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Multigenerational exposure of *Folsomia candida* to ivermectin – Using avoidance, survival, reproduction, size and cellular markers as endpoints

B. Guimarães[a,b], V.L. Maria[a], J. Römbke[b], M.J.B. Amorim[a,*]

[a] University of Aveiro, Department of Biology & CESAM, 3810-193 Aveiro, Portugal
[b] ECT Oekotoxikologie GmbH, Boettgerstr. 2-14, D-65439 Flörsheim, Germany

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ABSTRACT

In standard toxicity tests one generation of test organisms is used, and they are usually exposed only during a fraction of their life-cycle. This approach is very important but does not cover the potential effects of multi-generational (MG) exposure and may underestimate risks. Hence, the main aim of this study was to assess the MG impact of the veterinary pharmaceutical ivermectin (IVM) on *Folsomia candida* during three generations (F1–F3). Ivermectin is a veterinary medicine, persistent in the environment and toxic to non-target soil invertebrates. A suite of different endpoints was used including avoidance, survival, reproduction, size and cellular biomarkers (catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), acetylcholinesterase (AChE) and lipid peroxidation (LPO)). Survival and reproduction were affected (LC50: 40 mg/kg; EC50: 5 mg/kg), but no avoidance occurred, which poses additional ecological concern. Exposure throughout the generations showed similar toxicity in terms of survival and reproduction. Regarding size there was an impact, e.g., organisms were smaller and more abundant in F2 and larger and less abundant in F3. This can have implications in terms of risk as e.g. smaller organisms can respond differently to stress compared to larger organisms in future generations. The antioxidant mechanisms were dynamically activated along the generations, e.g. in F1 CAT was increased whereas in F3 there was increased GST activity, which resulted in damage (LPO) for F1 and F2 organisms but not for the F3 generation. The multi-endpoint approach proved to be beneficial for the interpretation of results and we recommend it, especially for persistent and/or highly adsorptive chemicals, but also endocrine disruptors. Moreover, the evaluation of size as an additional sub-lethal endpoint has significantly added to the relevance of this test. The relative proportion of small, medium and large animals may be an even more relevant aspect of this endpoint. This does not require guideline modifications and is hence easily implementable.

1. Introduction

Organisms are often exposed to contaminants during several generations although standard toxicity tests (e.g., OECD, 2009; ISO, 2004) are based on one generation, and usually exposure occurs during a fraction of the life-cycle. This is of course a good compromise for feasibility purposes but does not cover the potential effects of multi-generational (MG) exposure and may underestimate risks. Long term exposure in soils is of high concern because persistent chemicals can be deposited for long periods of time, accumulate in soil, undergo transformation, etc., while the organisms can be continuously exposed. There are still very few studies among terrestrial species that cover MG exposure, examples include the oligochaete species *Enchytraeus crypticus* (Bicho et al., 2017; Menezes-Oliveira et al., 2013), *Eisenia fetida* (Schnug et al., 2013), and the collembolan species *Folsomia candida* (Amorim et al., 2017; Campiche et al., 2007; Paumen et al., 2008). Results differed and this is not surprising since effects of multi-generational exposure of chemicals cannot be extrapolated from one endpoint to another due to biological and chemical differences.

In the present study we assessed the multigenerational effect of ivermectin (IVM), a high environmental concern parasiticide widely used in veterinary medicine. Ivermectin is partly metabolized by cattle, pigs and sheep and considerable amounts (up to 80% depending on the route of application and the treated farm animal) of the parent drug are excreted via faeces (Hennessy and Alvinerie, 2002), finally reaching the soil. Ivermectin is persistent in the environment (Kövecses and Marcogliese, 2005) and has been shown to be highly toxic to dung- (Madsen et al., 1990; Römbke et al., 2009, 2010a) and soil-inhabiting

* Corresponding author.
E-mail address: mjamorim@ua.pt (M.J.B. Amorim).

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invertebrates (Jensen and Scott-Fordsmand, 2012; Jensen et al., 2003; Römbke et al., 2010b). From standard laboratory as well as microcosm tests with ivermectin, it is assumed that collembolans are among the most sensitive soil organisms (Jensen and Scott-Fordsmand, 2012; Jensen et al., 2003; Römbke et al., 2010b). Ivermectin causes neurotransmission failure because of neuromuscular synapses interference (Ōmura, 2008), and is known to act by the interaction with glutamate-gated or γ-aminobutyric acid related chloride channels in synapse membranes (Campbell, 1985; Duce and Scott, 1985), hence behavioural effects, e.g., avoidance, are a relevant endpoint.

Therefore, we aimed to assess the effects of multigenerational exposure to ivermectin using the soil ecotoxicity model species *Folsomia candida* (Collembola) (OECD, 2009; ISO, 2004), in terms of survival and reproduction, along 3 generations. In order to increase mechanistic understanding and thus the relevance of this study, avoidance behaviour and cellular biomarkers involved in neurotransmission (AChE-acetylcholinesterase), biotransformation (GST-glutathione S-transferases), antioxidant defence (CAT-catalase, GPx-glutathione peroxidase, GR-glutathione reductase) and oxidative damage (LPO-Lipid Peroxidation) were also measured.

2. Materials and methods

2.1. Test organisms

The standard test species *Folsomia candida* (Collembola) was used. Cultures were kept on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio), at 20 ± 1 °C, under a photoperiod of 16:8 (light:dark). Food consisted of dried baker's yeast (*Saccharomyces cerevisae*) provided weekly. Age-synchronized juveniles (10–12 days) were used for the test.

2.2. Test substance, soil and spiking procedures

Ivermectin (IVM) (≥90% purity; Sigma-Aldrich) and the natural standard LUFA 2.2 soil (Speyer, Germany) were used. Soil characteristics are summarised as follows: pH (0.01 M CaCl2) of 5.5 ± 0.1, 1.61 ± 0.15% organic carbon, 7.9 ± 1.8% clay, 16.3 ± 2.5% silt, and 75.8 ± 3.9% sand.

Ivermectin is not water soluble, therefore acetone (100% purity; VWR Chemicals) was used as a solvent. Nominal test concentrations were 0-0.32-1-3.2-10-32-100 mg/kg soil dry weight (DW) for the survival, reproduction and avoidance tests and 0-1-3.2 mg/kg soil DW for the multigenerational test. The latter were selected based on the reproduction effect concentrations (0-EC10-EC50). Solutions were prepared and serially diluted and thoroughly homogenized with the soil. Acetone was left to evaporate overnight. Water was added to the soil in order to achieve 40–60% of the maximum water holding capacity (WHC). In addition to a water control, a solvent control was used in all tests, resembling the maximum added volume of solvent with the ivermectin spiking.

2.3. Experimental procedure

2.3.1. Avoidance test

The avoidance test guideline ISO 17512-2 (2011) was followed, using the 2 chamber option. Circular plastic boxes (Ø 8 cm × 4.5 cm) divided in the middle by a removable plastic barrier were used. Five replicates were done. Half of each of the containers were filled with 30 g of the control soil and the other half with 30 g of the spiked soil. After removal of the plastic barrier, 20 juveniles (10–12 days old) were placed in the middle. The test was conducted for 48 h, at 20 ± 2 °C, under a photoperiod of 16:8 h (light:dark). At the end of the test, the plastic wall was placed in the middle section of each box and the soil from each half of the container was separated and put into new vessels, flooded with water and the number of floating individuals was counted directly.

2.3.2. Reproduction tests

The standard guideline OECD 232 (2009) was followed. In short, 10 organisms were introduced into each test vessel, containing 30 g of moist soil. Five replicates were done. The test ran for 28 days at 20 ± 2 °C, under a photoperiod of 16:8 h (light:dark). Food and water loss were replenished weekly. At test end, test vessels were flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further automatic counting using the software ImageJ (Schneider et al., 2012). Two endpoints were evaluated: survival and reproductive output.

2.3.3. Multigenerational test

Each multigeneration test was conducted following of the same OECD guideline 232 (2009), except that at test end the juveniles were sampled and further exposed. In short, at test end, the similar flooding and photographing procedure for counting and measuring was done, both using the functions available in software ImageJ, and juveniles were transferred with a spoon to a box with a layer of Plaster of Paris (culture medium). For the exposure of the next generation, ten of the biggest juveniles (ca. 11 days old) were transferred to new test vessels, with freshly spiked soil. Additionally, 300 plus 150 juveniles were sampled in 2 microtubes, snap frozen in liquid nitrogen and stored at −80 °C, until further analysis. This was repeated for all 3 generations, i.e. 28, 56 and 84 days exposure for the three consecutive generations of juvenile collembolans. Five replicates were used for the controls and ten for each treatment, in order to ensure enough organisms to start the next generation tests and analysis. Three endpoints were evaluated: survival, reproductive output and size (area, mm^2).

2.3.4. Cellular markers analysis

Procedures followed the previously optimized methodology as detailed by Maria et al. (2014). The selected biomarkers were catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), acetylcholinesterase (AChE), glutathione S-transferase (GST) and lipid peroxidation (LPO). In short, pools of 300 juveniles were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4). For LPO, 4% BHT (2,6-dieter- butyl-4-metylphenol) in methanol was added to 150 µL of the homogenate and stored at −80 °C. The remaining 850 µL of the homogenate were centrifuged and the PMS (Post Mitochondrial Supernatant) was stored at −80 °C. Protein concentration was assayed using bovine γ - globuline as a standard adapted from literature (Bradford, 1976) in a 96-well flat bottom plate. For CAT, Clairborne (1985) was followed, as described by Giri et al. (1996). GPx, GR and GST activities were determined according to Mohandas et al. (1984), Carlberg and Mannervik (1975) and Habig et al. (1974), respectively, and as detailed in Maria et al. (2014). Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979) and Bird and Draper (1984), adapted by Filho et al. (2001). Acetylcholinesterase (AChE) activity was determined according to Ellman et al. (1961), adapted by Guilhermino et al. (1996).

2.4. Data analysis

Avoidance response (A) was calculated as the percentage of organisms that avoided the treated soil compared to the total number of organisms in the vessel, calculated as follows:

$$A = (C - T)/(N) \times 100$$

where C = number of organisms observed in the control soil; T = number of organisms observed in the test soil; N = total number of organisms per replicate. No avoidance or a non-response to the compound is considered when A is negative (ISO, 2011).

The Effect Concentrations (ECx) were calculated, based on nominal concentrations, using a logistic and threshold 2 parameters regression model (Toxicity Relationship Analysis Program (TRAP) – version 1.20, US EPA).