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Effects of Carbofuran on the Earthworm, *Eisenia fetida*, Using a Defined Medium

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The importance of sub-lethal effects of xenobiotics on the biology of non-target organisms can be determined from the contribution of these organisms on the ecology. This implies standardised tests and strict control over experimental conditions, facilitating correlation of results between different workers. The purpose of this study was to evaluate a chemically defined medium that supports growth and reproduction of the earthworm Eisenia fetida for 60 days and to quantify the sub-lethal effects of carbofuran on this representative of a non-target group of organisms. Carbofuran is a pesticide widely used in Southern Africa to control nematodes on maize.

Studies have already been made on the toxicity of this pesticide to the earthworm E. fetida on different media, which helped in the planning of the experiment as well as the evaluation of the usefulness of the medium. Stenersen (1979a) determined that levels of carbofuran of up to 64 mg kg^{-1} did not kill E. fetida. Gilman and Vardanis (1974) determined an LC-50 value of about 4 mg kg^{-1}. Haque and Ebing (1983) reported an LC-50 value of 566.2 mg kg^{-1} for a 5% carbofuran formulation which represents 28 mg kg^{-1} of active ingredient. The large differences in these results may be due to the different media employed by these authors. The use of a defined medium may eliminate such differences and, although predicting the results of field studies is not possible by direct extrapolation from the results of such a medium, it will give a good indication of the relative lethal and sub-lethal effects of xenobiotics.

MATERIALS AND METHODS

The chemically defined medium we employed consisted of 193 g MCU vermiculite (Micronized Products, Johannesburg, SA), 14 g cellulose (Merck, art. 2330), 0.8 g DNA (Merck, art. 42027), 0.7 g casein (BDH, 44018), 0.4 mg each of ascorbic acid, nicotine-amid and thiamonium-dichloride, 2.0 g humic acid and 80 ml of a 0.4% saline solution as used by Cole et al. (1976). One ml of a cow manure extract (1 g of cow manure from the culture medium shaken with 10 ml of water) and the saline solution were

added and the medium thoroughly mixed one day before the addition of three worms (twenty days old). Wide mouthed 1 L glass jars with a hole in the lid, were used as containers.

The worms were kept at a constant temperature of 25 °C and relative humidity of 80% in darkness (Reinecke and Kriel, 1980). Cocoons were collected and placed in separate compartments of replicate dishes. Hatchlings were removed every day and placed in a vermiculite medium with saline solution added only, no food. The hatchlings were able to survive for up to six weeks in this medium. As soon as enough hatchlings were collected, they were transfered to a cow manure medium in which they stayed for 20 days before being added to the jars.

Worm and media samples were analysed for carbofuran by using a Carlo Erba Series 2150 gas-chromatograph with a Ni-63 electron capture detector and 4 mm id, 1 or 1.5 m long glass columns packed with 3% OV 17 on Gaschrom Q. The test conditions were: injector 300 °C, oven 260 °C isothermal and detector 275 °C. The carrier gas was nitrogen at 30 - 40 ml per minute. Quantification was by measurement of peak heights and response curves.

The reagents used were: Florisil, 60-100 US mesh, washed in distilled water and reactivated at 680 °C and stored in a stoppered bottle at 130 °C. 1-Fluoro-2,4-dinitro benzene (BDH 44032) was used as a 1% solution (v/v) in acetone. Glycine (BDH 10119) was used as a saturated solution in distilled water. Potassium hydroxide was used as a 0.5 M solution in distilled water. Sodium sulphate (anhydrous) was dried for 12 hours at 400 °C and stored in a stoppered jar prior to use. Sodium tetraborate was used as a 5% solution (m/v) in distilled water. Carbofuran 99% pure was used (obtained from Dr. JE Oliver, USDA, Maryland, USA). Solvents used were acetone, chloroform, hexane and dichloromethane distilled in glass and ethylacetate used as received.

The method of extraction of carbofuran was an adaptation of the methods reported by Argauer (1969) and Gorder and Dahm (1981). After adding 10 ml of chloroform to a vial containing a known amount of the medium (5 - 10 g), it was mixed at a low speed in an ice-bath with a Virtis "45" homogenizer. The vial was capped and centrifuged at 1750 rpm for 5 min. The chloroform was decanted and the procedure was repeated. The extracts were collected in a 50 ml round-bottomed flask and evaporated at 30 °C with a rotary evaporator to almost dryness. 5 ml of a 1:9 mixture of ethyl acetate : hexane was then added.

The worms were kept on moist filter paper for 24 hours prior to extraction of carbofuran to allow the worms to pass out the gut contents. Because the contents of the gut also contained carbofuran, co-extraction would influence the data. After determining the wet weight of a worm, it was transferred to a clean vial, 5 g of washed sea-sand and 10 ml of chloroform were then added and the same procedure as for the medium extraction

was followed. The extract was cleaned up via a florisil step. A layer of sand was added in a 5 ml graduated pipette following a plug of glass wool, to support 1 g of florisil. The column was pre-washed using 5 ml dichloromethane, 5 ml water-saturated dichloromethane followed by 5 ml of the 1:9 mixture and the washings were discarded. The extract was transferred from the round-bottomed flask to the column. The column was then eluted by a further 20 ml of the 1:9 mixture and the eluate collected in a clean 50 ml round-bottomed flask. After evaporation to dryness, 5 ml of acetone was added. The determination of the extraction efficiency was done by adding a known amount of pesticide to a blank sample and mixing it thoroughly before extraction.

Because of the thermal instability of carbofuran, the pesticide was hydrolyzed to the phenol and derivatized to the electron-capturing 2,4-dinitrophenyl ether, a more stable compound, prior to gas-chromatographic analysis. This was done by adding 10 ml of distilled water, 2 ml of the sodium tetraborate solution, 2 ml of the KOH solution followed by 0.2 ml of the 1% FDNB solution. The flask was then stoppered securely and after being shaken for ten minutes on a mechanical shaker, heated to 80 °C for 60 minutes. The flask was cooled in a water-bath, 1 ml of the glycine solution added and the flask stoppered again and shaken for a further ten minutes. The contents of the flask was transferred to a 100 ml separating funnel, rinsed, first with 5 ml of acetone, then with 10 ml of hexane and the washings were added to the funnel. It was stoppered and shaken for 30 seconds, left to separate and the hexane phase was dried through a column of approximately 2 g of sodium sulphate. The water phase was extracted twice and the hexane extracts were combined, evaporated and the residue dissolved in a suitable volume of hexane. This was then analysed by gas-chromatography. A mean extraction efficiency of 92.0% (SD = 2.64) was obtained for the medium and 69.2% (SD = 0.08) for the worm sample. This was achieved for concentrations of 5 mg kg^{-1}. Single determinations of concentrations of 0.5 mg kg^{-1} yielded an efficiency of almost 100%.

Concentrations were used that might be expected to occur in soil. These were 10.0, 2.0 and 0.2 mg kg^{-1} (Kuhr and Dorough, 1976). The carbofuran was added dissolved in acetone, to the already mixed dry ingredients, and the solvent evaporated. Twelve replicates of each concentration as well as a control were prepared. This allowed for sampling of two jars per concentration every ten days. Because the other jars were not sampled, it minimized the disturbance of the earthworms. All the worms in the two sample jars as well as the medium could be used for analysis. If samples were taken periodically from each jar, disturbances and changes in volume would have altered the experimental conditions.

The worms were rinsed in water (25 °C) dried on damp filter paper and weighed in a vessel with water (25 °C) to prevent desiccation during weighing (adapted from Graff, 1978).

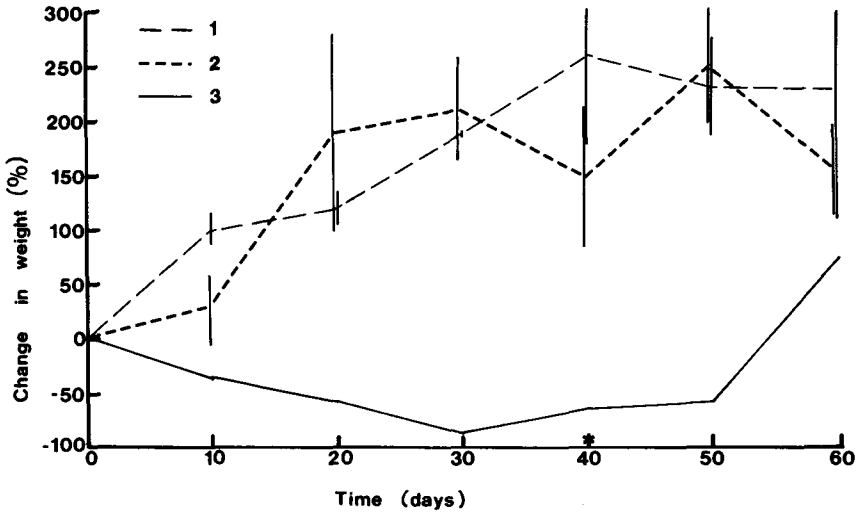

Figure 1. Influence of carbofuran on the change in weight of earthworms expressed as a percentage of the initial weight of the worms. Medium: 1 - control; 2 - 0.2 mg kg^{-1}; 3 - 2.0 mg kg^{-1}. Standard deviations are indicated.

RESULTS AND DISCUSSION

Despite the lack of toxicity of concentrations of up to 64 mg kg^{-1} reported for carbofuran by Stenersen (1979a), of the worms exposed to 10 mg kg^{-1}, 97% died before sampling. The majority of these died within the first 48 hours of the experiment. 69.7% of the worms exposed to 2.0 mg kg^{-1} died and the majority of these lost hydrostatic pressure before dying and decomposed very quickly. The other worms seemed to dehydrate since the integument stayed intact but was dry. The changes in live weight are summarised in Figure 1. Untreated worms and those exposed to 0.2 mg kg^{-1} did not significantly differ in weight. The survivors of the 2.0 mg kg^{-1} exposure lost weight consistently for 30 consecutive days then started to gain weight again and at the end of the experiment weighed more than initially.

The breakdown of the carbofuran was very rapid (Fig 2) and its concentration dropped to below 1 mg kg^{-1} after 30 days and below detection level after 10 days for the 0.2 mg kg^{-1} and after 50 days for the 2.0 mg kg^{-1} exposure. No carbofuran was found in the worms although a trace was detected in one individual that survived the 10 mg kg^{-1} exposure.

There was no significant difference in the rate of development of the clitellum between the control and the worms exposed to 0.2 mg kg^{-1} carbofuran. The worms exposed to 2.0 mg kg^{-1} carbofuran did not develop a clitellum or produce cocoons. The untreated worms

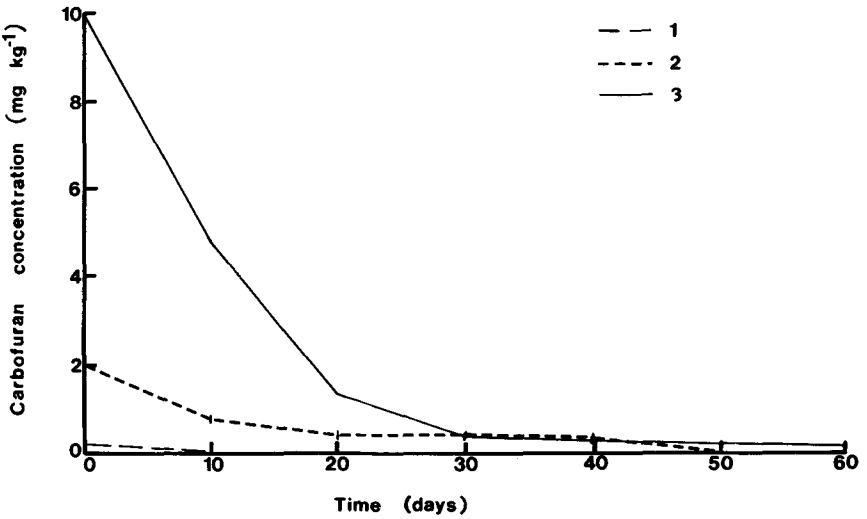

Figure 2. Decrease of the carbofuran concentrations in the media. Medium: 1 - 0.2 mg kg^{-1}; 2 - 2.0 mg kg^{-1}: 3 - 10.0 mg kg^{-1}. Standard deviations are indicated.

produced 99 cocoons with a combined mass of 0.87 g. The worms exposed to 0.2 mg kg^{-1} produced 124 cocoons with a combined mass of 1.21 g. A Mann-Whitney statistical test revealed no significant differences for either the number or the weight of the cocoons produced by the two groups.

The cocoons were monitored for number of hatchings and number of hatchlings. This was done by placing each cocoon in a cell of a replicate dish in 2 ml of distilled water. Few hatched and those that did hatch had few hatchlings. In the untreated and those treated with 0.2 mg kg^{-1} the percentage hatching were 17.2% and 53.2% respectively, with a mean of 2.1 and 1.8 hatchlings per hatched cocoon.

Changes in the pH and the water content were also measured. The pH of all the replicates fluctuated between 7.2 and 8.5. The water content (determined by oven-drying) increased slightly from a mean of 0.40 to 0.45 ml g^{-1} for all the carbofuran concentration levels. It was obvious that the pesticide had no influence on either the pH or the water content.

Much lower concentrations of carbofuran (LC-50 value between 0.2 and 2.0 mg kg^{-1} carbofuran for this study) were toxic to earthworms than reported by Stenersen (1979a). The biological availability of the pesticide might differ according to the physical but also the biological nature of the medium. Adsorption of the pesticide molecules by the particles or organic matter could have reduced

the amount of pesticide available for biological action. Because of biological activity, this might subsequently be released again as reported by Fuhremann and Lichtenstein (1978). Adsorption is usually correlated with the organic matter content, but this is not the norm, as reported by Lofs-Holmin (1981). She indicated that microbial activity should also be considered an important factor.

The previous exposure of the worms to pesticides may have had a direct influence on their susceptibility to carbofuran. The worms used in this study had no history of exposure to pesticides.

Stenersen (1979a) and also Haque and Ebing (1983) used worms acclimatized to European conditions while the worms used in the present study were acclimatized to local conditions. Physiological differences between different geographic populations of E. fetida exist (Graff, 1978) and could account for differences in susceptibility.

The most obvious sub-lethal response was the loss of weight by the worms that survived the 2.0 mg kg^{-1} exposure. The duration of the experiment was not long enough to determine whether the surviving worms would have reached maturity or not. Since optimal conditions were maintained, it is unlikely that these worms would have survived had harsher conditions prevailed.

All the worms exposed to 2.0 mg kg^{-1} carbofuran, except those collected after 60 days, showed a distinct coiling of the last few segments. After 24 hours on filter paper the coiling disappeared and normal behaviour was observed. This recovery may be due to excretion of the pesticide or metabolites from the body. This can also be correlated with the absence of parent compound (carbofuran) in the worms. The elimination of carbofuran from earthworms via excretion has been reported by Stenersen (1979b). With C-14 labelled carbofuran he found that almost 100% of the radio activity was eliminated within 24 hours. The same rapid elimination was also reported by Yu et al. (1974) from several animals in a model ecosystem. Thomson and Sans (1974) also reported no carbofuran residues in worms in a field experiment. The water solubility of the primary metabolite, 3-hydroxy-carbofuran, formed in E. fetida could account for the rapid elimination from the body (Gilman and Vardanis, 1974).

The degradation of carbofuran in E. fetida and subsequent elimination of the metabolites from the body may also acount for the decrease in the concentration of carbofuran in the medium. Although carbofuran is normally considered to be moderately persistent, relatively short breakdown periods have been reported. Wiliams and Brown (1976) reported a half-life of 28 days in loam soil at 20 °C and a pH of 6.0. Gorder et al. (1982) reported half-lives between 7 and 9 days for different soils. In this study the half-life was also in the order of 7 to 9 days.

Sub-lethal effects were not only observed in terms of weight

change; clitellum development and cocoon production were completely inhibited by concentrations of 2.0 mg kg^{-1} and higher. The single survivor in the 10 mg kg^{-1} medium not only lost 82% of its weight, but, similar to the survivors of the 2.0 mg kg^{-1} exposed group, it showed no development of a clitellum and produced no cocoons. Comparatively a reduction of 94.2% in the number of worms was observed 21 days after application of 3.4 kg ha^{-1} carbofuran, which represents 1.7 mg kg^{-1} (10 kg ha^{-1} = 5 mg kg^{-1}: Talekar et al., 1977), was reported for a field study by Tomlin and Gore (1974). Thomson and Sans (1974) reported a reduction of 60.4% in the biomass of worms and a reduction of 82.7% in the number of worms 21 days after application of 4.48 kg ha^{-1} carbofuran (it equals 2.24 mg kg^{-1}). These authors did not report the state of development of the worms. Stenersen (1979a) found E. fetida to be the least susceptible to pesticide action of all earthworm species he tested.

The unusually low percentage of cocoons that hatched and the number of hatchlings per cocoon may be related to the time the cocoons were in the culture medium before they were collected and not to the effects of the pesticide. In previous studies 80% or more hatched when they were collected not more than 2 days after they were produced (unpublished results). Some of the cocoons in this study remained in the medium up to 21 days. A decrease in the protective capabilities of the cocoon wall could be a reason.

Certain deficiencies in the experimental design should be mentioned. It would have been better to use two replicates of each pesticide concentration in order to monitor the growth of one group of worms while correlating it with the pesticide parameters in the other group. This would eliminate the need for expressing growth as a percentage of the initial weight. Of major importance, however, is that cocoons can be removed from the medium at the same time as the worms are being weighed.

This study demonstrated the feasibility of using an artificial culture medium to determine lethal and sub-lethal effects of pesticides on earthworms. It also showed that the use of carbofuran may have a serious effect on the earthworms in agricultural soils in which they are present. The sub-lethal effects of carbofuran on earthworms, combined with the harsh conditions that occur in South Africa, may diminish the contribution of earthworms on agriculture and ecology.

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REFERENCES

Argauer RJ (1969) Determination of residues of Banol and other carbamate pesticides after hydrolysis and chloracetelation. J Agric Food Chem 17:888-892

Cole LK, Metcalf RL, Sanborn JR (1976) Environmental fate of

insecticides in terrestrial model ecosystems. I J Environ Stud 10:7-14
Fuhremann TW, Lichtenstein EP (1978) Release of soil-bound methyl parathion residues and their uptake by earthworms and oat plants. J Agric Food Chem 26:605-610
Gilman AP, Vardanis A (1974) Carbofuran. Comparative toxicity and metabolism in the worm Lumbricus terrestris L and Eisenia foetida S . J Agric Food Chem 22:625-628
Gorder GW, Dahm PA (1981) Analysis of carbofuran in soil samples. J Agric Food Chem 29:629-634
Gorder GW, Dahm PA, Tollefson JJ (1982) Carbofuran persistence in cornfeld soils. J Econ Entomol 75:637-642
Graff O (1978) Physiologishe Rassen bei Eisenia foetida (Savigny, 1826) (Oligochaeta, Lumbricidae) ? Ein Beitrag zur Frage der Domestikation dieser Art. Rev Ecol Biol Sol 15:251-263
Haque A, Ebing W (1983) Toxicity determinations of pesticides to earthworms in the soil substrate. Z Pflanzenkrankheiten Pflanzenschutz 90:395-408
Kuhr RJ, Dorough HW (1976) Carbamate insecticides: Chemistry, biochemistry and toxicology. CRC Press, New York
Lofs-Holmin Astrid (1981) Influence in field experiments of benomyl and carbendazim on earthworms (Lumbricidae) in relation to soil texture. Swed J Agric Res 11:141-147
Reinecke AJ, Kriel JR (1980) The influence of temperature on the reproduction of the earthworm Eisenia foetida (Oligocheata). SA J Zool 16:96-100
Stenersen J (1979a) Action of pesticides on earthworms. Part I: The toxicity of cholinesterase-inhibiting insecticides to earthworms as evaluated by laboratory tests. Pestic Sci 10:66-74
Stenersen J (1979b) Action of pesticides on earthworms. Part II: Elimination of parathion by the earthworm Eisenia foetida . Pestic Sci 10:104-112
Talekar NS, Sun LT, Lee EM, Chen JS (1977) Persistence of some insecticides in subtropical soil. J Agric Food Chem 25:384-352
Thomson AR, Sans WW (1974) Effects of soil insecticides in southwestern Ontario on non-target invertebrates: Earthworms in pasture. Environ Entomol 3:305-308
Tomlin AD, Gore FL (1974) Effects of six insecticides and a fungicide on the number and biomass of earthworms in pasture. Bull Environ Contam Toxicol 12:487-492
Wiliams IH, Brown MJ (1976) Degradation of carbofuran by soil microorganisms. Bull Environ Contam Toxicol 15:244-249
Yu CC, Booth GM, Hasen DJ, Larsen JR (1974) Fate of carbofuran in a model ecosystem. J Agric Food Chem 22:431-434

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