Pergamon

PII: S0038-0717(96)00275-1

Soil Biol. Biochem. Vol. 29, No. 3/4, pp. 699–703, 1997

Printed in Great Britain
0038-0717/97 $17.00 + 0.00

EARTHWORM TOXICOLOGY: FROM ACUTE TO CHRONIC TESTS

ABDUL MOTALIB M. ABDUL RIDA* and MARCEL B. BOUCHÉ
Laboratoire de Zooécologie du Sol, CEFE, CNRS, BP5051 F-34033 Montpellier, France

(Accepted 23 September 1996)

Summary—The International Workshop on Earthworm Ecotoxicology (1991) offered the opportunity to synthesize the state of the art about the standardization of (eco)toxicological tests. It showed the need to improve the standardization of laboratory tests, especially for chronic assays. Such chronic tests need to avoid, or at least limit, the artefacts resulting from interferences between soil components (e.g. organic matter, sand, kaolinite, levilite), earthworm food, soil microorganisms and the chemical tested. The effects of test medium variables, especially pH changes and ionizable chemicals, are also crucial considerations. In order to ensure the goals of standardization and reproducibility for chronic tests, then one must provide an easy to prepare synthetic substrate and provide food in a non-aseptic environment using non-axenic earthworms. This paper deals with these improvements and describes a new synthetic medium, the procedure to make chronic tests and the monitoring of changes resulting from interferences due to the medium and chemicals. © 1997 Elsevier Science Ltd

INTRODUCTION

Many biomonitoring approaches and laboratory test methods have been proposed for the ecotoxicological assessments of chemicals on living organisms (Blandin, 1986; Salanki *et al.*, 1994). For earthworms, many different laboratory tests have been used for studing the (eco)toxicological effects of chemicals (pesticides, heavy metals), e.g.: artificial soil and filter paper contact (Edwards, 1983; Edwards, 1984); artisol (Ferrière *et al.*, 1981); immersing in diluted solutions of chemicals (Martin and Wiggans, 1959), injection of chemicals through the mouth (Stringer and Wright, 1976) or into the body cavity (Stenersen, 1979); immunotoxicity (Rodrigues-Grau *et al.*, 1989) or neurological assays (Drewes *et al.*, 1988; Drewes and Lingamneni, 1992). These tests are mostly designed to measure toxic effects (mortality) on earthworms as the main endpoints. Statistical treatments are used to evaluate some critical values for LC_{50} (concentration of a chemical substance which killed 50% of the test earthworms during the test period) or NOEC (no observed effect concentration). They are designed to contribute to the prediction of effects of chemicals on agro-ecosystems and they must lead to the evaluation, under standardized conditions, of deleterious biological consequences of chemicals. From an ecological or agronomical view point, these tests are only models of predictions which must be validated in the field (Bouché, 1992; Ramade, 1992; Abdul Rida, 1994). In addition, the major part of these methods present obvious disadvantages and shortcomings (Reinecke, 1992; Edwards and Bater, 1992). These tests expose earthworms to chemicals with very little relationship to what happens in the field. They also have to provide an adequate substrate and food for the acute and chronic tests (Kokta, 1992; Römbke *et al.*, 1992); the foods used in most toxicological test methods (such as cow or horse dung or mashed vegetables) are non-synthetic and very variable, i.e. non-reproducible.

Chronic tests need to select the earthworm species, the test conditions, the substrate types and the food. The choice of earthworm species is discused in depth by Bouché (1992) and Edwards and Coulson (1992). The laboratory test conditions and substrate types are described in the papers mentioned above. While the food is the critical point it is also necessary to state the conditions precisely. The interactions between chemical, food and substrate are time-dependent and could interfere with the direct effect of chemicals on the earthworms tested. The monitoring of these interactions was the key factor which led to our choice of medium materials for earthworm chronic tests.

In the present paper no attention is given to earthworm species and test conditions, we simply followed EEC (1982) and AFNOR (1984) organization guidelines. We focused our interest on attempts to make practical and more reproducible chronic earthworm tests. The artisol substrate (Ferrière *et al.*, 1981) is the earthworm medium chosen for all trials to minimize chemical interferences. We propose improvements for producing a

*Author for correspondence. Tel.: (33) 467 61 32 59. Fax: (33) 467 41 21 38.

Table 1. The simplified Winogradsky's solution used. Weights in mg l^{-1} of distilled water

Compound	Weight	Compound	Weight	compound	Weight
$Mg\ SO_4.\ 7H_2O$	600	$(NH_4)_2\ SO_4$	200	$Na_2HPO_4.\ 12\ H_2O$	4000
$Mn\ SO_4$	20	$Zn\ SO_4.\ 7H_2O$	20	$KH_2\ PO_4$	2100
$Fe_2\ (SO_4)_3$	80	$Cu\ SO_4$	20	$Ca\ CO_3$	2000

synthetic food made by microorganisms grown under standardized conditions. The mixed substrate and food produced are called "biosynthesol".

MATERIALS AND METHODS

The artisol test was carried out according to EEC (1982) and AFNOR (1984) guidelines. This test requires adult earthworms of the species *Eisenia fetida* to be kept for 14 days, at the end of which mortality is assessed, on a basic artificial substrate composed of (per container):

- 1425 g of glass balls, 2 cm, as a skeleton,
- 90 g (dry wt at 105°C) amorphous hydrated silica (trade mark "Livilite"),
- 215 ml deionized water.

The matrix of silica, deionized water and chemical test substance is mixed with glass balls and kneaded. This medium is placed in a test container and then 10 earthworms were introduced (which have been gut content cleared, washed, surplus water absorbed on filter paper and weighed). The containers are kept in a climatic chamber at a temperature of 20 ± 2°C in continuous darkness and aerated by satured air.

In this study, instead of deionized water as used for artisol, we used a salt mixture solution to promote microorganism growth and introduce energy into the system. The mineral compounds of this solution give a simplified Winogradsky medium (Pochon and Tardieux, 1962) present in Table 1. In our initial trial, the food was made in the container by microbial growth under septic conditions from three food sources; a natural soil solution as a microorganism inoculum and the Winogradsky's solution as a nutrient medium and an energy and nitrogen source. This last point was particularly critical, so, we varied the energy and nitrogen sources and chose three organic sources; glucose, cellulose powder and dry yeast. In order to simplify the following trials, we eliminated the soil solution.

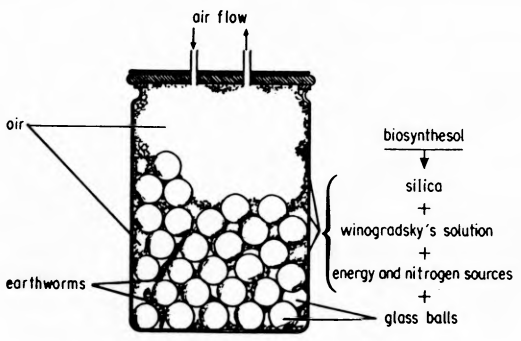

Fig. 1. The glass jar in which biosynthesol is tested.

Glass balls, silica, simplified Winogradsky solution, nitrogen and energy sources (= biosynthesol) were poured into glass jars and then well mixed (Fig. 1). This process coats glass balls and jar walls with the medium, and creats an air porosity for microorganisms and earthworms. Ten adult earthworms (*Eisenia fetida andrei*, Bouché, 1972) were introduced, initially, or after some days of incubation (depending of the trial), in each container, and the earthworm developments were followed. The organic sources induce microbial activity which produce CO_2 and change the biosynthesol pH and the test atmospheric compositions. These changes could induce earthworm mortality. After few trials, pH was used as the criterion to control the biosynthesol change. The main problem was in calibrating the organic sources, to initiate microorganism activities, and to achieve a stable medium. This can be carried out by searching for an equilibrium between quickly available and slowly biodegradable energy sources. We present in this paper three trials (Table 2) in which earthworms were introduced after 3 days, and changes were followed for 76 days.

RESULTS

Figure 2 presents the variations and stabilization of biosynthesol pH during the experiments. It shows an unstable period of 10 days in all trials and then, a trend to stabilization. Figure 3 presents the mean body weight changes of 10 adult earthworms (two replicates per trial) during the three selected trials. It shows an initial decrease and then, an increase of earthworm biomass up to 40 days. After this, earthworm growth was more or less stable.

DISCUSSION

Advances in the design of experimental approaches to toxicological earthworm testing were reviewed during the first International Workshop on Earthworm Ecology (1991) (Greig-Smith *et al.*, 1992). Earthworm acute tests provide information only concerning mortality, which was usually observed after 2 weeks. They allow the estimations of some standard relative index as such as LC_{50}, LC_5 and NOEC. Thus, food is not necessary in this type of earthworm tests. In contrast, chronic tests

Table 2. Energy and organic nitrogen sources used in the three selected trials, in g per container

Energy sources	Trial 1	Trial 2	Trial 3
Glucose	2	1.5	1
Dry yeast	2	1.5	1
Cellulose powder	2	3	4

are made to detect earthworm physiological disorders due to different chemical concentrations during the middle term. They observe the harmful effects of chemicals on cocoon production, hatchability, burrowing behaviour and flaccidity–tonicity (Texier-Pecheul, 1993). These changes must be observed over a period of 1 or 2 months, so, a food source is necessary for these chronic tests. They are carried out in conditions where the chemicals must be acting on earthworms (i.e. without uncontrolled interferences of the chemicals with test components other than the earthworms).

Thus, chronic tests need a synthetic substrate to avoid interference with the other medium components and a food source to provide the energy requisite for earthworm development. The production of an artificial food is possible out of the medium test. However, the input of this food to the substrate induces a disequilibrium due to changing physico-chemical properties of the medium and microbial activities. The food must also be well mixed with test components to avoid a substrate selection by earthworms. In order to avoid these disadvantages, food must be produced directly in the test medium. We must also wait until the new medium reaches equilibrium before earthworms are introduced. At first sight, the food production is controlled better under aseptic conditions. In fact, the absence of axenic earthworms forbids such a food production and we must work in septic conditions which induce an easy-to-reproduce method and lead us to eliminate the soil solution.

Our numerous trials were made to simplify the test components and to minimize the initial stabilization time. In such a synthetic substratum the energy-producing carbon source induces microorganism activities which, in turn, produce much CO_2. This last decrease medium pH and expose earthworms to harmful effects. This CO_2 production is particularly accelerated by glucose, an easily biodegradable organic matter. However, glucose is needed for its starter role for microorganism growth. We must also provide a slowly biodegradable energy source which remains available to microorganisms at least for some weeks. For this, cellulose powder was used as the slowly biodegradable energy source. Dry yeast was also added to the medium as a nitrogen source. An equilibrium between fast food production, minimal stabilization time and availability of nitrogen and energy sources during the test period is essential. Trials 1, 2 and 3 have been allowed us to choose these factors. The composition of the "food" in trial 2, and better, trial 3 gave us a short initial stabilization time and good earthworm weight control.

The biosynthesol, made from glass balls, levilite, simplified Winogradsky's solution and energy and nitrogen sources, yielded a synthetic and convenient medium for earthworm chronic tests. This biosynthesol is practical and easy to make. It allows chronic deleterious effects to be studied with a good reproducibility, a cost–benefit efficiency by serial planning, and an appraisal of ecosystem risk through a relative index of susceptibility.

Microbial development in the biosynthesol induces changes in gas pressures and pH (Fig. 4). These changes affect both, earthworm physiological activities and the direct chemical–earthworm inter relationships. Despite the great simplification of the biosynthesol substrate, in comparison with the various soil materials and complex natural food media used by other workers, some interference is unavoidable. These effects could be reduced if the time of interference is reduced. This could be done by changing the biosynthesol and chemical period-

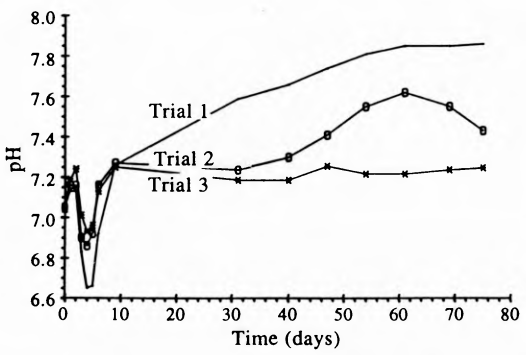

Fig. 2. Evaluation of pH values in the three selected trials.

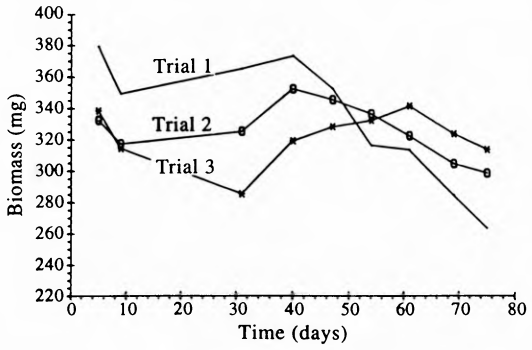

Fig. 3. Mean earthworm weight changes in the three selected trials.

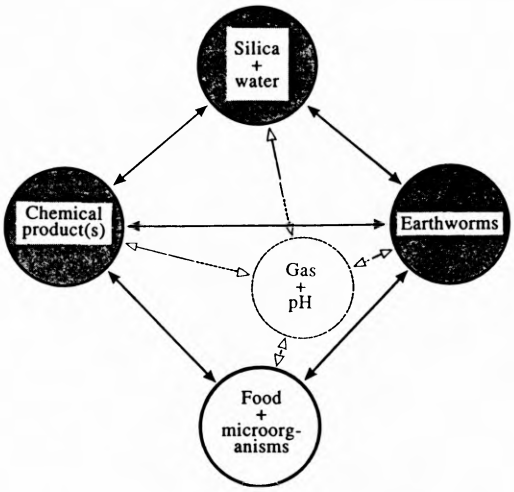

Fig. 4. Interactions between chemicals and substrate components in artisol (gray) and in biosynthesol (gray and white).

ically, but keeping the same earthworm individuals (Fig. 5). The frequency of change would depend on the interference of the specific chemical with the biosynthesol, and could be detected, for instance, by pH variations. It should be noted that these periodic medium changes allow biological effects, such as earthworm growth, time to reach sexual maturity and cocoon production, to be monitored.

The often claimed need of "ecological realism" in the design of microcosm-based toxicological tests does not fit with the need for the test to display reproducibility, economy, significance, easy of handling, and reduced or no interference. Microcosms that offer these advantages are not themselves models of the ecosystem, but serve only as tools to evaluate chemical effects in standard conditions. This is true for all earthworm laboratory tests. The aim of such tests is just to build a relative scale of earthworm chronic susceptibility to a given series of chemicals under comparable conditions (Bouché, 1984). Ecological realism is only

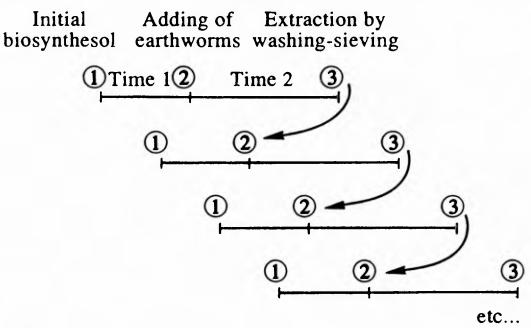

Fig. 5. Changes of chemical and biosynthesol during chronic tests. Time 1 = stabilization period. Time 2 = period of earthworm chemical exposing. Arrows indicate earthworm transfer to new biosynthesol and associated physiological controls.

achieved if we make studies in ecosystems (Abdul Rida, 1992; Abdul Rida and Bouché, 1995). Ecotoxicological studies ultimately aim at a protection of the environment which can only be assessed in the field.

Acknowledgements—Financial support for this study has been provided by French Ministry of Environment.

REFERENCES

Abdul Rida A. M. M. (1992) Biosurveillance de la contamination du sol: apport de l'étude des lombriciens à l'évaluation des risques liés aux éléments traces. *Documents Pédozoologiques* **1,** 1–233.

Abdul Rida A. M. M. (1994) L'écotoxicologie terrestre est sans "éco". *Ecologie* **25,** 140–142.

Abdul Rida A. M. M. and Bouché M. B. (1995) The eradication of an earthworm genus by heavy metals in southern France. *Applied Soil Ecology* **2,** 45–52.

AFNOR guideline (1984) Déterlination de la toxicité d'une substance *vis-à-vis* des lombriciens (espèce *Eisenia fetida*), méthode artisol. *Norme* X31-250, 1–5.

Blandin P. (1986) Bioindicateurs et diagnostic des systèmes écologiques. *Bulletin d'Ecologie* **17,** 215–307.

Bouché M. B. (1984) Ecotoxicologie des lombriciens. I. Ecotoxicité contrôlée, *Acta Oecologica. Oecologia Applicata* **5,** 271–287.

Bouché M. B. (1992) Earthworm species and ecotoxicological studies. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 20–35, Intercept, Andover.

Drewes C. D. and Lingamneni A. (1992) Use of earthworms in eco-neurotoxicity testing: sublethal effects of carbofuran in *Lumbricus terrestris* L. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 63–72. Intercept, Andover.

Drewes C. D., Vining E. P. and Callahan C. A. (1988) Electrophysiological detection of sublethal neurotoxic effects in intact earthworms. In *Earthworms in Waste and Environmental Management* (C. A. Edwards and E. F. Neuhauser, Eds), pp. 355–366. SPB Academic Publishing, The Hague.

Edwards C. A. (1983) Development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms, pp. 1–141. Commision of the European communities, Doc EUR 8714 EN.

Edwards C. A. (1984) Report of the second stage in development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms, pp. 1–99. Commision of the European communities, Doc EUR 9360 EN.

Edwards C. A. and Bater J. E. (1992) The use of earthworms in environmental management. *Soil Biology & Biochemistry* **24,** 1683–1689.

Edwards P. J. and Coulson J. M. (1992) Choice of earthworm species for laboratory tests. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 36–43. Intercept, Andover.

EEC guideline (1982) Toxicity for earthworms, artisol tests. EEC Directive 79/831 Annex V, part C, level 1, DG XI/129/82. 1–9.

Ferrière G., Fayolle L. and Bouché M. B. (1981) Un nouvel outil, essentiel pour l'écophysiologie et l'écotoxicologie, l'élevage des lombriciens en sol artificiel. *Pedobiologia* **22,** 196–201.

Greig-Smith P. W., Becker H., Edwards P. J. and Heimbach F. (Eds) (1992) *Ecotoxicology of Earthworms*, pp. 1–269. Intercept, Andover.

Kokta C. (1992) Measuring effects of chemicals in the laboratory: effect criteria and endpoints. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 55–62. Intercept, Andover.

Martin L. W. and Wiggans S. C. (1959) The tolerance of earthworms to certain insecticides, herbicides and fertilizers, pp. 1–334. Oklahoma State University Experiment Station. Processed Series.

Pochon A. and Tardieux P. (1962) *Technique d'Analyse en Microbiologie du Sol.* La Tourelle Publication, Saint Mandé.

Ramade F. (1992) *Précis d'Ecotoxicologie.* Masson, Paris.

Reinecke A. J. (1992) A review of ecotoxicological test methods using eathworms. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 7–19. Intercept, Andover.

Rodriguez-Grau J., Vanables B. J., Fitzpatrick L. C., Goven A. J. and Cooper E. L. (1989) Suppression of secretory rosette formation by PCBs in *Lumbricus terrestris*: an earthworm assay for humoral immunotoxicity of xenobiotics. *Environmental Toxicology and Chemistry* **8,** 1201–1207.

Römbke J., Vickus P. and Bauer C. (1992) Experiences and problems with the OECD-earthworm acute test in routine testing. In *Ecotoxicology of Earthworms* (P. W. Greigh-Smith, H. Becker, P. J. Edwards and F. Heimbach, Eds), pp. 209–212. Intercept, Andover.

Salanki J., Jeffrey D. and Hughes G. M. (Eds) (1994) *Biological Monitoring of the Environment: a Manual of Methods.* CAB International, Wallingford.

Stenersen J. (1979) Action of pesticides on earthworms. Part I: The toxicity of cholinesterase-inhibiting insecticides to earthworms as evaluated by laboratory tests. *Pesticide Science* **10,** 104–112.

Stringer A. and Wright M. A. (1976) The toxicity of benomyl and some related 2-substituted benzimidazoles to the earthworm Lumbricus terrestris. *Pesticide Science* **7,** 459–464.

Texier-Pecheul C. (1993) Etude expérimentale de divers modes d'action de l'aldicarbe sur *Aporrectodea caliginosa* (Oligochaeta, Lumbricidae) Ph.D. thesis, University of Rennes, France.