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Growth and biochemical responses of earthworms (*Dendrobaena veneta*) exposed to tetracycline

ŽALTAUSKAITĖ J. [*]**, MIŠKELYTĖ D., SUJETOVIENĖ G., DIKŠAITYTĖ A., JANUŠKAITIENĖ I., KACIENĖ G., DAGILIŪTĖ R.**

Vytautas Magnus University, K. Donelaičio 58, LT-44248 Kaunas, Lithuania

*corresponding author:
e-mail: e-mail: jurate.zaltauskaite@vdu.lt

Abstract Veterinary antibiotics have received growing attention in recent years as emerging terrestrial contaminants. Livestock, animal manure and slurry land application are the main routes for veterinary antibiotics to enter the soil environment. Widespread use and distribution of tetracyclines poses a serious risk to soil dwelling organisms. Being soil keystone species, earthworms perform a number of essential functions in the soil and occupy important position in the terrestrial food chain. However antibiotic effects on earthworms are still poorly understood. Earthworms *Dendrobaena veneta* were exposed to tetracycline for 56 days. Mortality, body weight, and biochemical responses including the activity of antioxidant enzymes (catalase, superoxide dismutase, gluthathione-S-transferase) and oxidative damage (lipid peroxidation) were investigated. Tetracycline did not induce acute lethal toxicity to *D. veneta*, though impaired weight growth, antioxidant enzymes system and induced oxidative stress.

Keywords: antioxidant system, earthworms, mortality, growth, tetracycline

1. Introduction

Antibiotics are important tools to prevent, control and treat diseases in food animal production. Wastewater, sewage sludge and manure soil application are the major antibiotic entrance sources into environment (Grenni et al. 2018). Although antibiotics are less persistent in the environment than classical persistent pollutants (Daughton and Ternes 1999), high biological activity at low concentrations and increasing release pose significant risk to soil dwelling and aquatic organisms.

The most frequently used veterinary antibiotics in Europe were tetracyclines accounting for approximately 37% of total consumption (Carvalho and Santos 2016). Tetracyclines are broad spectrum antibiotics having vast veterinary and aquaculture applications. Approximately 25-90% of the dose admitted is excreted via urine and feces (Daghrir and Drogui 2013). Due to their extensive usage, solubility in water, low octanol-water partition coefficient (K_{ow}) and higher adsorption capacity tetracyclines residues widely occur in the environment.

The presence of tetracyclines has been reported in surface and ground waters (MacKie et al. 2006; Xu et al. 2021), sediments (Luo et al. 2011) and soils, especially in those amended with manure (Hamscher et al. 2002; Hu et al. 2010).

Despite the widespread tetracycline distribution in the environment, information of its short-term and long-term effects on soil dwelling organisms is lacking. Being the soil keystone species, earthworms play a significant role in the soil community structure and ecosystem functions. Since earthworms are a significant part of the diet of many terrestrial vertebrate species, they are an important vector of pollutants transfer in terrestrial food web. Earthworms are recognized as relevant bioindicators of soil quality and health and are extensively used in ecotoxicological bioassays. The aim of this study was to assess tetracycline chronic toxicity to earthworm *Dendrobaena veneta* by measuring earthworm mortality, growth and biochemical response.

2. Materials and methods

Chronic toxicity of tetracycline (TC) to *D. veneta* earthworms was assessed according to the modified OECD guidelines for the testing of chemicals (OECD 222).

The air-dried constituents of artificial soil (70 % quartz sand, 20 % kaolin clay and 10 % Sphagnum peat) were mixed thoroughly and weighted (500 g) into plastic containers. Soil was spiked with solutions of tetracycline (Apollo Scientific, Cheshire, UK) to obtain the final required water content (50 % of the maximum water holding capacity) and TC concentrations in soil: 0 (control), 1, 10, 100, 250, 500 and 750 mg kg^{-1}. Each treatment was prepared in triplicate.

Ten adult earthworms with well-developed clitellum and of similar weight were added to each covered container. The earthworms were exposed to TC for eight weeks at 20 ºC under constant light (600 lux). The water content in each container was checked weekly. The earthworms were weekly fed with oatmeal (approximately 0.5 g per earthworm). Survival and body weight were measured on the 4^{th} and 8^{th} week by counting and weighing the surviving earthworms in each container. The earthworms were considered alive if they were able to respond to mechanical stimulus. After eight weeks of exposure, the earthworms were removed from the soil, cleaned and placed on moistened filter paper in Petri dishes for 48 h to void their gut content. After the depuration, the earthworms were frozen at -80°C until further chemical analysis.

All biochemical analysis procedures were carried out at 4°C. Earthworms tissues were homogenized with TRIS buffer (pH 7.4) containing 1 mM EDTA and 250 mM sucrose and 1 mM DDT. The homogenates were centrifuged at 10000g for 15 min at 4 °C and the supernatant was used for further analysis of proteins, antioxidative enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and gluthathione-S-transferase (GST) and lipid peroxidation. Protein concentration was determined according to the method of Lowry et al. (1951).

SOD activity was determined according to Dhindsa et al. (1981) by measuring the inhibition of NBT reduction. CAT activity was assayed by monitoring H_2O_2 decomposition at 240 nm (Aebi 1984). GR activity was measured as NADPH oxidation in the presence of 0.2 mM NADPH (Murshed et al. 2008), GST activity was measured by the methos described by Habig et al. (1974). The content of malondialdehyde (MDA), the biomarker of lipid peroxidation, was estimated by formation of thiobarbituric acid reactive substances according to the method described by Murshed et al. (2008). All measurements were made using SPECTROstar® Nano microplate reader (BMG LABTECH, Offenburg, Germany).

A one-way analysis of variance (ANOVA) was used to assess the concentration effect on measured parameters. Significant differences between treatments were determined by Fisher's Least Significant Difference (LSD) test. The differences were considered significant if $p < 0.05$. All analyses were performed by STATISTICA 8 software.

3. Results and discussion

TC had no significant effect on *D. veneta* survival during the first four weeks of exposure to TC (ANOVA, F=1.34, p>0.05), though after eight weeks of exposure mortality was recorded from 50 mg kg^{-1} TC concentration (Fig. 1). The survival of earthworms in the treatments of 250-500 mg kg^{-1} of tetracycline has fallen to 30 % implying high lethal TC toxicity. Our data are in line with the results of other studies showing no acute lethal TC toxicity to earthworms (Dong et al. 2012; Havelkova et al. 2016). As chronic TC toxicity to earthworms was not studied, we cannot compare our results.

No weight loss was observed during the whole period of earthworms' exposure to TC, however the weight gain in TC treatments was somewhat lower compared to the control.

Tested TC concentrations had only minor inhibitory effect on the fresh weight of *D. veneta* (Fig. 2). The fresh weight was reduced by 8-11.5 % in the treatments with 50-500 mg kg^{-1}.

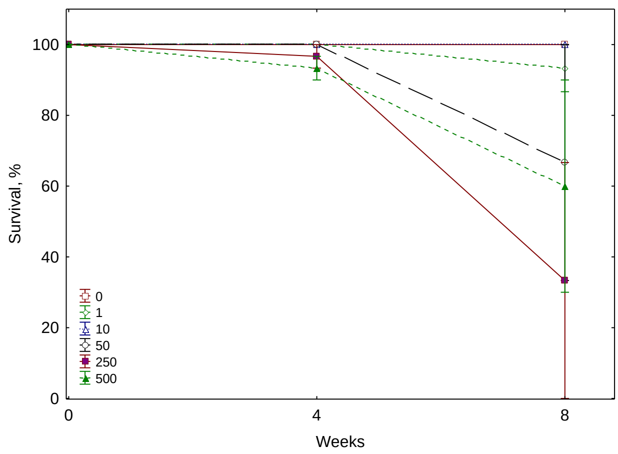

Figure 1. Survival of *D. veneta* exposed to tetracycline for four and eight weeks

Earthworm fresh weight growth corresponded well with the data of survival. Weight reduction was observed in those treatments where lethal toxicity was determined as well.

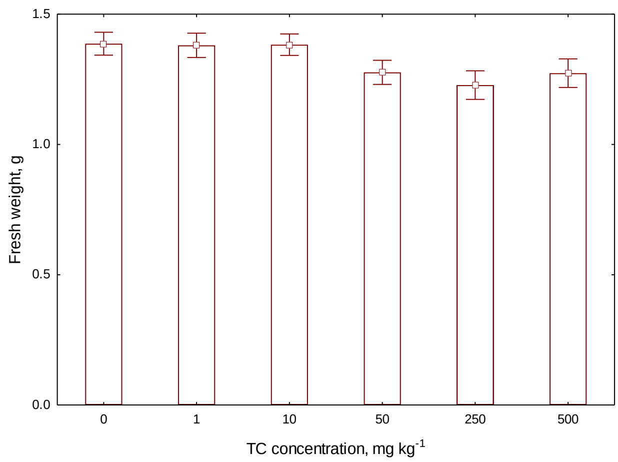

Figure 2. Fresh weight of *D. veneta* exposed to tetracycline for eight weeks

Low TC toxicity to earthworm survival and growth could be partly attributed to poor TC absorption in the gut of animals and relatively rapid biodegradation in the soil (Conde-Cid et al. 2020; Pino et al. 2015).

The antioxidant system assessment indicated that CAT and SOD exhibited similar responses to TC exposure (Fig. 3). Slight stimulation of CAT and SOD, by 14.7% and 10.3%, respectively, compared with the control was found in the treatment of 1 mg kg^{-1}. Exposure to 50-250 mg kg^{-1} of TC resulted in significantly suppressed SOD activity (up to 34%, $p<0.05$), while at 500 mg kg^{-1} it rose again to higher than control level. The activity of CAT was also suppressed at 250 mg kg^{-1}, and reached the control level at 500 mg kg^{-1}, as well. The activity of GST was suppressed in all TC treatments (by 34.4-46.5%), though GST did not vary with TC concentration. No stimulation of CAT and SOD was observed after *Eisenia fetida* exposure to TC for

28 days (Dong et al. 2012). Chlortetracycline, another first generation tetracycline, has stimulated CAT and SOD enzyme activities in *E. fetida* (Lin et al. 2012). GST activity was reduced in all TC treatments, reaching only 53.5% of the control at the highest 500 mg kg^{-1} concentration.

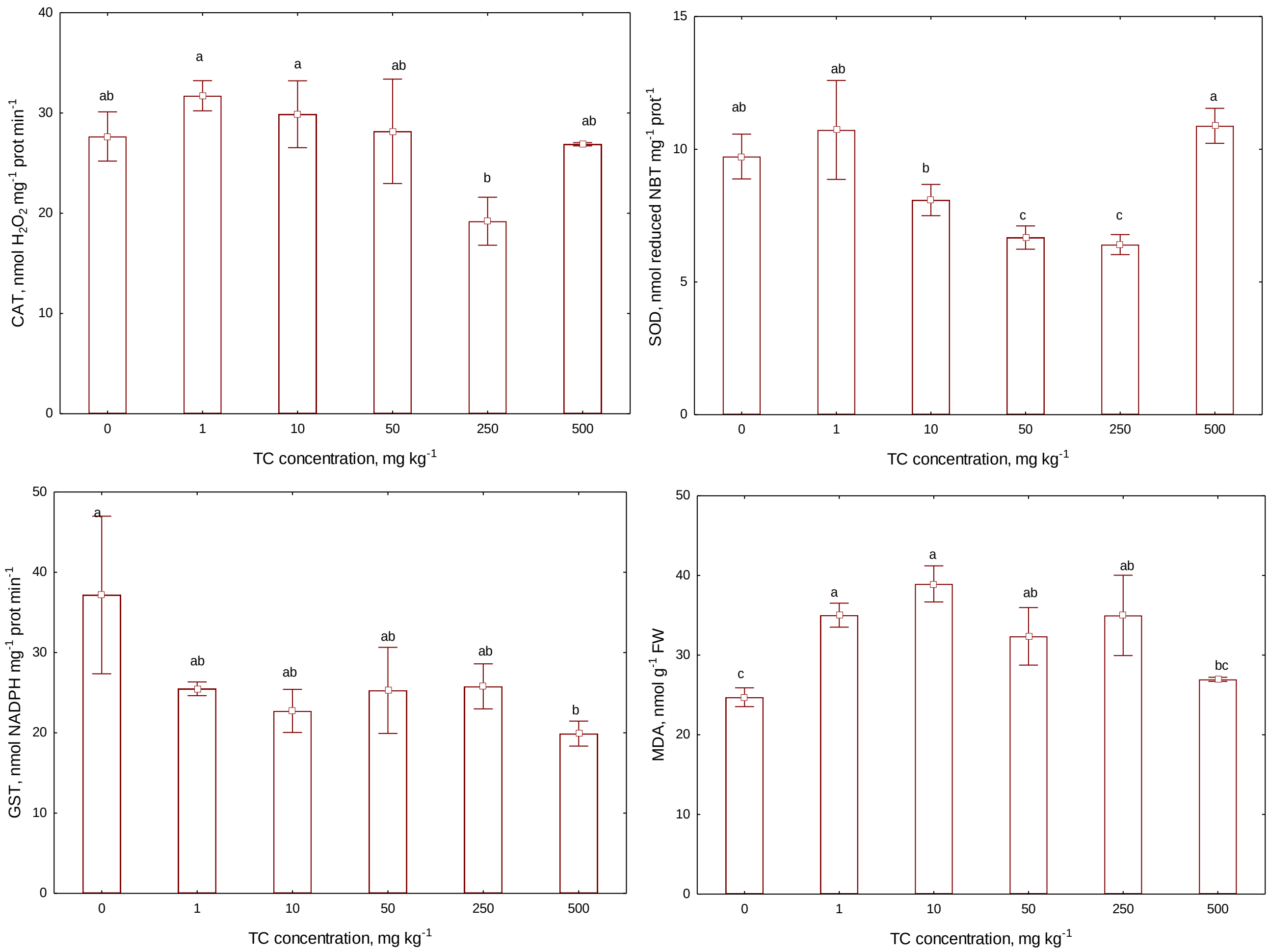

Figure 3. Activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and gluthathione-S-transferase (GST) and concentration of malondialdehyde (MDA) in *D. veneta* exposed to tetracycline for eight weeks. Different letters indicate significant difference ($p < 0.05$) among treatments (Fisher's LSD)

An induction of lipid peroxidation, assessed through the concentration of MDA, was observed, though the response was not dose dependent (Fig. 3). MDA concentrations corresponded well to the pattern of the changes in antioxidant enzymes. Slight decrease in MDA in the treatment of 500 mg kg^{-1} could be partly attributed to an increase in SOD and CAT activities. TC has been shown to induce oxidative stress in *Danio rerio* embryos exposed to TC. Though significant effect was seen only from 10 µg l^{-1} (Zhang et al. 2015).

4. Conclusions

This study has demonstrated that tetracycline has no acute lethal toxicity, though chronic exposure to tetracycline has reduced the survival of *D. veneta*. TC slightly reduced the growth of earthworms. Chronic exposure to tetracycline caused changes in antioxidant enzymes leading to oxidative damage to cell membranes.

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