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Modifications to a defined medium for the study of the biology and toxicology of the earthworm *Eisenia fetida* (Oligochaeta)

Henk Bouwman *

School for Environmental Sciences and Development (Zoology), North-West University (Potchefstroom Campus), Private Bag X6001, Potchefstroom 2520, South Africa

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Abstract

Defined and standardised media for the investigation of growth and reproduction of earthworms from a juvenile stage have been developed, but still retain a measure of components that are difficult to characterise completely. A defined medium, based on vermiculate, cellulose, humic acid and other components that are commercially available, and that supports growth and reproduction of *E. fetida*, added as juveniles, has previously been developed. However, one of the nutrients is not available anymore, and modifications were required. A series of various combinations were evaluated using commercially available nutrients such as fatty acids, organic phosphates and oxy-humic acids. All of the media supported growth (excepting one containing creatinine), but cocoons were only produced in some. It was shown that the growth and reproduction of *E. fetida* could be studied in a defined medium for up to 70 days (starting with 20-day old juveniles) without additional feeding. The various defined media reported here can be adopted to study specific aspects of earthworm biology and ecotoxicology in the laboratory, but would not replace the existing OECD methods.

Keywords: *Eisenia fetida*; Defined medium; Juvenile test; Growth; Reproduction; Earthworms

1. Introduction

Earthworms are an important ecological component of many soils, and consequently, a wide variety of tests has been developed to determine the effects of pollutants on these animals (Edwards and Bohlen, 1996). The OECD artificial-soil test to determine the toxicity of substances to earthworms (OECD, 1984; Edwards, 1984) is well established and has been in place for decades. In this test, 10 adult earthworms (*Eisenia fetida*) are exposed for 14 days to chemicals in a standardised medium consisting of a mixture of finely ground sphagnum peat (10%), kaolinitic clay (20%), industrial sand (69%), and calcium carbonate (1%), with a water content between 25 and 42% of the dry weight. The worms should weigh between 0.3 and 0.6 g, should be at least 2 months old and have clitella (OECD, 1984). Further work has been done on the artificial soil test to allow the assessment of effects on of sub-lethal levels of pollutants on growth and reproduction, mainly by food supplementation (alfalfa or manures) to soils or artificial soils, placed either on top, in holes, or mixed into the media (Van Gestel et al., 1991; Gibbs et al., 1996; OECD, 2004). In the OECD (2004) earthworm reproduction test, the 10 clitelate worms to be added (from a synchronised culture between 2 and 12 months old) should weigh between .25 and 0.6 g.

However, the reproducibility of standardised or artificial soils as media sustaining growth and reproduction of earthworms for sub-lethal laboratory tests,

* Tel.: +27 18 2992377; fax: +27 18 2992503.
E-mail address: drkhb@puk.ac.za.

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has been an issue addressed in one way or another, by various authors (Arnaud et al., 2000; Spurgeon et al., 2003; Kuperman et al., 2004; Van Gestel and Weeks, 2004; Spurgeon et al., 2004). Ideally, a defined medium should contain pure, well-characterised compounds that are commercially available. A defined medium that allowed the study of growth and reproduction of juvenile *E. fetida* over 50 days, has been developed and tested using carbofuran at sub-lethal levels (Bouwman and Reinecke, 1987; Bouwman and Reinecke, 1991). The original medium (Bouwman and Reinecke, 1991) consisted of an inorganic vermiculite matrix, with cellulose as the main carbohydrate source. Other defined nutrients were milk casein, DNA (salmon), vitamins, and humic acid. Growth and reproduction were supported for up to 56 days after addition of 20-day old worms, with a cocoon production rate of 0.239 cocoons per day, when compared to a rate of 0.546 cocoons per worm per day for controls kept on cow manure.

The initial medium that was developed (Bouwman and Reinecke, 1991), because of its well-defined nature, would be useful as a reference medium, not only for ecotoxicological studies, but also to investigate the negative or positive effect of any other substance such as for waste material conversion, or biological investigations of *E. fetida* or other worms. The usefulness of this medium, however, depends on the availability of the components used. Since the publication of the two papers (Bouwman and Reinecke, 1987, 1991), some products have become unavailable. One of these was raw DNA extracted from salmon. The cost of alternative DNA products prohibited the use of these at the amounts required (0.8 g per container). Bouwman and Reinecke (1991) speculated as to the contribution of this compound on growth of the worms. It has been found that riboflavin, for which guanine is a precursor, is present in large amounts in the coelomic fluid of *E. submontana* (Wenig and Kubista, 1949). It might be that the guanine, N, phosphate or carbohydrate residues of the DNA were responsible for the increase in growth rate, more than might be expected from its energy contribution alone (Bouwman and Reinecke, 1991). This was partially supported by the findings of active assimilation of microbial biomass, which of course contains DNA (Neuhauser et al., 1980b; Flack and Hartenstein, 1984). The availability of water-soluble vitamins also turned out to be problematic.

The use of a medium that supports growth and reproduction of earthworms allows the measurement of weight gain or loss, clitellum development, onset and rate of cocoon production, and the number of cocoons and hatchlings. One of the recommendations of the 1st International Workshop on Ecotoxicology of Earthworms (Grieg-Smith, 1992), and repeated at the 3rd (Van Gestel and Weeks, 2004), was that juvenile worms should be considered for use in toxicological tests. Growth would then be an endpoint, with the additional advantage that weight changes, due to cocoon production, do not complicate analysis. The use of adult worms as in the OECD standard test (OECD, 1984), with a varying rate of cocoon production (Venter and Reinecke, 1988), would also complicate the interpretation of cocoon data. From the outset, the initial defined medium (Bouwman and Reinecke, 1987) was developed with juvenile worms in mind, and therefore had to accommodate growth and reproductive requirements over a period much longer than 14 or 28 days, without the addition of supplemental food, that could otherwise complicate interpretation of results.

This paper reports on the modification of the initial defined medium to accommodate the problems experienced with the availability of its constituents. The aim was also to simplify the components, in a series of five investigations, to identify commercially more readily available organic phosphates to replace DNA, and to eliminate the vitamins. The opportunity was also taken to investigate the effect of combinations of different components, as well as to add substances such as fatty acids (as suggested by Bouwman and Reinecke, 1991) and another type of humic acid, to investigate its effects on growth and reproduction.

2. Materials and methods

All experiments were carried out at 25°, in darkness, with an RH of about 75%, using wide-mouthed 1 l round glass jars (115 mm × 110 mm), with a copper screw lid with a single hole. MCU grade commercial vermiculite from Micronised Products (South Africa), with a water holding capacity of 0.48 ml g^{-1} was used as a matrix throughout (Bouwman and Reinecke, 1991). The protocol of treatments is presented in Table 1. Defined medium 1 (DM1) was considered as the base medium. All treatments were done concurrently in triplicate.

The organic components used were: cellulose, Merck art. 102330; casein, BDH 44018; DNA, Merck art. 42027; humic acid, sodium salt, Aldrich H1,675-2; stearic acid, Merck art. 800673; palmitic acid, Merck art. 800508; lecithin (phosphatidyl choline) (soyabean), BDH 29863; β glycerophosphate disodium salt pentahydrate, Merk art. BB103953M; ATP Sigma A3377; Phytin (calcium inositol hexaphosphate), BDH 38045 (alternative Sigma P 8810); oxy humic acid as liquid