or the bacteria might be adventitious residents whose presence is not associated with the addition of pesticides to the pit. However, most of the isolates that we tested grew in filter-sterilized pit liquid, and it is possible that they did play a significant role in pesticide degradation. Our data indicate substantial biological activity as evidenced by the numbers and the fluctuation of numbers of bacteria in the pit. In addition, gas chromatographic analyses performed on extracts of the pit liquid and soil indicated that there has been no long-term accumulation of any pesticide (G. Junk and J. J. Richard, Iowa State University, personal communication).

Several aspects of pesticide disposal by the pit method are in need of evaluation. An understanding of the effects of the presence and types of soil and (or) rocks is necessary. Our data show that the inclusion of soil and rocks probably resulted in the presence of a more diverse flora than if the pit contained only liquid. It also may be worthwhile to analyze the effects of temperature, pH and the addition of organisms capable of transforming pesticidal compounds. Also, the addition of analogs of pesticides that are readily metabolized may facilitate pesticide degradation.

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