Gehlen, H.; Moeckel, K. "Oxidation of Aldehyde Semicarbazones to 2-Amino-1,3,4-oxadiazoles and Their Conversion into 1-Acylsemicarbazides". *Ann.* **1962**, 651, 133–136; *Chem. Abstr.* **1962**, *57*, 3424i.
Gibson, M. S. "Hydrazones. III. "Intramolecular 1,3-Dipolar Addition Involving Nitro and Carbonyl Groups". *Tetrahedron* **1962**, *18*, 1377–1380.
Hoggarth, E. "Compounds Related to Thiosemicarbazide. Part III. 1-Benzoyl-S-methylisothiosemicarbazides". *J. Chem. Soc.* **1949**, 1918–1923.
Horsfall, J. G. "Quantitative Bioassay of Fungicides in the Laboratory". *Bot. Rev.* **1945**, *11*, 357–397.
Rothwell, K.; Wain, R. L. "Plant Growth-Regulating Substance. XVII. S-Esters of Dithiocarbamates Derived from Amino Acids". *Ann. Appl. Biol.* **1963**, *51*, 161–167; *Chem. Abstr.* **1964**, *60*, 1041c.
Singh, S.; Yadav, L. D. S.; Singh, H. "Synthesis & Antifungal Activity of Some 1,3,4-Thiadiazolo[3,2-a]-*s*-triazine-7-thiones & N-Acyl-N′-(5-aralkyl/Aryl-1,3,4-thiadiazol-2-yl) thioureas". *Indian J. Chem., Sect. B* **1981**, *20B*, 518–520.
Singh, H.; Yadav, L. D. S.; Sharma, K. S. "1,3-Cycloaddition of Cyclic Isothioureas to Heterocumulenes and Fungitoxicity of the Resulting 1,2,4-Triazolo[3,4-c]-1,2,4-dithiazoles". *Agric. Biol. Chem.* **1983a**, *47*, 1017–1020.
Singh, H.; Yadav, L. D. S.; Sharma, K. S.; Singh, A. K. "Synthesis of 1,3,4-Oxadiazolo[3,2-a]-*s*-triazine-7-thiones via 1,4-Dipolar Cycloaddition of 1,3,4-Oxadiazol-2-yl Isothiocyanates to Aceto-/Benzo-nitrile & Their Antifungal Activity". *Indian J. Chem., Sect. B* **1983b**, *22B*, 1177–1178.

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Coumaphos Degradation in Cattle-Dipping Vats

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Coumaphos [*O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate] is used for the control of cattle ticks. Cattle are dipped in large vats containing a solution of coumaphos at 0.1%–0.2% (ai). Recently, several vats (problem vats) have experienced unexplained and persistant losses of coumaphos. Experiments were initiated with cattle dip from five sites in Texas (four nonproblem and one problem vat) to obtain information on the fate of coumaphos. Aerobically, coumaphos was degraded in all vat samples. Anaerobically, coumaphos was reductively dechlorinated to potasan [*O,O*-diethyl *O*-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate] only in high-use vats (including the problem vat). Samples from the problem vat were also obtained biweekly over the lifetime of the vat. Initial concentrations of coumaphos decreased with time, while concentrations of potasan increased. Aerobically, coumaphos was degraded in all samples. Anaerobically, the rate of reductive dechlorination increased with vat age. Experiments with [*benzo ring*-U-^{14}C]coumaphos demonstrated that the aromatic portion of coumaphos was mineralized.

Microbial degradation of pesticides is an important process in the removal of these compounds from the environment. Compounds that are not readily degraded by soil and water microorganisms tend to accumulate and persist in the biosphere. Alternatively, biodegradation of a pesticide at too rapid a rate may cause a loss of efficacy for that particular pesticide and other structurally related compounds. Over the last several years, this rapid or enhanced metabolism has been documented in the "problem" soils phenomenon seen with soil-incorporated herbicides and insecticides (Harris et al., 1984; Read, 1983).

Recently, a loss of efficacy has been observed with the organophosphate pesticide coumaphos in cattle-dipping solutions. Coumaphos [*O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate] is used as an acaricide for the control of the southern cattle tick (*Boophilus microplus*) and the cattle tick (*Boophilus annulatus*) by the USDA Animal and Plant Health Inspection Service (APHIS) in its Tick Eradication Program. Annually, several hundred thousand cattle are dipped for tick control along the U.S.–Mexican border by APHIS, in one of 42 vats. Each of these vats contains approximately 12 000 L of a solution of Co-Ral flowable cattle insecticide (42% coumaphos, 58% inert ingredients) mixed with water to give a final concentration of 0.1%–0.2% (ai). The concentration of coumaphos in each vat is monitored weekly by onsite personnel using a colorimetric assay kit supplied by the manufacturer of coumaphos and is confirmed by APHIS' Analytical Chemistry Laboratory in Ames, IA. Normally, the levels of coumaphos in the vats are very stable over time such that any decrease in coumaphos concentration is due to removal by cattle. These vats are emptied, cleaned, and recharged annually because of fouling by soil and animal wastes. Recently, however, several vats (problem vats) have experienced unexplained and persistent losses of coumaphos that are probably due to microbial degradation. Since these vats are constantly monitored and the losses of pesticide are well documented over several years, they can serve as excellent experimental systems for the study of how microbial degradation affects the efficacy of a pesticide that must persist for a finite period of time.

The purpose of these experiments was to study the role of biological degradation in "problem vats" and determine the metabolic fate of coumaphos in several working dip vat solutions.

METHODS AND MATERIALS

Chemicals. Analytical-grade and formulated coumaphos, potasan [*O,O*-diethyl *O*-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate], and chlorferon (3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-ol) (Figure 1) were gifts from Animal Health Division, Mobay Corp. (Shawnee, KS 66201). [*benzo ring*-U-^{14}C]Coumaphos (sp act. 21.1 mCi/mmol) was also a gift from Mobay. Labeled

Pesticide Degradation Laboratory, U.S. Department of Agriculture—Agricultural Research Service, Building 050 BARC-West, Beltsville, Maryland 20705.

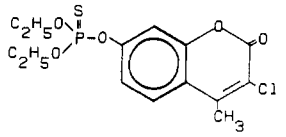

Figure 1. Structure of coumaphos. Potasan is the dechlorination product of coumaphos (the chlorine is replaced by a hydrogen). Chlorferon is the hydrolysis product of coumaphos (the diethyl thiophosphate moiety is replaced by a hydroxyl group).

coumaphos was purified (99.5%) by thin-layer chromatography (TLC).

Sampling. Samples of dip vat material were obtained by APHIS personnel at each site by rapidly immersing a 4-oz plastic container in the vat to a depth of about 4 ft immediately after the vat contents were mixed. The containers were tightly closed and shipped from City Vat, Laredo, TX; San Andreas Vat, Laredo, TX; Tordillo Vat, Carrizo Srings, TX; Vat 8, Zapata, TX; and Vat 9, Zapata, TX. These vats are designated LAR, SAN, TOR, ZAP8, and ZAP9, respectively. The initial pH values of samples were 7.0–7.2. SAN, TOR, ZAP8, and ZAP9 were sampled once. LAR was sampled on a biweekly basis for an additional 6 months.

Incubations. For aerobic incubations, 50 mL of dip vat solution was dispensed into duplicate 250-mL flasks and incubated at 27 °C on a rotary shaker at 120 rpm. For anaerobic incubations, 50 mL of dip vat solution was dispensed into duplicate 125-mL Wheaton serum bottles, sparged vigorously with N_2 passed through a high-capacity and an indicating O_2 trap to give an O_2 concentration <0.1 ppm (Chemical Research Supplies, Addison, IL 60101), and sealed with 1-cm butyl rubber stoppers. Serum bottles were incubated statically at 27 °C.

Radiolabeled experiments were conducted by adding approximately 10^6 dpm [*benzo ring*-U-^{14}C]coumaphos in 1 mL of methanol to duplicate biometer flasks (Bartha and Pramer, 1965) and allowing the methanol to evaporate. Twenty milliliters of dip vat solution was added, and the samples were incubated as previously described.

Analytical Methods. For determination of coumaphos, potasan, and chloroferon, 1 mL of dip vat solution was diluted with 9 mL of methanol, shaken vigorously, and centrifuged for 10 min at 2000*g*; the supernatant was stored at 4 °C until analysis. Coumaphos, potasan, and chlorferon were quantified on a Waters HPLC system (Waters Associates, Inc., Milford, MA 01757) consisting of M 6000 A pumps, 721 system controller, Data Module, radial compression module, and 712 WISP autosampler, with a Perkin-Elmer LC-95 UV/visible variable-wavelength detector set at 320 nm. Separations were achieved on a Waters C-18 Nova-Pak (4 μm) radially compressed cartridge with a mobile phase of 80% methanol and 20% 0.75 mM phosphoric acid; the flow rate was 2.0 mL/min.

$^{14}CO_2$ production from [*benzo*-U-^{14}C]coumaphos was monitored by periodically removing the 10 mL of 0.2 N KOH from the side arm of the biometer flask, mixing 1-mL aliquots with 10 mL of Beckman Ready-Solv HP aqueous scintillation cocktail, and counting in a Beckman Model LS 6800 scintillation counter. Disintegrations per minute were calculated from a quench curve against a set of Beckman ^{14}C quenched standards. The production of ^{14}C metabolites was determined by removing 1-mL samples of solution, mixing with 3 mL of methanol, centrifuging at 2000*g* for 10 min, and storing the supernatant at 4 °C until analysis. Analysis for ^{14}C metabolites was performed on a Gilson HPLC system, Model 42, equipped with a programmable UV detector, Model 116, a Model 231-401 sample injector, and IBM PC AT system controller.

Table I. Location, Vat Age, and Number of Cattle Treated for the Five Vats Tested

vat	location	desig-nation	vat age, weeks	no. of cattle treated
City Vat[b]	Laredo	LAR	17	1154
San Andreas Vat	Laredo	SAN	9	98
Tordillo Vat	Carrizo Springs	TOR	33	2908
vat 8	Zapata	ZAP 8	37	240
vat 9	Zapata	ZAP 9	20	227

[a] In Texas. [b] Problem vat.

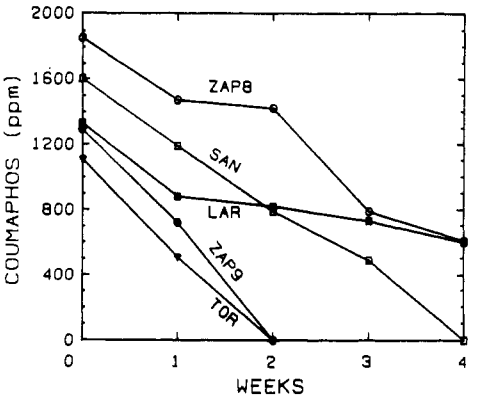

Figure 2. Degradation of coumaphos under aerobic conditions in vat dip solutions.

Fractions were collected in scintillation vials at 15-s intervals with a Gilson Model 202C fraction collector, and radioactivity was determined as previously described. Separations were achieved with an Axxion (5 μm, ODS) 10 mm × 25 cm (semipreparative) column and a gradient mobile phase of 40% methanol–60% 0.75 M phosphoric acid (pH 2) (0–7 min), increasing in a linear gradient over 2 min to 80% methanol for the continuation of the run; flow rate was 4.5 mL/min.

RESULTS AND DISCUSSION

Dip vat solutions from five separate vats were examined with respect to coumaphos degradation. These included one problem vat and four nonproblem vats; Table I provides information on vat location and past history. Our initial assumption was that problem vats were the result of enhanced microbial activity. This proved to be an oversimplification. As demonstrated in Figure 2, coumaphos degradation occurred in all five vat samples, problem (LAR) and nonproblem alike, under aerobic conditions. The rate of coumaphos degradation during the first week was almost identical in all five samples. Potasan, the dechlorination product of coumaphos, was degraded to below the detection limit (1 ppm) by the end of the first week of incubation in all samples (data not shown). Initial concentrations of potasan (ppm): LAR, 335; TOR, 300; ZAP8, 60; ZAP9, 40; San, undetectable. These data show that each vat possesses the potential for coumaphos degradation under conditions of continuous aeration. In the field, however, degradation potential is not necessarily expressed. This is clear, since with the exception of LAR none of the vats examined had a history of coumaphos degradation in the field. Vats are mixed (aerated) only before cattle are to be dipped, so that vats are likely to be in a microaerophilic or anaerobic state much of the time. High-use vats (LAR, TOR) are likely to be aerated more frequently than low-use vats (SAN, ZAP8, ZAP9).

Since vats are likely to be in an anaerobic state a substantial portion of the time, anaerobic incubations were also conducted. The concentration of coumaphos in low-

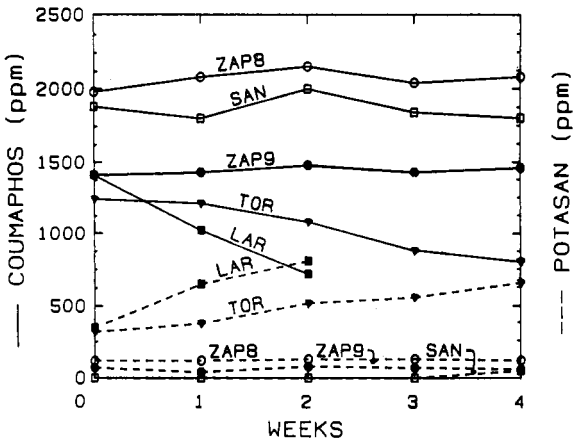

Figure 3. Disappearance of coumaphos and appearance of potasan under anaerobic conditions in vat dip solutions.

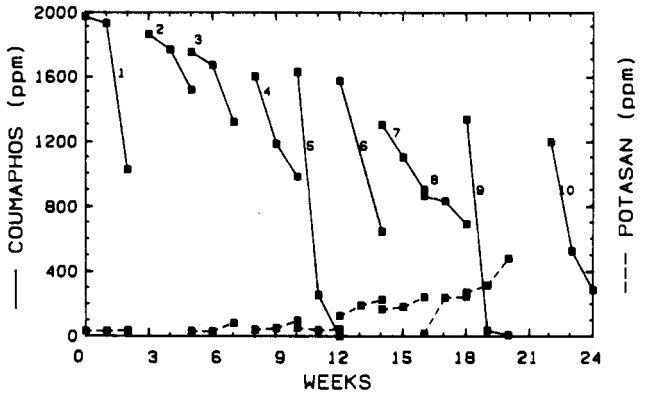

Figure 4. Degradation of coumaphos under aerobic conditions and appearance of potasan under anaerobic conditions in LAR vat dip solutions.

use vats (SAN, ZAP8, and ZAP9) was stable throughout the 4-week incubation, and there was at most only a slight increase in potasan concentrations (Figure 3), indicating that coumaphos was not readily susceptible to hydrolysis or dechlorination by chemical means. In high-use vats (LAR, TOR) there was a decrease in the concentration of coumaphos with a concomitant increase in the concentration of potasan (74% recovery of potasan from coumaphos in LAR, 78% in TOR), indicating the occurrence of reductive dechlorination. The phenomenon of reductive dechlorination has been thoroughly documented (Murthy et al., 1979; Attaway et al., 1982; Suflita et al., 1982; Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Gibson and Suflita, 1986) and occurs under conditions of anaerobiosis and low E_h. Reductive dechlorination only occurred in high-use vats, presumably because of the significant accumulation of organic matter and anaerobic organisms in these vats.

To obtain a better understanding of how problem vats develop, samples from City Vat, Laredo (LAR), were obtained on a biweekly basis and incubated under aerobic and anaerobic conditions. It is clear why LAR was considered a problem vat. There was a decline in the initial concentration of coumaphos and an increase in potasan concentration in samples with time (Figure 4). The loss of coumaphos was, in fact, even more dramatic than these data indicate, since operators in the field were continuously adding Co-Ral in an attempt to maintain an efficacious level of coumaphos in the vat. Under aerobic conditions coumaphos degradation occurred in all samples, although the rates were variable. Potasan was degraded to below 20 ppm in all samples by the end of the first week of incubation and to below the detection limit (1 ppm) by the

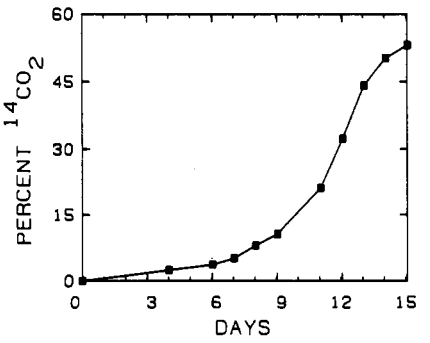

Figure 5. Production of $^{14}CO_2$ from [*benzo ring*-U-^{14}C]coumaphos in LAR vat dip solution.

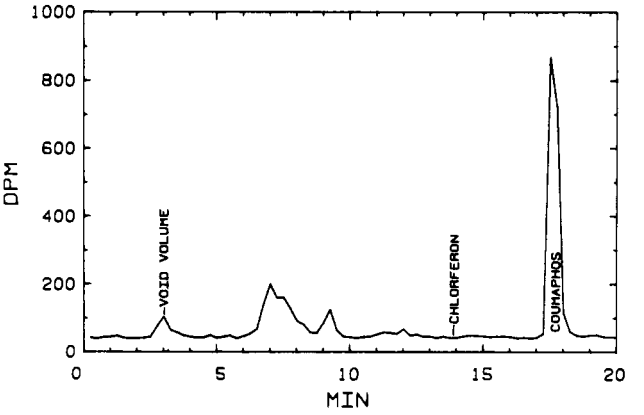

Figure 6. Chromatogram of LAR vat dip solution showing the presence of ^{14}C polar metabolites. Fractions were collected at 15-s intervals.

end of the second (data not shown).

Under anaerobic conditions, the rate of reductive dechlorination (80%–100% recovery of potasan from coumaphos) increased with the age of the vat (Figure 4). Data for sample 10 are not included because of an apparent loss of anaerobiosis; the initial concentration of potasan was 541 ppm.

Previous work with organophosphate pesticides has shown that the initial step in degradation is hydrolysis of the phosphate ester (Munnecke et al., 1982). Chlorferon, the hydrolysis product of coumaphos, was observed to accumulate to 50 ppm in LAR samples 1 and 2 after 1 weak of aerobic incubation; however, no chlorferon was detected in samples 3–10 (data not shown). An experiment with [^{14}C]coumaphos was initiated in which 100 ppm of unlabeled chlorferon was added to flasks containing LAR dip vat solution at time zero in an attempt to determine whether [^{14}C]chlorferon would accumulate (assuming chlorferon to be a freely diffusable intermediate). However, all the added chlorferon was metabolized before any significant coumaphos degradation occurred (1 week). In this same experiment 53% of the ^{14}C from the ring of labeled coumaphos was recovered as $^{14}CO_2$, indicating mineralization of the aromatic portion of the coumaphos molecule (Figure 5). Experiments with SAN, TOR, and ZAP9 also resulted in the release of $^{14}CO_2$ from [^{14}C]-coumaphos; recoveries were 53%, 27%, and 37%, respectively, after 21 days of incubation. In a separate experiment samples of LAR solution were removed after 8 days and assayed for labeled metabolites. Polar metabolites were observed in chromatograms of these samples (Figure 6). Of the [^{14}C]coumaphos 42% was recovered as $^{14}CO_2$, 27% was present as coumaphos, and 13% was recovered as polar metabolites. Presumably, the remaining 18% (which was accounted for in solution) was present as biomass.

The exact cause(s) of problem vats remains to be elucidated; however, these data offer some significant insights. Since the potential for coumaphos degradation existed in all samples, it would appear that the presence of organisms able to metabolize coumaphos is not the sole controlling factor. Rather, the existence of the proper physical/chemical environment in the vats, allowing expression of the degradative capacities of the organisms, is of critical importance in determining whether or not rapid coumaphos degradation will occur. The fact that LAR had the highest rate of reductive dechlorination suggests that there may be some correlation between reductive dechlorination and loss of coumaphos in problem vats. The fact that potasan was consistently degraded to low levels during the first week of aerobic incubation suggests that potasan may be degraded more rapidly than coumaphos. However, the initial concentration of potasan in the vat samples was always much less than the concentration of coumaphos; thus, the total quantity of coumaphos degraded was always equal to or greater than the amount of potasan degraded. The rate of reductive dechlorination in the LAR vat samples (Figure 4) (as judged by potasan accumulation in anaerobic incubations) does not appear to be rapid enough to account for the total decline in the coumaphos concentration in the vat. Samples were taken in Texas and mailed to Maryland where the tests were performed. Loss of anaerobiosis during sampling and shipping may have adversely affected strict anaerobes in the vat material. Alternatively, possible metabolism of potasan under anaerobic conditions may give an artificially low estimate of the rate of reductive dechlorination of coumaphos to potasan. Aeration allowed for very rapid coumaphos degradation. The fact that LAR is a high-use vat means that the frequency of aeration was likely to have been higher than in lower use vats. The accelerated loss of coumaphos in the LAR vat may be simply a result of the increased rate of aeration. However, the TOR vat, which is also a high-use vat, has no history of coumaphos degradation in the field.

To definitively determine the factors that are required for the phenomenon of problem vats, more data are required. Specifically, more intensive sampling of coumaphos and potasan in fresh vat samples as well as determination of the oxygen concentrations in the vats are needed. Information on the population densities of coumaphos-degrading organisms in different vats will also be useful. We have isolated two separate bacteria from enrichment cultures using Co-Ral (42% coumaphos) as carbon and energy sources capable of metabolizing coumaphos. These isolates are currently being characterized. We intend to use these isolates as a tool for studying the microbial ecology of cattle-dipping vats.

ACKNOWLEDGMENT

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LITERATURE CITED

Attaway, H. H.; Paynter, M. J. B.; Camper, N. D. "Degradation of Selected Phenylurea Herbicides by Anaerobic Pond Sediment". *J. Environ. Sci. Health, Part B* **1982**, *B17*, 683–699.

Bartha, R.; Pramer, D. "Features of a Flask and Method for Measuring the Persistence and Biological Effects of Pesticides in Soil". *Soil Sci.* **1965**, *100*, 68–69.

Boyd, S. A.; Shelton, D. R. "Anaerobic Biodegradation of Chlorophenols in Fresh and Acclimated Sludge". *Appl. Environ. Microbiol.* **1984**, *47*, 272–277.

Gibson, S. A.; Suflita, J. M. "Extrapolation of Biodegradation Results to Groundwater Aquifers: Reductive Dehalogenation of Aromatic Compounds". *Appl. Environ. Microbiol.* **1986**, *52*, 681–688.

Harris, C. R.; Chapman, R. A.; Harris, C.; Tu, C. M. "Biodegradation of Pesticides in Soil: Rapid Induction of Carbamate Degrading Factors after Carbofuran Treatment". *J. Environ. Sci. Health, Part B* **1984**, *B19*, 1–11.

Mikesell, M. D.; Boyd, S. A. "Reductive Dechlorination of the Pesticides 2,4-D, 2,4,5-T and Pentachlorophenol in Anaerobic Sludges". *J. Environ. Qual.* **1985**, *14*, 337–340.

Munnecke, D. M.; Johnson, L. M.; Talbot, H. W.; Barik, S. "Microbial Metabolism and Enzymology of Selected Pesticides". In *Biodegradation and Detoxification of Environmental Pollutants*; Chakrabarty, A. M., Ed.; CRC: Boca Raton, FL, 1982.

Murthy, N. B. K.; Kaufman, D. D.; Fries, G. F. "Degradation of Pentachlorophenol (PCP) in Aerobic and Anaerobic Soils". *J. Environ. Sci. Health, Part B* **1979**, *B14*, 1–14.

Read, D. C. "Enhanced Microbial Degradation of Carbofuran and Fensulfothion after Repeated Applications to Acid Mineral Soil". *Agric., Ecosyst. Environ.* **1983**, *10*, 37–46.

Suflita, J. M.; Horowitz, A.; Shelton, D. R.; Tiedje, J. M. "Dehalogenation: A novel Pathway for the Anaerobic Biodegradation of Haloaromatic Compounds". *Science (Washington, D.C.)* **1982**, *218*, 1115–1117.

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