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Original article

Effects of fluoranthene on the growth, bioavailability and anti-oxidant system of *Eisenia fetida* during the ageing process

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ABSTRACT

Studies were conducted to assess the effects of certain concentrations of fluoranthene on the growth, bioavailability, and anti-oxidant system of *Eisenia fetida* during the ageing process. The concentration of fluoranthene in the soil decreased rapidly during the first 25 days (27.0% decrease) followed by a slower decrease in the next 90 days (11.2% decrease). The increase in the weight ratio (WR) was initially rapid and then slowed with ageing in a manner consistent with the rate of decrease in fluoranthene concentration. The bioconcentration factor (BCF) and the concentration of fluoranthene in the earthworms' epidermis and intestines increased prior to 25 days but decreased thereafter. This result confirmed that the bioavailability of fluoranthene decreases as time increases, except when increased toxicity caused the earthworms to die at the beginning of the experiment. The fluoranthene concentration and the BCF in earthworms' intestines were higher than those in the epidermis throughout the whole experiment, which suggested that earthworms accumulate fluoranthene mainly by ingesting it with soil particles rather than by penetrating it through their epidermis. The effects of fluoranthene on anti-oxidative enzyme activity (superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, POD and polyphenol oxidase, PPO) and malondialdehyde (MDA) content in earthworms were also investigated. The activities of SOD, POD, and PPO and the content of MDA decreased gradually, but the activity of CAT decreased initially and then increased thereafter. The WR, POD, and PPO sensitivities of the earthworms were closely related to the decrease in fluoranthene concentration, while the sensitivity to SOD lagged behind.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental contaminants that mainly originate from the incomplete combustion and pyrolysis of organic matter [36]. Human activities, especially the combustion of fossil fuel, the production of automobile exhaust and the refining of coal, significantly increase the quantity of PAH in the environment [19]. Because these pollutants exert toxic, mutagenic, and carcinogenic effects, their presence in soil represents a public health hazard and attracts great concern [12]. Fluoranthene, a typical representative of PAH, is listed as a priority control organic pollutant by the U.S. Environmental Protection Agency [29]. The PAH in the soil in southern China are mainly in 3 and 4 annuluses which include naphthalene, phenanthrene, fluoranthene and benzofluoranthene [8]. When PAH enter the soil, they undergo a number of loss or transport processes, such as volatilization, leaching, and chemical and biological degradation. In addition, sorption- and transport-related processes may make the chemicals become increasingly solvent non-extractable or 'irreversibly bound' within the soil matrix. As the soil–PAH contact time increases, there is a corresponding decrease in the extractability and bioavailability of PAH from the soil. This phenomenon has been termed the "ageing effect" [34].

Earthworms have relatively high body-lipid content, and a variety of PAH have been detected in their tissues. There have been a number of studies that use accumulation in earthworms as an assay of PAH bioavailability [25,33,41]. Generally, earthworms accumulate contaminants through two major routes. The first route is simple and passive diffusion through the epidermis from aqueous phases. The second involves the ingestion of contaminants together with soil, resulting in the diffusion of the contaminants across the gastrointestinal tract and accumulation in the lipid-rich tissues [23,40]. The bioavailability of PAH to earthworms has been

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widely investigated, but research on the bioavailability of PAH to the epidermis and intestine of earthworms is extremely limited. However, this research is becoming increasingly important because it can provide information regarding the way in which earthworms absorb pollutants.

Biochemical responses in organisms against environmental stress are regarded as early warning indicators of pollution in the environment. Enzymes involved in detoxification can be used as important biomarkers for environmental contamination. These enzymes (SOD, POD, CAT and PPO) possess anti-oxidant activities and can protect cells against the adverse effects of reactive oxygen species (ROS). Saint-Denis et al. (1998) found that the enzymes CAT, glutathione peroxidase, glutathione S-transferase, and glutathione reductase are involved in anti-oxidant defence systems in *Eisenia fetida* and are mainly localized in the cytosol [37]. However, information on the effect of fluoranthene on anti-oxidative enzymes in earthworms is lacking.

The objectives of the present study were to (1) determine the ageing rate of residual fluoranthene in soil under sterile conditions, (2) examine fluoranthene bioaccumulation in the epidermis and intestine of earthworms at different time intervals, and (3) investigate the effects of ageing fluoranthene on anti-oxidant enzyme activities and MDA content in earthworm tissues.

2. Materials and methods

2.1. Materials

2.1.1. Soil samples

Yellow-brown soil at a depth of 0–20 cm was obtained from Nanjing, Jiangsu Province in China. The soil was air-dried for 15 days and passed through a 2-mm sieve to remove stones and roots. The physical and chemical properties of the soil are shown in Table 1.

The pH of the soil was determined using a calibrated pH electrode in water (1:2.5 soil:water). Organic matter in the soil was analyzed by the $K_2Cr_2O_7$ oxidation method. The soil CEC was measured by the CH_3COONH_4 method. The clay content was determined by sedimentation using 40 g of air-dried soil. All of the measurements were performed using standard laboratory techniques [28].

2.1.2. Earthworm samples

Adult earthworms (*E. fetida*) with well-developed clitella were obtained from the Nanjing Special Animal Factory in China. They were laboratory-bred in the soil at 20 °C in the dark and fed on cattle manure, and the soil moisture (70% of water-holding capacity) was balanced with tap water. All earthworms were allowed to acclimatize to laboratory conditions for at least 2 weeks before they were used in the experiments. Earthworms with approximately the same age and size (0.25–0.30 g fresh weight, 6–8 cm long) were used in the accumulation tests.

2.1.3. Reagents

Fluoranthene (purity ≧ 98%) was purchased from Alfa Aesar Corporation. Methanol was of chromatographic purity, and other reagents for enzyme activity measurements were analytically pure.

Table 1
Physiochemical properties of soil.

Soil type	pH	TOC ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	Clay (%)	Sand (%)	Silt (%)
Yellow-brown soil	6.12	15.3	20.6	24.7	13.4	61.9

2.2. Experimental design

2.2.1. Soil contamination

Soil (2.4 kg) was divided evenly among 24 narrow-mouthed glass bottles (150 mL), with 100 g of soil per bottle, and immediately sterilized using r-irradiation (2.5 Mrad) with a ^{60}Co source [2]. Under sterile conditions, fluoranthene was dissolved in acetone, added by droplets to the soil, well shaken, and after mixing, volatilized fully overnight to produce an initial fluoranthene concentration of 150 $mg\ kg^{-1}$ (dry mass of soil). Sterile water was added to give a moisture content of 70% of water-holding capacity (WHC), which was suitable for earthworm incubation [7]. The bottles were sealed with paraffin and placed in the dark at 22 ± 2 °C to allow for ageing throughout the test period. Each treatment was performed using four replicates. These treatments were sampled destructively, and sub-samples (3.0 g) were collected for fluoranthene analysis at 0, 10, 25, 45, 75, and 115 days. At each of the time points, no clones emerged on trypticase-soy agar, confirming the sterility of the soil.

2.2.2. Bioavailability experiments

At 0, 10, 25, 45, 75, and 115 days after sampling, the remaining soil samples were transferred into 150 mL Erlenmeyer flasks for cultivation of earthworms. Ten adult earthworms of similar ages and sizes were placed into a 150-mL Erlenmeyer flask that was sealed with foil, and eight pinholes were made in the foil to allow for aeration. Sterility was no longer maintained, and no additional food was added to feed the earthworms. There were 240 individuals in total. After being incubated in the dark for 7 days at 22 ± 2 °C, five earthworms were selected, rinsed with distilled water, and placed on moist filter papers for 24 h to empty their intestines. They were then dissected in wax cylinders, and the epidermis and intestine were separated. Each sample was mixed with anhydrous sodium sulphate in an amount three times the sample weight and then crushed using a mortar and pestle. The ground earthworm tissues were packed in filter paper and stored at 4 °C for extraction. The remaining earthworms were used to determine other biochemical indicators, such as enzyme activities and MDA content.

2.2.3. Fluoranthene concentrations in soil and in earthworms: Soxhlet extraction

For Soxhlet extraction, a sample of soil (3.0 g) or worm tissue was introduced into a cellulose extraction thimble (25 mm × 80 mm, Whatman International, Maidstone, England) and extracted for 10 h with 100 mL of dichloromethane and hexane (1:1 v/v, HPLC grade) in Soxhlet extractors at a rate of 5–6 min $cycle^{-1}$. The extracts were concentrated to near dryness in a rotary evaporator at 45 °C, and the residue was dissolved in 5 mL of methanol. This solution was passed through a 0.22-μm Teflon filter to remove particulate matter prior to analysis.

2.2.4. Determination of fluoranthene concentration: high performance liquid chromatography (HPLC)

The HPLC system was equipped with a UV–visible detector (LC-20AT, SPD-20A/20AV) and a reverse-phase C_{18} column (4.6 mm × 250 mm). The mobile phase was 100% methanol with a flow rate of 1.0 mL min^{-1}. The wavelength used for detection was 254 nm, and the injections were 20.0 μL. The HPLC column was heated constantly at 30 °C.

2.2.5. Determination of enzyme activity and MDA content

Earthworms were placed into a pre-chilled mortar and pestle under ice-cold conditions in a 50 mmol L^{-1} potassium phosphate buffer (1:8 w/v), pH 7.0. The homogenate was centrifuged at 10,000 rev min^{-1} at 4 °C for 10 min. The supernatant was used to assay enzyme activity and to determine the MDA content.