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Toxicity of the ionophore antibiotic lasalocid to soil-dwelling invertebrates: Avoidance tests in comparison to classic sublethal tests

Suzana Žižek [a,*], Primož Zidar [b]

[a] Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia
[b] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

HIGHLIGHTS

- We assessed the toxicity of the veterinary antibiotic lasalocid to soil organisms.
- Standard toxicity tests were compared to avoidance behaviour essays.
- Lasalocid is more toxic to earthworms than isopods.
- Avoidance is a much more sensitive endpoint compared to reproduction or growth.
- Lasalocid could impair the habitat function of agricultural soils.

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ABSTRACT

Lasalocid is a veterinary ionophore antibiotic used for prevention and treatment of coccidiosis in poultry. It enters the environment with the use of contaminated manure on agricultural land. Despite its extensive use, the effects of lasalocid on non-target soil organisms are poorly explored. We used classical sublethal ecotoxicity tests to assess the effects of lasalocid on earthworms (*Eisenia andrei*) and isopods (*Porcellio scaber*) and compared the results with tests using avoidance behaviour as the endpoint. The results showed that avoidance is a much more sensitive endpoint. For earthworms, EC_{50} for avoidance (12.3 mg kg^{-1} dry soil) was more than five times lower than EC_{50} for reproduction (69.6 mg kg^{-1} dry soil). In isopods the sensitivity of the behavioural response test was even higher. While the highest lasalocid concentration 202 mg kg^{-1} had no significant effects on isopod growth or survival, already the lowest used concentration in the behavioural assay (4.51 mg kg^{-1}) caused significant impact on isopod behaviour. Using the avoidance test results for calculating the predicted no-effect concentration (PNEC) of lasalocid to soil invertebrates, the value is close to the predicted environmental concentration (PEC). This indicates that the use of lasalocid-contaminated manure could potentially impair the habitat function of agricultural soils.

1. Introduction

The growing needs of the human population drive modern agriculture into using increasing amounts of pesticides and fertilisers, thereby increasing the threat of contamination to soils and underground water. Intensive use of manure burdens the environment not only with large amounts of nitrogen and metals, but also with veterinary pharmaceuticals and feed additives. Manure from treated animals in farms and droppings in pastures usually contain numerous pharmaceuticals, as well as their metabolites, which can also be bioactive (Celiz et al., 2009). Their introduction into the environment is not controlled, a problem that has been neglected for decades (Boxall, 2004).

Coccidiosis is a protozoal infection in poultry causing diarrhoea and dysentery. It is often fatal and spreads rapidly. Coccidiostats are authorised in the European Union as feed additives for poultry. Overall in the EU, of the estimated 40.65 million tonnes of feed produced, some 18.33 million tonnes is manufactured with an in-feed added coccidiostats (EC, 2008). Broilers and turkeys are treated with coccidiostats almost their entire life. The most frequently used coccidiostats in Slovenia and in northern Europe (Hansen et al., 2009a) salinomycin, monensin, and lasalocid are natural ionophore antibiotics produced by bacteria of the genus *Streptomyces*. In treated animals these substances are only partially metabolised and are thus excreted predominantly in the active form. EFSA reported that 74–83% of lasalocid in broiler excreta is in the active

* Corresponding author. Address: Laboratory of Forensic Toxicology and Ecotoxicology, Institute for pathology, forensic and administrative veterinary medicine, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 47 79 851; fax: +386 1 28 34 091.
E-mail addresses: Suzana.Zizek@vf.uni-lj.si (S. Žižek), Primoz.Zidar@bf.uni-lj.si (P. Zidar).

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form (EFSA, 2004). When in the environment, lasalocid, as other ionophores, undergoes both biotic and abiotic degradation, with microbial degradation being the prevalent (Vertesy et al., 1987; Sassman and Lee, 2007; Hansen et al., 2009b, 2012). The rate of biological decay, which is much slower in anaerobic conditions, depends on soil properties such as organic content, moisture, temperature and pH (Sassman and Lee, 2007). The reported half-life of lasalocid is from 0.6 to 14.2 days (EFSA, 2004; Sassman and Lee, 2007).

Although lasalocid has been on the market for decades, the available toxicological effect data for non-target soil organisms are very scarce. EFSA (2004, 2010) reported that lasalocid affects earthworm (*Eisenia foetida andrei*) survival, growth and reproduction. LC_{50} is 71.8 mg kg^{-1} and NOEC for weight change is 75 mg kg^{-1} soil (EFSA, 2004). The NOEC for reproduction was determined to be 41.2 mg lasalocid sodium per kg soil. To the best of our knowledge the only other information on lasalocid toxicity to soil animals is the survival of face fly (*Musca autumnalis*) larvae (Broce et al., 1988). The mortality of larvae was increased when cattle dung was spiked with lasalocid in concentration 41 mg kg^{-1}.

Acute toxicity tests with mortality as the end point indicate the maximum damage to the test organisms but are insufficiently sensitive and of low ecological relevance. On the other hand, chronic tests such as the earthworm reproduction test give more relevant information on the sublethal environmental effects of chemicals, but they can be very labour-intensive and time-consuming. As an alternative to standard ecotoxicity tests and a rapid and cost-effective first screening tool for soil assessment, avoidance behaviour tests with different soil organisms have been proposed (Yeardley et al., 1996; Natal da Luz et al., 2004; Loureiro et al., 2005; Amorim et al., 2008). Behaviour tests are sensitive and yield results at low cost and in short time. They are ecologically relevant as soil functioning (nutrient cycling) is reduced when animals escape contaminated soils (Yeardley et al., 1996).

The aims of the present study were to determine the acute and chronic toxicity of lasalocid to earthworms and woodlice, the two detritivorous species with an important role in decomposing plant material and recycling nutrients in terrestrial ecosystems. We performed the earthworm reproduction test (OECD, 2004) and a modified woodlice test proposed by Hornung et al. (1998). To measure the effects of lasalocid on avoidance behaviour, we used the ISO test for earthworm avoidance (ISO, 2008) and a modified test proposed by Loureiro et al. (2005) for woodlice. The new information obtained in this way was used to reassess the risk of lasalocid entering soil ecosystems and to evaluate the usefulness and sensitivity of the avoidance tests for testing ionophore antibiotics.

2. Materials and methods

2.1. Test species

The earthworm species used in the experiments was *Eisenia andrei* (Oligochaeta: Annelida, Lumbricidae) from the laboratory culture at the Veterinary Faculty, University of Ljubljana. Animals were kept in a climate chamber at 20 ± 1 °C with a 12/12 h light/dark period and 80% relative humidity (RH). Plastic containers were filled with a bedding of potting soil and peat, adjusted to pH 6. The cultures were regularly fed with ground dried horse faeces. Sexually mature animals with a visible clitellum and weighing between 200 and 300 mg were used in the experiments.

Porcellio scaber, Latr. (Isopoda, Crustacea), originated from the laboratory culture at the Department of Biology, University of Ljubljana. Animals were kept in a climate chamber at 20 ± 1 °C with a 16/8 h light/dark period, caged in glass containers with moist sand and peat on the bottom. They were fed fallen leaves from various trees, with periodical additions of potatoes, fresh vegetables, apples and commercial pet rabbit food (Hobby Vit, HP Hobby program, Hoče, Slovenia), which contained no coccidiostats. All tests were performed with adult animals of both sexes weighing between 30 and 45 mg. Pregnant females were excluded.

The experimental animals used in this study were treated in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.2. Soil preparation

The standardised natural soil Lufa 2.2 (Speyer) was used in all the tests. It is a loamy sand with 2.09% organic matter, 6.4% clay and a pH (OECD, 2004) of approx. 5.8 (1 M KCl). The lasalocid A sodium salt (99.3% purity) was obtained from Alpharma (Belgium).

Lasalocid was introduced to the soil dissolved in acetone. Control soils were treated with the same amount of acetone. A small portion of the soil required for the experiment (approx. 25%) was spiked with the acetone solution (5 mL solution per 100 g soil), thoroughly mixed and left overnight in a fume hood. After evaporation of the acetone, the remainder of the soil was added, carefully mixed and the moisture content was adjusted to 50% of the Water Holding Capacity.

2.3. Experimental design

2.3.1. Earthworm reproduction test

The test was performed in accordance with the OECD test No. 222 (OECD, 2004). Glass jars (1 L) were filled with 500–600 g moist soil. Ten adult pre-weighed (non-depurated) earthworms were randomly introduced into the test containers. There were four replicates per test concentration and control. Incubation took place in a climate chamber at 21 °C, with 80% RH and a 16/8 h light/dark cycle. A small amount of finely ground dried horse manure from animals not treated with any pharmaceuticals was added as food once a week and water loss was compensated if necessary. After 28 d, the test containers were emptied and the surviving adults were counted and weighed (non-depurated) and their combined weight was recorded.

The soil was returned into the test containers to allow for hatching of the cocoons for another 28 d. To count the number of juveniles produced, the test containers were placed in a water bath at 55 °C; after approx. 15 min, juvenile earthworms appeared on the soil surface and were gently transferred to a separate jar and counted.

The test was run with four replicates per treatment. The nominal concentrations of lasalocid used in the test were 0, 5, 30, 60, 125 and 200 mg kg^{-1} dry soil.

2.3.2. Earthworm avoidance test

The earthworm avoidance test was performed in accordance with the ISO standard No. 7512-1:2008 (ISO, 2008). Rectangular polypropylene vessels (160 × 110 mm) with a volume of 1.5 L were divided into two equal sections by a vertically introduced divider (thin sheet of metal). They were filled with soil to a height of 5–6 cm (approximately 0.5 kg of wet soil in each section). One half of the vessel was filled with the contaminated soil and the other with control soil, treated only with the corresponding amount of acetone. Then the separator was removed and 10 worms were placed onto the separating line. The containers were covered with perforated transparent lids and wrapped in aluminium foil to avoid lateral effects of light. Incubation took place in a climate chamber at 21 °C, with 80% RH and a 16/8 h light/dark cycle. At the end of the test period (48 h) the control and test soils in each vessel were again separated by inserting the divider. The number of worms in each section was counted.