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Determination of a Suitable Sterilization Method for Soil in Isoproturon Biodegradation Studies

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It is always desirable to distinguish biological from chemical degradation during laboratory degradation studies of pesticides in soil. Historically, this distinction has been made by comparing degradation rates in non-sterilized and sterilized soils (Fletcher and Kaufman 1980). Despite various sterilization processes causing considerable changes in chemical and adsorptive soil properties (Lehr et al. 1996), sterile conditions are always required in studies where distinction has to be made between abiotic and biotic processes. The most common method of sterilization is by autoclaving (Jonston and Camper 1991; Uan-Boh Cheah et al. 1998). However, other methods, like use of chemicals such as 0.1% solution of sodium ethyl mercurothiolate 2-benzoate (Munier-Lamy et al. 2002) and 1000ppm of $HgCl_2$ solution (Alvey and Crowley 1995) have been applied in soil sterilization. Gamma radiation with 2.5Mrad from a ^{60}Co-source (Hans-Holger Liste and Alexander 2000) has been used. The results from these methods are varied, with different degrees of microbial elimination. In most of these methods, soil physical and chemical properties were altered. Due to incomplete removal of the microbes, it has not been possible to exactly determine what proportion of degradation of the pesticides is due to abiotic processes in most of the studies.

In the present study both gamma radiation from ^{60}Co-source and autoclaving were evaluated as methods of sterilization. The duration of the experiment was restricted to 12 days in order to avoid any likely growth of microbes in the abiotic set-ups.

MATERIALS AND METHOD

^{14}C-ring uniformly labeled isoproturon [(N, -(4-isopropylphenyl)-N',N' -dimethylurea); radiochemical purity >99% (International Isotopes, Munich). Non-labeled isoproturon (purity>99.5%) was purchased from Promochem (Wessel, Germany). Monodemethyl-isoproturon [N-(4-isopropylphenyl)-N'-methylurea] and didemethyl-isoproturon [N-(4-isopropylphenyl urea] were donated by Rhone-Poulenc (Lyon, France), 1-OH-isoproturon [N-(4-(1-hydroxy-isopropyl)-phenyl)-N'N'-dimethylurea] and isopropyl aniline were donated by Ciba Geigy (Basel, Switzerland). A tropical soil with a clay texture with the following physical and chemical properties, organic carbon (OC) 2.07%, pH 6.08, clay content 68%

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sand content 28%, silt content 12%, N 0.19%, P 80ppm, Na 0.95%, K 1.85%, Ca 10.5%, Mg 4.95%, Mn 0.51%, Fe 226.96% and electrical conductivity (EC) 0.62μS/cm was used in the study. A dry sample of the soil was crushed in a mortar with a pestle and sieved through a 2-mm sieve. Prior to starting the laboratory experiment, the soil was equilibrated for 10 days at 20±1°C and at 60% of the maximum water holding capacity (WHC). Three different sets of experiments were set up with the soil. Two sets of experiments were abiotic. One set of the soil was sterilized by autoclaving at 120°C with pressure of 1.2 bars for 30 minutes in three consecutive days. Another set was sterilized by irradiation with gamma radiation from ^{60}Co-source, which supplied a total of 2.5Mrad at a rate of 39.9 grays/min (1 gray (gy) = 100 rads) for ten hours. Another set of experiment, which was biotic acted as control and contained soil, which was not sterilized by any method. Each set of experiment was replicated three times. Each incubation flask was filled with moistened soil of equivalent dry weight of 20g. The soil in the incubation flask was spiked with 80μl of isoproturon pesticide solution with a Hamilton syringe. The pesticide solution consisted of both labelled and non-labelled isoproturon in the ratio of 16.5:83.5, respectively. The soil was homogenized in the incubation flask, which was closed immediately and connected to the incubation system as described by Lehr et al. (1996). The initial radioactivity and pesticide concentration in soil in each of the incubation flask was 2997684dpm and 21.5μg/g, respectively. The soil samples were incubated at 20±1°C for 12 days. Humid and CO_2-free air (0.8l/h) was sucked three times a week (Monday, Wednesday and Friday) for one hour through the incubation flasks to avoid the drying of the soil during the course of the experiment and to flush volatile ^{14}C-compounds and $^{14}CO_2$ to the traps. The first trap contained 10ml of ethyleneglycolmonomethylene ether (EMME), which adsorbed volatile ^{14}C-compounds. The second and third traps, each contained 10ml of 0.1N NaOH, which adsorbed $^{14}CO_2$ resulting from the mineralization of ^{14}C-uniformly ring-labelled isoproturon. Sampling from the traps was done after sucking humid and CO_2-free air through the incubation flasks. All the 10ml of EMME was emptied from the trap into a 20-ml vial to which was added 10ml of a scintillation cocktail of Ultima Gold (Packard, Germany), mixed and scintillation counted. 2ml aliquot of NaOH solution was mixed with 3ml of Ultima Flo scintillation cocktail before scintillation counting. The experiment was discontinued after 12 days before microbes could develop in the abiotic set-ups.

To determine radioactivity in the extractable residue in the soil, 15g of dry soil from each incubation flask was extracted by accelerated solvent extraction, which was performed in an ASE 200 (Dionex, Germany). The extraction experiments were conducted in three replicates. 0.5ml of the methanol extract was mixed with 10ml of Ultima Flo cocktail to determine radioactivity by Liquid Scintillation Counting (Tricarb 1900 TR, Packard, Germany). To determine non-extractable (bound) residue, the extracted soil was air-dried and a sub-sample of 0.3g was weighed in paper cones, mixed with sucrose solution before combustion in an oxidizer (Oxidizer 306, Packard, Germany). The $^{14}CO_2$ released was trapped in 7ml of Carbsorb E (Packard, Germany), which was mixed with 7ml of Permafluor E (Packard, Germany) prior to scintillation counting. A ^{14}C-mass balance was established at the end of the experiment.

To determine metabolites formed in soil, the methanol extract was concentrated to 5ml under vacuum in a rotary evaporator. The extract was diluted with deionized water to total volume of 250ml and cleaned by solid phase extraction (SPE), which was prepared first by conditioning the SPE column. 1ml of methanol was drawn through the column, followed by an equal amount of deionized water under vacuum. The sample was applied and drawn through the SPE column at a rate of 20ml/minute. The column was flushed with deionized water followed by methanol. The SPE column was dried by purging it with nitrogen gas. The compounds in the SPE column were eluted with 7ml of HPLC grade methanol. The methanol was reduced to 0.5ml, from which 20µl was injected into HPLC. The HPLC separation was carried out under the following conditions, column: 250x4mm Lichrosper 100 RP-18, 5µm (Merck, Darmstadt, Germany), detector: UV-VIS 240 nm detector (Merck, Darmstadt, Germany), Radio-activity monitor LB 506 Cl (Berthold, Wildbad, Germany), mobile phase: A= water (chromatography grade, Merck, Darmstadt, Germany), B = acetonitrile (HPLC grade, Riedel-de Haen, Seelze, Germany) At time = 0 (T0), A= 95%, B= 5%; T20: A= 70%, B=30%; T30: A=40%, B=60%; T35: A= 40%, B=60%; T40: A=95%, B= 5%; T50: A=95%, B=5% injection volume: 20µl. Flow rate was 0.8ml/min.

RESULTS AND DISCUSSION

Figure 1 shows the amount of accumulated $^{14}CO_2$ evolved during mineralization of ^{14}C-uniformly ring-labelled isoproturon from soil with different treatments. Autoclaving was more effective than gamma radiation in sterilizing the soil. Only 0.02±0.006% of $^{14}CO_2$ was evolved from autoclaved soil in 12 days of incubation. 0.07±0.01% of $^{14}CO_2$ was evolved from gamma-irradiated soil. The soil, which was not sterilized, encouraged high microbial activity in the soil resulting in 0.56±0.01% of ^{14}C-isoproturon mineralized. The mineralization curve of isoproturon in non-sterilized soil showed a steady increase of $^{14}CO_2$ production with time. Even autoclaving method did not totally eliminate the microbes but only substantially reduced them in the soil. This was why the experiment was discontinued on the 12th day because thereafter, microbes would have multiplied in the abiotic set-ups and would have interfered with results of abiotic processes. Effects of autoclaving as a method of sterilizing soil has been shown to significantly increase extractable Mn levels in the soil (Wolf et al. 1989). The soil in the present study had Mn concentration of 0.51% but the effect of autoclaving on the extractability of Mn was not investigated in this study. In studies involving the use of gamma-irradiated seep sediments as a sorbent in phenanthrene biodegradation, mineralization of ^{14}C-phenanthrene was drastically reduced but not completely eliminated. Reduction of mineralization was attributed to changes induced by gamma irradiation on the sorptive properties of the seep sediment (Sandoli et al. 1996).

The distribution of ^{14}C in various forms of isoproturon in soil is shown in figure 2. There was good recovery of the total radioactivity from soil after 12 days of incubation. The highest recovery was made from the autoclaved soil, where

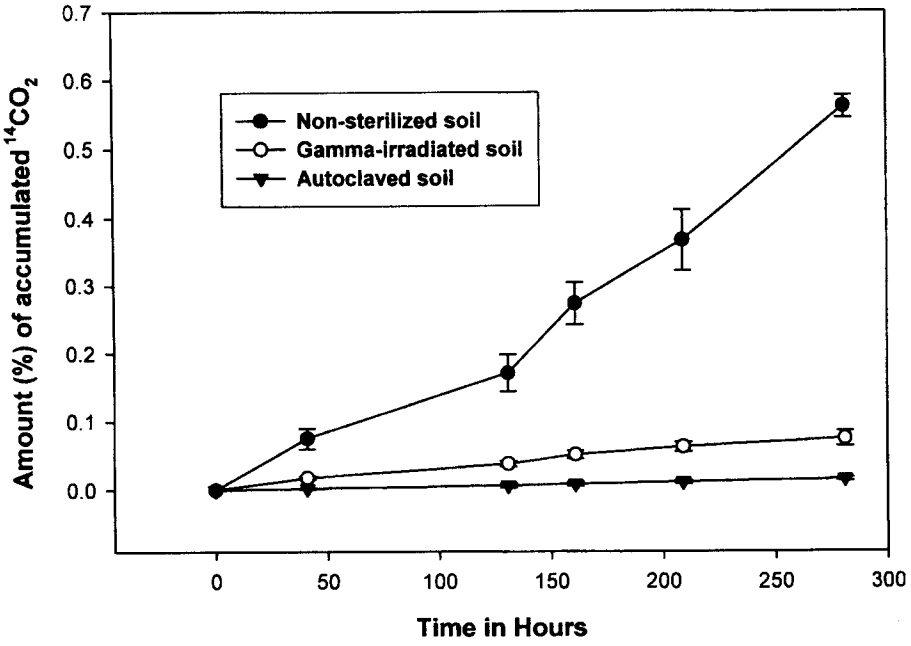

Figure 1. Mineralization of ^{14}C-ring labeled isoproturon in soil with different sterilization methods.

97.2±0.8% of the total radioactivity was recovered. The lowest recovery of 93.3±6.8% was registered in the soil, which was not sterilized. The recovery from gamma-irradiated soil was intermediate with a value of 96.3±6.5%. The distribution of ^{14}C in various forms of isoproturon in soil with different treatments (figure 2) shows that the extractable form constituted the highest proportion of all forms of ^{14}C-isoproturon in all the experimental set-ups. Highest amount (89.0±0.2%) of extractable residue was found in autoclaved soil, in which microbial activity was substantially reduced. It is in the same soil, where the least amount of $^{14}CO_2$ was evolved and the least amount of non-extractable residue was also formed. In non-sterile soil, the highest amount of $^{14}CO_2$ was evolved and the highest amount of non-extractable residue was formed. However, the least amount of extractable residue was found in the soil. The results showed that non-extractable (bound) residue formation and the amount of $^{14}CO_2$ evolved was determined by the level of microbial activity in soil. In the study of the biodegradation of ^{14}C-glyphosate, the least amount of extractable residue of glyphosate was formed in the sterile soil while the highest amount was formed where the highest amount of $^{14}CO_2$ was evolved, in one of the soils with compost amendment (*unpublished*). In a study by Abdelhafid et al. (2000) on the biodegradation of ^{14}C-atrazine in soils with different organic amendments, the soil in which the highest amount of $^{14}CO_2$ was evolved had the least amount of extractable residue. However, the amount of non-extractable residue was not as high as could be expected in a soil where microbial activity was highest, probably due to the mineralization of the non-extractable residue in the soil. The present

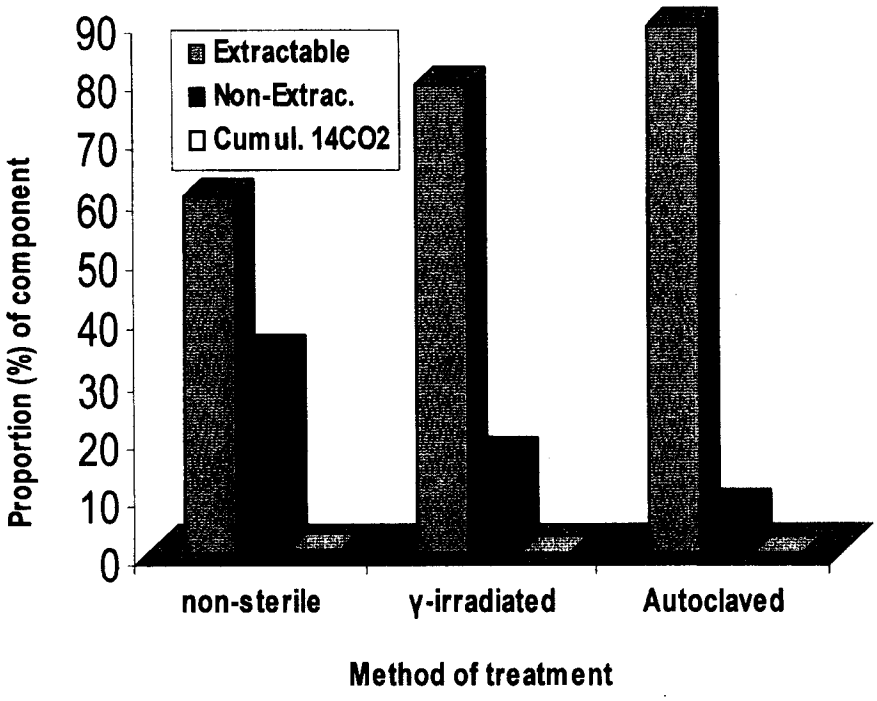

Figure 2. The distribution of ^{14}C in various forms of isoproturon in soil.

study confirmed that non-extractable (bound) residue formation is a biotic degradation process, which took place parallel to $^{14}CO_2$ formation. The gamma-irradiated soil with moderate amounts of microbes had intermediate values between the extremes of non-sterile and autoclaved soils.

Isoproturon and its degradation products were analyzed by HPLC. The residues in the methanol extracts consisted of the parent compound and eight metabolites from non-sterile soil as shown in table 1. Only three peaks (1, 5 and 6) of the metabolites in addition to that of the parent compound, isoproturon (peak 7) were identified in the methanol extract from non-sterile soil, when compared with retention times of authentic reference standards. In methanol extracts from gamma-irradiated soil seven peaks were recorded of which only 6 and 7 peaks were identified. In the methanol extract from autoclaved soil, Peaks 5, 6 and 7 were the only ones identified from eight peaks. The concentrations of the metabolites were higher in non-sterile soil than they were in either autoclaved or the gamma-irradiated soil. Further more, there were more peaks in the non-sterile soil than they were in either autoclaved or gamma-irradiated soil. This shows that the formation of the metabolites from the parent compound, isoproturon, was entirely due to biodegradation. The results showed that the level of microbial activity in soil influenced the number and concentration of the metabolites formed. This is consistent with data presented in figures 1 and 2, which show the

Table 1. Isoproturon and its soluble metabolites in non-sterile soil.

Peak	1	2	3	4	5	6
R_t (min)	18.52	21.28	22.97	29.23	31.60	33.07
%	0.3	3.6	5.5	0.8	0.7	6.6
Peak	7	8	9			
R_t (min)	34.45	37.32	38.13	-	-	-
%	78.6	0.5	0.8	-	-	-

Key: 1=1-OH-monodemethyl-isoproturon, 2 =unknown metabolite, 3=unknown metabolite, 4=unknown metabolite, 5=didemethyl–isoproturon, 6=monodemethyl-isoproturon, 7=isoproturon, 8=unknown metabolite, 9= unknown metabolite.

amounts of $^{14}CO_2$ evolved and the proportions of various forms of isoproturon in soil during mineralization. Dorfler et al. (2002) got ten metabolites together with the parent compound, isoproturon in Bligh-Dyer-Solution extract from wheat plants after an incubation period of 67 days. The parent compound was predominant followed by 2-OH-isoproturon and 1-OH-isoproturon combined. In the present study the main compound was isoproturon (78.6%) followed by monodemethyl-isoproturon. The concentration of 1-OH-monodemethyl-isoproturon was the least in the extract from non-sterile soil.

Both methods could not completely eliminate all microbes in the soil. However, autoclaving method was more effective than gamma-irradiation. The gamma-radiation from the ^{60}Co-source required a longer period of exposure. It was not possible therefore to deduce what proportion of the bound residue of isoproturon was due to abiotic process.

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REFERENCES

Abdelhafid R, Houot S, Berriuso E (2000) Dependence of atrazine degradation on C and N availability in adapted and non-adapted soils. Soil Biol Biochem 32:389-401

Alvey S, Crowley DE (1995) Influence of organic amendments on biodegradation of atrazine as a nitrogen source. J Environ Qual 24: 1156-1162

Fletcher CL, Kaufman DD (1980) Effects of sterilization methods on 3-chloroaniline behavior in soil. J Agric Food Chem 28: 667-671

Hans-Holger L, Alexander M (1999) Accumulation of phenanthrene and pyrene in rhizosphere soil. Chemosphere 40: 11-14

Johnston WH, Camper ND (1991) Microbial degradive activity in pesticide pretreated soil. J Environ Sci Health 26:1-14

Lehr S, Scheunert I, Beese F (1996) Mineralization of free and cell-wall-bound isoproturon in soils in relation to soil microbial parameters. Soil Biol Biochem 28:1-8

Manier-Lamy C, Feuvrrrier MP, Chone T (2002) Degradation of ^{14}C-atrazine bound residues in brown soil and rendzina fractions. J Environ Qual 31: 241-247

Sandoli RL, Ghiorse WC, Madsen EL (1996) Regulation of microbial phenanthrene mineralization sediment samples by sorbent-sorbate contact time, inoculation and gamma irradiation–induced sterilization artifacts. Environ Toxicol Chem 15: 1901-1907

Uan-Boh Cheah, Kirkwood RC, Keng-Yeang Lum (1998) Degradation of four commonly used pesticides in Malaysian agricultural soils. J Agric Food Chem 46: 1217-1223

Wiren-Lehr S, Scheunert I, Dorfler U (2002) Mineralization of plant-incorporated residues of ^{14}C-isoproturon in arable soils originating from different farming systems. Geoderma 105:351-366

Wolf DC, Dao TH, Scott HD, Levy TL (1989) Influence of sterilization methods on selected microbiological, physical, and chemical properties. J Environ Qual 18:39-44