Table I. Coumestrol Content of Alfalfa Haylages as Analyzed by LC

sample no.	coumestrol, ppm
1	37.7
2	20.9
3	10.1
4	53.3
5	184.0

as coumestrol. The samples were therefore analyzed by their fluorescence output and quantitated by the ratio of standards vs. samples and then the extraction efficiency, their respective moistures, and volume changes taken into account (Lookhart et al., 1978).

The haylage samples varied in coumestrol concentration from 10.1 to 184 ppm (Table I). The haylage (sample 1) fed to cattle implanted with Synovex estrogens that had shown physical effects of high estrogen intake, i.e., bulling of steers and udder development and prolapsed vagina, cervix, and rectum of heifers contained 37 ppm coumestrol or more. Of the 170 animals fed sample 1, 150 were heifers and 20 were steers. All of the heifers showed udder development and over 100 animals showed prolapse. Six of these animals had to be marketed early after being sewn up a second time and their flesh was noted as being very soft.

When animals fed sample 1 (37.7 ppm) plus the corn (no coumestrol) and high-energy mixture (5.8 ppm coumestrol) showing effects of high estrogen intake were switched to sample 2 (20.9 ppm) plus the corn and high-energy mixture, the incidence of the deleterious effects gradually subsided (Turner, 1978). Animals not implanted and fed haylage from which sample 3 was taken showed no estrogen effects. Nonimplanted animals fed up to 30% of their intake as haylage from which sample 4 was taken also showed no ill effects. Animals not implanted and fed haylage from which sample 5 was taken showed the effects of high estrogen levels (Jones, 1978). Our results indicate that cattle fed haylage containing 37 ppm coumestrol or more as their major feed will show deleterious estrogenic effects.

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Effect of Sterilization Methods on 3-Chloroaniline Behavior in Soil

The persistence and degradation of 3-[U-^{14}C]chloroaniline (3-CA) was examined in nonsterile, autoclaved and potassium azide, mercuric chloride, and ethylene oxide treated, Hagerstown silty clay loam. Treatments were monitored for ^{14}C volatilization, the formation of organosoluble products, and soil-bound residues. Highest levels of ^{14}C volatilization occurred from autoclaved and potassium azide treated soils, whereas lower ^{14}C volatilization occurred from nonsterile and mercuric chloride and ethylene oxide treated soils. ^{14}C volatilization from autoclaved and potassium azide, mercuric chloride, and ethylene oxide treated soils occurred primarily as 3-[^{14}C]CA, whereas both $^{14}CO_2$ and 3-[^{14}C]CA were evolved from nonsterile soil. Organosoluble products accounted for 22–31% of the ^{14}C recovered, whereas soil binding accounted for 34–80% of the total ^{14}C applied. The distribution of ^{14}C in organosoluble degradation products varied with each treatment. Alkaline extraction of the soil-bound residues released 5–7% of the total ^{14}C applied. Distribution of the residual ^{14}C in fulvic and humic acids and humin soil organic matter fractions varied with the soil sterilization method.

Pesticides may be degraded in soil by a variety of processes. One of the methods frequently used to distinguish biological reactions in soil is by comparison of nonsterile and sterilized soils. Soil sterilization procedures most commonly used, however, generally destroy the soils integrity to the extent that not only are biological reactions

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eliminated, but nonbiological or chemical reaction processs may also be altered or prevented. This has been exemplified by the investigations of Kaufman et al. (1968) and Skipper and Westermann (1973). Skipper and Westermann (1973) compared the effects of propylene oxide, sodium azide, and autoclaving on selected soil properties. They observed that propylene oxide induced the least soil chemical alterations upon sterilization and consequently considered it to be an appropriate sterilant to study nonbiological transformations in soil although germination and growth of wheat and alfalfa were retarded in propylene oxide treated soil. Kaufman et al. (1968) demonstrated that autoclaving soil destroyed the free radical mechanisms involved in the nonbiological degradation of amitrole (3-amino-*s*-triazole). Amitrole degradation occurred readily in soils sterilized with ethylene oxide. Thus, if an accurate distinction is to be made concerning mechanisms of degradation in soil, the proper choice of sterilization procedures is of paramount importance. The purpose of this investigation was to examine the effects of soil sterilization methods on 3-chloroaniline (3-CA) degradation and persistence in a silty clay loam.

MATERIALS AND METHODS

The chemical and physical characteristics of Hagerstown silty clay loam soil are as follows: sand, 17.0%; silt, 50.6%; clay, 32.4%; organic matter, 2.5%; moisture content at 0.33 bar tension, 25.8%, pH, 6.8; and cation-exchange capacity (mequiv/100 g), 14.7. Fifty grams (dry weight) of fresh seived soil was placed in biometer flasks (Bartha and Pramer, 1965) for sterilization treatment. Soil sterilization methods were as follows: (a) steam sterilization by autoclaving at 121 °C, 15 lb of pressure for 0.5 h on each of two consecutive days; (b) gaseous sterilization with ethylene oxide by exposure of soil in flasks to an ethylene oxide atmosphere for 24 h; and (c) chemical treatment by the addition of 1000 ppm of potassium azide or mercuric chloride. The potassium azide and mercuric chloride were added directly to the soil and mixed by shaking and stirring.

After sterilization, the soil in each flask was treated with 400 ppm of 3-CA containing approximately 1 μCi of 3-[U-^{14}C]CA (sp act., 9.48 mCi/mM) dissolved in 0.1 mL of ethanol. The 3-CA solution was added dropwise over the soil surface and then mixed in by shaking and stirring. The soil was brought to 60% of 0.33 bar moisture tension by the addition of sterile, distilled water and allowed to incubate for 64 days at 25 °C. At regular intervals, the carbon dioxide trapping solution (10 mL of 0.1 N KOH) was removed and the volatilized ^{14}C was measured by liquid scintillation counting. Volatilized 3-CA in the trapping solution was measured colorimetrically by the method of Pease (1962).

At the conclusion of the 64-day incubation period, all treatments were extracted twice with 75 mL of benzene and then twice with 75 mL of methanol. The extracts were combined, the volume was recorded, and the ^{14}C extracted was determined by liquid scintillation counting. The extracts were then concentrated on a rotary evaporator and submitted to thin-layer chromatographic (TLC) analysis on silica gel HF254 chromatoplates with benzene/acetone (99:1) as the solvent system.

Residual or bound ^{14}C was determined by fractionation of the bound ^{14}C as outlined in U.S. EPA Pesticide Registration Guidelines (1975), and combustion of the air-dried soil, and liquid scintillation counting of the evolved $^{14}CO_2$.

Possible effects that the different soil sterilization procedures may have on cell-free soil biochemical processes were examined at the enzymatic level. Cell-free soil peroxidases were extracted from the Hagerstown silty clay loam with 0.1 M phosphate buffer at pH 8. This was accomplished by shaking 50 g of soil in 50 mL of buffer for 5 min, followed by filtration through Whatman No. 1 filter paper to remove soil particles. To assure that the enzyme activity was indeed cell-free, the enzyme preparation was passed through an Amicon Ultrafilter equipped with an XM-100 ultrafilter disc. The effects of propylene oxide, ethylene oxide, potassium azide, and autoclaving on the soil peroxidase and a commercially available horseradish peroxidase (HRP) activity were then compared. Inhibition of HRP activity was measured with 0.1 M pyrogallol and H_2O_2 as substrates in buffer solution at pH 6.1 and color development determined at 402 nm. Inhibition of extracted soil peroxidase activity was measured with 0.1 M *o*-dianisidine and H_2O_2 as substrates in buffer solution at pH 8. Color development was determined at 460 nm (Meudt, 1974).

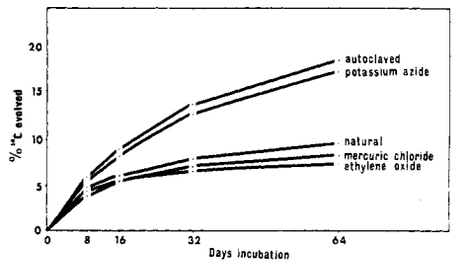

Figure 1. ^{14}C volatilization from Hagerstown silty clay loam.

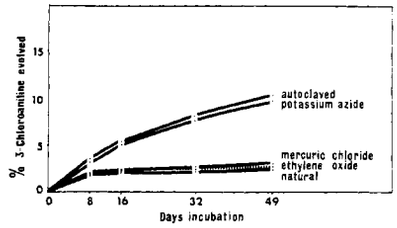

Figure 2. 3-Chloroaniline volatilization from Hagerstown silty clay loam.

RESULTS AND DISCUSSION

The percent ^{14}C and 3-CA volatilized during the incubation period is shown in Figures 1 and 2. In attempting to measure $^{14}CO_2$ it was essential to distinguish between $^{14}CO_2$ evolved and volatilized 3-[^{14}C]CA. $^{14}CO_2$ is readily adsorbed by the alkaline trap solution, whereas the alkaline solution has a lesser affinity for 3-[^{14}C]CA. Similar results with volatilized 3-[^{14}C]CA were obtained wiith acidic trapping solutions (0.1 N H_2SO_4). Thus, this is not an accurate measure of total 3-[^{14}C]CA volatilization, but rather a measure of the amount of 3-[^{14}C]CA in equilibrium with the soil air space and the alkaline trapping solution. However, one can see that the ^{14}C and 3-CA volatilization from potassium azide treated soil resembles the volatilization from the autoclaved soil, whereas the other treatments, ethylene oxide and mercuric chloride, resemble the nonsterile or natural soil. The establishment of sterility by these sterilization methods has been determined in previous investigations (Kaufman et al., 1968; Skipper and Westermann, 1973).

Distribution of the total ^{14}C recovered at the conclusion of the experiment is presented in Table I. Volatilized ^{14}C includes all volatile 3-[^{14}C]CA products in addition to any $^{14}CO_2$. Organosoluble products accounted for 21–30% of the total ^{14}C recovered, which indicates no significant quantitative difference. ^{14}C dissolved in the alkaline trap solution accounted for 7–18% of the original amount. The treatment similarities indicated by Figures 1 and 2 are also

Table I. Distribution of ^{14}C from 3-[^{14}C]Chloroaniline in Sterile and Nonsterile Hagerstown Silty Clay Loam

sterilization method	% ^{14}C recovered as volatile[a]	organo-soluble[b]	residual[c]	total recov
potassium azide	16.9	22.4	33.7	73.0
autoclave	18.2	27.1	40.4	85.4
mercuric chloride	8.0	22.9	79.6	108.8
ethylene oxide	7.1	21.4	69.1	91.4
none	9.4	30.8	73.3	110.2

[a] $^{14}CO_2$ + 3-chloroaniline. [b] Benzene-methanol extracts. [c] Determined by combustion.

apparent in the residual ^{14}C. The amount of ^{14}C residual material in the mercuric chloride and ethylene oxide treated soil was similar to the nonsterile soil, whereas the potassium azide treated soil residue level was similar to the autoclaved soil. By comparing this with the volatilization data, one can conclude that where there is low measured volatility there is higher adsorption to the soil material. The total recovery yields are erratic, we believe, because of the ^{14}C that volatilized, but was not trapped.

The distribution of ^{14}C in 3-CA products extracted from the soils is listed in Table II. There were numerous bands appearing in extracts from each of the various treatments and the products distribution was different for each method. Two chromatographic regions are of particular interest. The first, R_f 0.11, contains the 3′-chloroacetanilide which was present only in the nonsterile soil. Acylation frequently occurs during the microbial metabolism of chloroanilines. Acetylated *p*-bromoaniline was identified from microbial metabolism of metobromuron [3-(*p*-bromophenyl)-1-methoxy-1-methylurea] (Tweedy et al., 1970). 4′-Chloroacetanilide was identified as a metabolite of 4-chloraniline (Kaufman et al., 1973). The presence of 3′-chloroacetanilide in only the nonsterile soil would support its formation as a result of microbial metabolism.

The second region (R_f 0.88) of particular interest involves the 3,3′-dichloroazobenzene detected in the nonsterile soil relative to the other treatments and its complete absence in the autoclaved soil. Actually, there are several complex products which frequently appear in this band, which have also been eliminated by these treatments. When this band was scraped from TLC plates, eluted, and rechromatographed in hexane/benzene (70:30), it separated into two–six individual bands identified as follows: *trans*-3,3′-dichloroazobenzene (R_f 0.50), 3,3′-dichloroazoxybenzene (R_f 0.45), *cis*-3,3′-dichloroazobenzene (R_f 0.18), and several unidentified bands (R_f 0.38, 0.20, and 0.12. TLC, gas–liquid chromatographic, and mass spectrographic characteristics of the azo- and azoxybenzenes were identical with known standard samples. Tentative identification of one of the unidentified products suggests a similarity to the three ring compounds [4-chloro-4′-(4-chloroanilino)azobenzene or 4-(3,4-dichloroanilino)-3,3′,4-trichloroazobenzene] described by Rosen and Siewierski (1971) and Bordeleau and Bartha (1972a,b). Mass spectral analysis of our compound indicated the presence of three aromatic rings, each having a single chlorine (Table III). This compound is tentatively identified as 4-(3-chloroanilino)-3,3′-dichloroazobenzene. Efforts are being made to confer a more positive identification on this and the other unidentified products.

From these results it would appear that these products were not biologically formed, or that some potential for biological-type activity still remained in the sterilized soils. One cannot discount the possibility, however, that these compounds are either autoxidation products of 3-CA adsorbed to some soluble lignin degradation product, or 3-CA itself adsorbed to soluble lignin degradation products, neither requiring a microbiological or enzymatic system. These products will receive further investigation in the future. The significant point to be made here is that the product distribution is different for each sterilization method.

Fractionation of the residual ^{14}C was achieved by alkaline extraction of the remaining soil (Table IV). A large percentage of the residual ^{14}C was alkali extractable (fulvic plus humic acids) from autoclaved and potassium azide treated soils, whereas the larger percentage of residual ^{14}C in the mercuric chloride and ethylene oxide treated and nonsterile soils remained in the humin fraction.

Further partitioning of the alkaline extract into fulvic and humic acid fractions yielded a similar distribution of residual ^{14}C (Table IV). In mercuric chloride and ethylene oxide treated and nonsterile soil, the larger percentage of ^{14}C was associated with humic acid fraction, whereas the larger percentage of the extractable ^{14}C in the potassium azide treated and autoclaved soil was present in the fulvic acid (aqueous plus ether) fraction. Since it was possible that the use of 0.5 N NaOH to remove the fulvic–humic acid fraction from soil, or the acidification required to precipitate the humic acids, could have liberated some of the adsorbed ^{14}C, the fulvic acid fraction was further partitioned with ether. Although the amount of ^{14}C associated with the fulvic acid fraction of the nonsterile soil was slightly lower than in all other treatments, the distribution of ^{14}C between the ether and acid phases was essentially the same for all treatments. Thus, it is reasonable to conclude that the extraction process affected

Table II. Distribution of ^{14}C in TLC of Solvent Extracts from Sterile and Nonsterile Soils

R_f	% ^{14}C present in extracts of soil sterilized by KN_3	$HgCl_2$	ethylene oxide	autoclaved	nonsterile	aromatic[a] amine reaction	standard
0.88	7.6	3.9	8.1		25.6	+	3,3′-dichloroazobenzene
0.85		5.9	5.5			+	
0.83	7.4	7.8	5.5		10.6	+++	
0.75	7.7				5.2		
0.72	7.4	11.7	5.3	8.1	5.4	++	
0.65	12.0	11.7	16.7	7.9	10.4	+	
0.57		3.9	8.1	8.1	10.2	+	
0.46	11.6		8.1	7.9	3.5		
0.24	7.7	3.9	5.5	8.1	5.2	++	3-chloroaniline
0.16	7.7	11.7	5.3	12.2	3.4	+	
0.11					6.7	+	3′-chloroacetanilide
0.05	7.6	27.4	16.2	23.8	3.5	+	
origin	23.2	12.0	15.9	23.8	10.2	++++	

[a] Relative intensity of positive reaction to modified Bratten-Marshall reagents (Pease, 1962): (+) weak, (++) intermediate, (+++) strong, and (++++) intense.

Table III. Mass Fragmentation of Compound Tentatively Identified as 4-(3-Chloroanilino)-3,3′-dichloroazobenzene

m/e	chemical composition
375	$C_{18}H_{12}N_3Cl_3$
264	$C_{12}H_8N_3Cl_2$
250	$C_{12}H_6N_2Cl_2$
236	$C_{12}H_8NCl_2$
201	$C_{12}H_8NCl$
166	$C_{12}H_8N$
139	$C_6H_4N_2Cl$
127	C_6H_6NCl
111	C_6H_4Cl

Table IV. Distribution of ^{14}C in Soil Organic Matter Fractions

	% original ^{14}C present in			
	fulvic acid			
	aqueous	ether	humic acid	humin
autoclave	7.8	7.4	8.2	16.9
potassium azide	7.2	6.2	7.7	12.6
mercuric chloride	7.7	7.0	17.6	48.1
ethylene oxide	7.5	6.8	15.3	39.5
none	5.6	4.6	14.7	48.4

Table V. Inhibition of Peroxidase Activity by Soil Sterilants

	horseradish peroxidase		soil peroxidase	
sterilization method	OD 402	% inhib	OD 460	% inhib
none	0.164	0	0.105	0
ethylene oxide	0.160	2.3	0.100	4.8
propylene oxide	0.146	11.0	0.096	8.6
potassium azide	0.022	86.4	0.000	100.0
autoclave	0.000	100.0	0.000	100.0

all treatments equally, and that the differences observed in ^{14}C distribution amoung the fulvic, humic, and humin fractions are indeed real.

The degradation of some pesticides is known to occur by cell-free biochemical mechanisms in soil. Getzin and Rosefield (1968) isolated a heat-labile substance from soil which was capable of rapidly degrading several organophosphate pesticides. Burge (1972) obtained an acylamidase from soil by sonication which hydrolyzed propanil (3′,4′-dichloropropionalide). Peroxidases were believed to be active in the reactions (Bartha and Bordeleau, 1969; Bordeleau et al., 1972; Linke et al., 1969) associated with chloroanilines in soil. The possibility that the various sterilization methods may have had different effects on soil biochemical processes was examined at the enzymatic level. The effect of propylene oxide, ethylene oxide, potassium azide, and autoclaving on peroxidase enzymes isolated from the Hagerstown silty clay loam soil, and a commercially available peroxidase were examined (Table V). This experiment revealed that both potassium azide and autoclaving inactivated or inhibited both horseradish and extracted soil peroxidases, whereas ethylene and propylene oxides caused only a very slight inhibition of these enzymes. These results would seem to explain, at least in part, the results obtained in previous experiments. The partial failure of potassium azide to completely inhibit 3,3′-dichloroazobenzene formation in our soil experiments could be attributed to the different nature of the reaction medium (soil vs. aqueous system), or to its own subsequent degradation in the soil system. It is also quite possible that other mechanisms of azobenzene formation exist in the soil which are unaffected by the azide.

Many pesticides are degraded chemically or biologically to phenolic intermediates or to aromatic amines. Phenolic compounds can be polymerized by chemical reagents as well as by enzymatic activity. In nature biological oxidation and coupling of phenols are key reactions which result in the formation of lignins, melanins, tannins, alkaloids, and antibiotics. Although it is well established that naturally occurring aromatic compounds are polymerized in the formation of humus by the activity of phenol oxidases (Brown, 1967), there are only a few studies that have attempted to establish the importance of phenol oxidases in soil (Kiss et al., 1975). Sjoblad and Bollag (1977) isolated an extracellular phenol oxidase from the soil fungus *Rhizoctonia practicola* which transformed 4-methoxyaniline to 2-amino-5-*p*-anisidinobenzoquinonebis(*p*-methoxyphenyl)imine. This enzyme was ineffective in polymerizing mono-substituted chloro- and bromoanilines.

Other investigators, however, have attributed the behavior of chloroanilines in soil to their interactions with peroxidase enzymes. Chloroazobenzenes and other products have been reported. Holland and Saunders (1968) described the formation of polymeric compounds with both anil- and quinoid-type bonds. Hsu and Bartha (1976) also suggested that chloranilines may be bound in soil by anil- and quinoid-type bonding. The formation of quinoid bonds, however, would require an initial oxidation of the substituted aromatic ring. The role of peroxidases in oxidizing these aromatic substituents is well known. The results of our experiments would suggest, therefore, a possible role of peroxidases in both binding of chloroanilines to soil particles, as well as the previously ascribed role of chloroazobenzene formation. Greater levels of binding occurred in ethylene oxide treated and nonsterile soils than in azide-treated or autoclaved soils. In the absence of binding processes, greater amounts of 3-chloroaniline would be available for volatilization, such as occurred in autoclaved and azide-treated soils.

The differences in ^{14}C levels in fulvic and humic acids could also be attributed to peroxidase activities. In nonsterile and ethylene oxide treated soil, slightly more than one-half of the bound ^{14}C activity extracted with the soil organic matter was associated with the humic acid fraction. In azide treated and autoclaved soils, however, two-thirds of the ^{14}C activity was associated with the fulvic acid fraction. These results would indicate that chloroaniline-peroxidase reaction products may have a somewhat greater tendency to bind to humic than fulvic acids, or themselves act as humic-type materials.

While more rigorous experimentation is necessary to substantiate these conclusions, they do suggest a further role of peroxidase enzymes in degradation and binding of chloroaniline-based pesticide residues, as a potential tool for examining and possibly delineating the binding mechanisms of chloroanilines and other aromatic compounds in soil.

In summary, we have examined the effects of several sterilization procedures on 3-chloroaniline degradation and behavior in soil. All affected 3-chloroaniline degradation differently, i.e., product composition, volatilization, and adsorption and/or binding phenomena. All soil sterilization methods are known to affect soil chemical and physical characteristics in unique ways. While the use of various soil sterilization procedures would appear to be a valuable tool from the academic viewpoint of sorting out the various nonbiological processes affecting behavior of chemicals in soil, any single method would appear to be of questionable value in terms of determining pesticide behavior in "sterile vs. nonsterile soils". Comparisons of autoclaved or "sterile" soils with nonsterile soils are fre-

quently used as the sole criterion for concluding that a chemical is microbially degraded in soil. Such casual conclusions may indeed be in error and credit microorganisms with reactions which they are only indirectly responsible for, or which are actually nonbiological in nature.

Unfortunately, scientists examining the degradation of chemicals in soil are continually encouraged to fall into this trap (U.S. EPA, 1978). While this conclusion may seem somewhat negative in its outlook, it is important to realize that the state of the art in this aspect of soil research is still somewhat deficient. Although other methods of soil sterilization such as air-drying, heat, microwaves, γ irradiation, or treatment with methylbromide, chloroform, or formaldehyde might also be considered, the authors are not presently aware of any studies which fully characterize the effects of these methods on soil chemical and/or physical processes, or how they might affect a given chemical's behavior in soil.

In the past, the authors have generally compared the degradation of a selected chemical in at least two different sterilized soil samples (generally autoclaved or azide-treated, and ethylene oxide treated) with its degradation in a nonsterile soil in preliminary experiments. On the basis of careful consideration of the chemicals' inherent reactivity or suspected lability in a chemical milieu such as soil, the presence or absence of apparent differences in degradation rates and products, the possible presence or absence of a lag phase in the chemicals degradation, and the possible isolation of a soil microorganism effective in degrading the chemical in question, the authors have been willing to conclude the potential role of the soil microbial population in that chemical's degradation in soil. In more recent investigations, however, the use of individual and combined microbial inhibitors such as cycloheximide and streptomycin sulfate have been examined (Anderson and Domsch, 1974; Barnett and Kaufman, 1979; Barnett et al., 1979). Although the possible influence of these antibiotics on other soil processes is still not fully understood, they would appear to offer certain advantages. In addition to specifically inhibiting the soil microbial activity, the selective nature of these compounds facilitates characterization of that segment of the soil microbial population (i.e., bacteria, fungi, etc.) which may actually be primarily involved in that chemical's metabolism in soil. Such methods may ultimately be more meaningful in interpreting the actual role of soil microorganisms in metabolizing a given chemical in soil, and more closely in line with the actual intent of protocols proposed for characterizing degradation of chemicals in soil.

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