Effects of the antibiotics oxytetracycline and tylosin on soil fauna

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Abstract

Antibiotics may enter the terrestrial environment when amending soils with manure. A Note of Guidance on ecological risk assessment of veterinary medicines was issued in January 1998. Hardly any information about ecotoxicological effects of already existing substances are available. This study has tested the effects of two widely used antibiotics, tylosin and oxytetracycline, on three species of soil fauna: Earthworms, springtails and enchytraeids. Neither of the substances had any effect at environmentally relevant concentrations. The lowest observed effect concentration was 3000 mg kg^{-1} and in many cases no effect was seen even at the highest test concentration of 5000 mg kg^{-1}. © 2000 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

Veterinary medicinal products such as antibiotics help to protect the health and ensure the well being of domestic animals. Veterinary medicinal products are licensed for use by regulatory authorities if they comply with scientific criteria on quality, efficacy and safety. The authorities consider safety to the treated animal, to the consumer, and to the individuals handling the product during treatment. In addition to these criteria the environmental risk of veterinary medicinal products has recently become a matter of increasing public scrutiny and legal requirements. The environmental impact of veterinary medicines is assessed after different regulations depending whether the application is therapeutic or nontherapeutic. The legislation in the European Union on the environmental risk assessment of veterinary medicines is part of the Commission Directive 92/18EEC.

This directive outlines the basic requirements for conducting an environmental risk assessment of veterinary medical products. A detailed evaluation to assess the environmental risk of new veterinary medical products is given in a Technical Guidance Document by the European Agency for the Evaluation of Medicinal products (EMEA), EMEA/CVMP/055/96 (EMEA, 1997). However, the environmental risk assessment only concerns new products sold after the 1st of January 1998. There is currently no European initiative to assess environmental risk of veterinary medicinal products already on the market.

Very little is known about the ecotoxicological effects of antibiotics. However, antibiotics are specifically designed to control bacteria in animals. Obviously this makes them potentially hazardous to bacteria and other micro-organisms in the environment (Warman, 1980; Pursell et al., 1995). For soil fauna and plants hardly any information is available. This paper describes the direct effects of antibiotics on soil living fauna. Three soil living invertebrate species (earthworms, springtails and enchytraeids) have been exposed to two widely used

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antibiotics in controlled laboratory experiments. Oxytetracycline (OTC), is a broad spectrum antibiotic with a long history in veterinary medicine for the treatment and control of a wide variety of bacterial infections. Tylosin is a macrolide antibiotic that is active mostly against Gram-positive bacteria and mycoplasmas. Tylosin is used in pigs, cattle and poultry for the treatment of conditions caused by sensitive micro-organisms.

2. Materials and methods

2.1. Animals and stock culture

Folsomia fimetaria (Linné, 1758) (Collembola: Isotomidae) is a euedaphic, nonpigmented, eyeless springtail that reproduced sexually. A laboratory culture was established from field-collected animals that were mass reared on moistened substrate of plaster of Paris/charcoal. The collembolans were fed dried baker's yeast. Every 2–4 weeks the animals were moved to another Petri dish with fresh food and substrate, which stimulated egg production. A synchronised culture was produced by collecting approximately 1-week-old eggs, which were allowed to hatch over 3 days (unhatched eggs were removed after this period) (Krogh, 1995). Animals 23–26 days old were used in the experiments. The sex was discerned by size, as females are bigger than males.

Enchytraeus crypticus (Enchytraeidae: Oligochaeta) were collected from the field and cultured in laboratory conditions in order to get worms of the same size for the experiments. Oat flakes were used for food and to avoid infections with mites it was boiled. Adult worms, with eggs in the clitellum and approximately the same size, were used in the experiments.

The earthworm *Aporrectodea caliginosa* (Savigny, 1826) (Annelida: Oligochaeta) were collected in the field from an uncontaminated agricultural soil. Adult animals (with clitellum) were maintained under laboratory conditions in the same test soil-substrate for one week before use (van Gestel et al., 1989). A mixture of test soil and finely ground cattle manure (1:1 dry vol.%) subsequently moistened to 50% water content of fresh weight was used as food and applied on the surface of the test soil ad libitum. Adult earthworms were used in the experiments.

2.2. Test substances and soil

Oxytetracycline Dihydrate, $C_{22}H_{24}N_2O_9$ $2H_2O$ (num. CAS 6153-64-6), and Tylosin Tartrate (num. CAS 74610-55-2) with a potency of 898 mg tylosin/mg, both by Sigma®, were used in the experiments.

In order to reach the optimal conditions for the different animal's species two kind of soils were used. A sandy soil, composed by 66.9% coarse sand, 15.8% fine sand, 3.3% coarse silt, 5.3% fine silt, 6.2% clay, 2.7% humus, 1.5% total carbon and a pH-H$_2$O of 5.5, was used in the springtail and enchytraeid experiments. A sandy–loamy soil (38.4% coarse sand, 23.6% fine sand, 10.0% coarse silt, 12.3% fine silt, 13.0% clay, 2.8% humus, 1.6% total carbon and a pH-H$_2$O of 6.2) was used in the earthworm tests. In order to eliminate undesired soil fauna, both soils were dried at 80°C and stored at 5°C until use. Thereafter soils were sieved through a 2 mm mesh net.

2.3. Test substance concentrations

In order to determine the range of antibiotic concentrations for use in the final tests, preliminary range-finding tests were performed in each experiment (Løkke and van Gestel, 1998). Acute mortality tests were carried out with *F. fimetaria* (10 female and 10 male collembolans were exposed for one week), *E. crypticus* (10 adult worms were exposed two weeks) and *A. caliginosa* (5 adult earthworms exposed for one week). Five concentrations and a control (two replicates per treatment) of both antibiotics were tested.

On the basis of these preliminary range-finding tests, the test species were exposed to the following OTC concentration series given as mg/kg soil (dry weight): 0, 500, 1000, 2000, 3000 and 5000; The following test concentrations of Tylosin were used: *F. fimetaria* and *A. caliginosa*, 0, 500, 1000, 2000, 3000 and 5000; *E. crypticus*, 0, 1000, 2000, 3000, 4000 and 5000. Four replicates per concentration were done.

2.4. Test procedures

The sublethal toxicity tests with *F. fimetaria* were done following the guideline described by Wiles and Krogh (1998). To each test container 30 g sandy moist soil (27 g dry soil and 3 g demineralised water) was added. The antibiotics were dissolved in water and mixed homogeneously into the dry soil one day before the start of the experiments. 10 males and 10 females, 23–26 days old, were added to each test container. 2 mg dried baker's yeast was added to the soil surface in each container at the start of the experiments and again after two weeks. The test containers were weighed initially and after two weeks so that water lost by evaporation could be compensated for. Test containers were incubated for 21 days at 20°C ± 2°C with 12:12 light:dark cycle. After incubation the animals were extracted for 48 h in a high-temperature gradient soil fauna extractor of the MacFadyen type (Petersen, 1978). The extraction lasted 48 h starting at 25°C at the surface of the soil. Every 12 h, the temperature at the soil surface was increased by 5°C up to 40°C during the last 12 h. The collembolans were collected into containers with a 0.5

cm layer of plaster of Paris/charcoal. The surface of the plaster was kept constantly at 3°C during the extraction. The animals were stored at 5°C until automatic counting (Krogh et al., 1998). Measured parameters were survival and reproduction of the collembolan *F. fimetaria* (number of juveniles produced).

With regard to the enchytraeid tests, 23.2 g sandy moist soil (20 g dry soil and 3.2 g demineralized water) was added to each test container. The antibiotics were dissolved in water and mixed thoroughly into the dry soil one day before the start of the experiments. Then 10 sexually mature enchytraeids, with eggs in the clitellum and approximately the same size, were added to each test container. A small amount of boiled oat flakes (around 25 mg dry weight) was added and mixed with the soil in each container just before the worms were added to soil. Food supply was added weekly at the soil surface to avoid harming the worms. The test vessels were weighed initially and once a week the weight loss was replenished with the appropriate amount of de-ionised water. Test containers were covered with plastic and perforated lids and incubated for 21 days at 20°C ± 2°C in a controlled light dark cycle of 12:12 light:dark cycle. After 21 days, the test substrates were carefully searched and surviving adults were removed and counted. The test substrates, including cocoons hatched juveniles, were incubated under the same test conditions for additionally 21 days. After two weeks 25 mg (d.w.) of food was added to each test container. The containers were weighed once a week and the weight loss was replenished with the appropriate amount of de-ionised water. At the end of the incubation period the worms were extracted using the wet funnel method (O'Connor, 1985). The enchytraeids were collected into plastic containers and stored at 5°C until counting. In order to facilitate the counting of the high number of worms after the exposure time, the procedure was as follows: first, the enchytraeids were transferred to graduate-counting Petri dishes with a thin layer of water, and their movement were decreased by adding 1–2 drops of glycerol. Afterwards 2–3 drops of Bengalred were added in order to increase the contrast and improve visualisation of the worms.

The sublethal toxicity tests with the earthworm *A. caliginosa* were carried out following the guideline described by Kula and Larink (1998). 800 g dry sandy–loam soil was weighed into each test container. Four replicates for each concentration were used in all tests. Solutions of OTC and Tylosin were used to give the required antibiotic concentration and percentage water content in the test soil. The solutions were mixed homogeneously into the soil the day before the start of the experiments. The same volume of de-ionised water (160 ml) was added to the controls. At the end of the acclimatisation period (one week), four earthworms were collected by hand, carefully washed with tap water,

softly blotted on absorbent paper to remove excess water and weighed. In order to ensure that the replicate units for each treatment cover a similar range of initial weights, a 'ranking and blocking' procedure was done and the earthworms were assigned the different treatments, one in each test container (McIndoe et al., 1999). Test containers were incubated for 21 days at 15°C ± 1°C. Food (test soil mixed with cattle manure) was applied on the surface of the test soil ad libitum in each container at the start of the experiments and again after two weeks. The test containers were weighed initially and evaporated water was replaced after two weeks. Test containers were incubated for 21 days at 15°C ± 1°C. At the end of the test the substrates were carefully searched for surviving earthworms and cocoons. The earthworms were washed, blotted, weighed and counted. Cocoons were counted and maintained in Petri dishes with wet filter paper and were incubated at 20°C for further nine weeks to allow hatching. For each test, survival, growth, reproduction (number of cocoons produced) and cocoon viability were measured. As chemicals in line with other stresses may impact the scope for growth of organisms, that is the energy available for growth and reproduction, the total biomass produced during the exposure time was used as the most ecological relevant endpoint of growth (Holmstrup, personal communication). The growth of earthworms was calculated as the final biomass (FB), including body growth and the numbers (NC) and mean weight of cocoons (MCW) produced in each replicate, i.e. $FB = FW + (NC \times MCW)$. To normalise the weight data to a uniform weight class all weights were divided by the weight of the corresponding controls from the same block. Cocoons viability was estimated with regard to the number of cocoons produced in each replicate and a hatch rate (HR) was calculated as the ratio between the number of hatched cocoons (HC) during the nine weeks of incubation and the number of produced cocoons (PC) during the three weeks of exposure i.e. $HR = HC/PC$.

3. Statistics

Estimation of the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) was done by comparing the control with each of the concentrations by a Dunnett's test in an ANOVA (SAS, 1989). For all tests the level of significance was 0.05. The validity of the ANOVA has been tested by SAS/LAB (SAS, 1992), which check for homogeneity of variances (Levene's test), outliers and normality. Calculation of EC_{10} and EC_{50} values for reproduction, growth and cocoon viability was done by using the ICp approach (Norberg-King, 1993). Lethal concentration (LC_x) values for adults were estimated by use of PROBIT analysis (SAS, 1989).

4. Results

The toxicity of OTC and tylosine to the three tested soil animals was generally very low. The lowest observed significant effects were found at 3000 mg kg^{-1} and in many cases no effects were observed at the highest test concentration of 5000 mg kg^{-1} (Figs. 1–3). Reproduction was generally a more sensitive endpoint than survival (Tables 1–4). Growth and fertility, expressed as the number of cocoons hatched during a nine weeks post exposure period, were a slightly more sensitive endpoints than survival of earthworms. Estimated EC$_{10}$ values were found in the range of 134 to more than 5000 mg kg^{-1}, whereas all EC$_{50}$ values were above 2000 mg kg^{-1}.
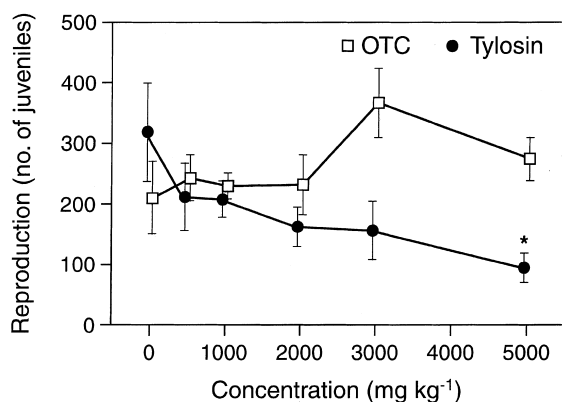

Fig. 1. Effects of the antibiotics oxytetracycline (rombs) and tylosin (squares) on the reproduction (number of offspring) of the springtail *F. fimetaria*. Data significantly different from controls are marked with an asterisk (*). Error bars = SEM. $N = 4$.
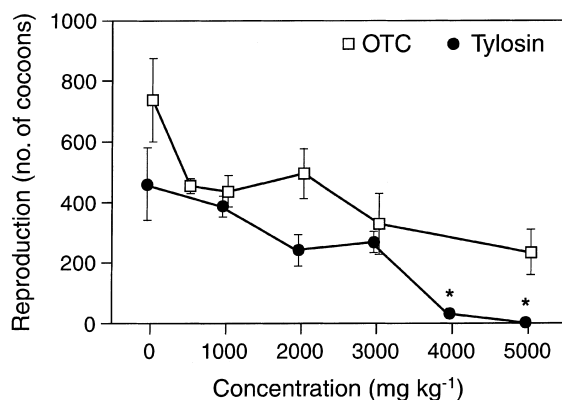

Fig. 2. Effects of the antibiotics oxytetracycline (rombs) and tylosin (squares) on the reproduction (number of offspring) of the enchytraeid *E. crypticus*. Data significantly different from controls are marked with an asterisk (*). Error bars = SEM. $N = 4$.
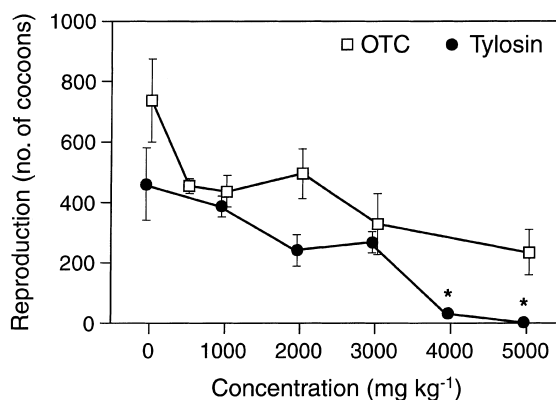

Fig. 3. Effects of the antibiotics oxytetracycline (rombs) and tylosin (squares) on the reproduction (number of coccoons) of the earthworm *A. caliginosa*. Data significantly different from controls are marked with an asterisk (*). Error bars = SEM. $N = 4$.

Generally large confidence intervals were found in the interpolation of results.

5. Discussion

Veterinary medicines may be spread to the environment, either directly when using the drugs (minor) or by subsequent excretion from the animals (major). Before entering the environment, the substances may be metabolised in the animal. Reactions may consist of oxidation, reduction, hydrolysis or conjugation. The metabolisation changes the physical, chemical and ecotoxicological properties of the substance (see, e.g., Gibson and Skett, 1986). It is, however, shown that metabolites may be reconverted to their parent compounds after leaving the animals. Berger et al. (1986) showed that the metabolites chloramphenicol glucoronide and *N*-4-acetylated sulphadimidine were converted to the parent compounds chloramphenicol and sulphadimidine in samples of liquid manure.

The dominating pathway of environmental release in the terrestrial compartment is by amendment of arable soil with manure or slurry. Very few studies concerning the levels of veterinary medicines in soil after manure amendment have been reported. Warman and Thomas (1981) found chlortetracyclines in soil amended with chicken manure, and Shore et al. (1988) found testosterone and estrogene in manure from American chickens. Van Goll (1993) estimated that if the total amount of growth promoters used in the Netherlands were spread over all the two million hectares of Dutch arable land, an annual average of 130 mg antibiotics and antibiotic metabolites per m^2 of arable land would be found, corresponding to approximately 0.9 mg/kg of dry

Table 1
Collembola. Effects of oxytetracycline and tylosin on the survival and reproduction of *F. fimetaria*[a]

| | Oxytetracycline | | Tylosin | |
	Survival	Reproduction	Survival	Reproduction
NOEC	≥5000	≥5000	≥5000	3000
LOEC	>5000	>5000	>5000	5000
LC/EC$_{10}$	>5000	>5000	>5000	149 [100,789]
LC/EC$_{50}$	>5000	>5000	>5000	2520 [638,4666]

[a] All concentrations in mg kg^{-1} d.w. Numbers in brackets are 95% confidence interval.

Table 2
Enchytraeids. Effects of oxytetracycline and tylosin on the survival and reproduction of *E. crypticus*[a]

| | Oxytetracycline | | Tylosin | |
	Survival	Reproduction	Survival	Reproduction
NOEC	3000	2000	2000	3000
LOEC	5000	3000	3000	4000
LC/EC$_{10}$	4201 [2832–9144]	134 [68; 695]	2501 [2143; 2745]	632 [−14; 1885]
LC/EC$_{50}$	>5000	2701 [−843; 5619]	3381 [3185; 3581]	3109 [329; 3609]

[a] All concentrations in mg kg^{-1} d.w. Numbers in brackets are 95% confidence interval.

Table 3
Earthworms. Effects of oxytetracycline and tylosin on the survival and reproduction of *A. caliginosa*[a]

| | Oxytetracycline | | Tylosin | |
	Survival	Reproduction	Survival	Reproduction
NOEC	≥ 5000	3000	≥ 5000	3000
LOEC	>5000	5000	>5000	5000
LC/EC$_{10}$	>5000	1954 [588; 4229]	>5000	3306 [−1651; 3657]
LC/EC$_{50}$	>5000	4420 [2592; 5246]	>5000	4530 [not estimated]

[a] All concentrations in mg kg^{-1} d.w. Numbers in brackets are 95% confidence interval.

Table 4
Earthworms. Effects of oxytetracycline and tylosin on the growth of *A. caliginosa* and the hatchability of produced cocoons[a]

| | Oxytetracycline | | Tylosin | |
	Growth	Hatchability	Growth	Hatchability
NOEC	3000	≥ 5000	3000	3000
LOEC	5000	>5000	5000	5000
LC/EC$_{10}$	3251 [−615; 5536]	2127 [−376; 4247]	3477 [2251; 4149]	3288 [−1262; 3576]
LC/EC$_{50}$	>5000	>5000	>5000	4823 [not estimated]

[a] All concentrations in mg kg^{-1} d.w. Numbers in brackets are 95% confidence interval.

soil. Such a uniform distribution is unlikely and local concentration significantly higher than this must be expected. A uniform procedure for estimating the predict environmental concentrations (PECs) for veterinary medicines is suggested by Spaepen et al. (1997). Empirical estimations predict environmental concentrations in the range of 1–5 mg/kg soil.

As soil dwelling organisms potentially will be exposed to antibiotics it is important to know whether effects on non-target organisms are likely to occur. Very little is known about the possible side effects of antibiotics to soil fauna and plants. Batchelder (1981, 1982)

tested the effects of the antibiotics chlortetracycline and oxytetracycline on plants when grown in both a nutrient solution media and in soils. When grown in a nutrient solution of 160 mg/l all plants died and in lower concentrations both the growth of roots and the dry weight of the shoots were significantly reduced (approximately 60–90%). In the study using soil as growth media a large variation of the sensitivity among plant species was found (Batchelder, 1982). Lanzky and Halling-Sørensen (1997) showed that *Chlorella sp.* are very sensitive (EC$_{10}$ = 2.03 mg/l and EC$_{50}$ = 12.5 mg/l) to the antibiotics metronidazole. Studies on the effect of antibiotics

to soil invertebrates have not yet been published. The present study shows that the antibiotics oxytetracycline and tylosin have a low toxicity to soil dwelling fauna. EC_{10} values were found from approximately 150 mg kg^{-1}. Due to variation in the data this is markedly below the NOEC values. A common situation in ecotoxicology. Many scientists have therefore recommended the use of EC_{10} values instead of NOEC values in, e.g., the risk assessment procedure (e.g., Hoekstra and van Ewijk, 1993).

Information from the aquatic environment shows that antibiotics may be toxic for other organisms than target bacteria. Acute toxicity (LC_{50}) of furazolidone, which are largely used in medicated fish feed, was found at 40 mg kg^{-1} for the mosquito larvae *Culex pipiens* (Macrí et al., 1988). In general antibiotics has to be considered moderately toxic to aquatic invertebrates or fish. Acute toxicity studies typically show EC_{50} values in the range of 25 to more 500 mg l^{-1} (Halling-Sørensen et al. 1998).

The main objective of these experiments has been to study the acute effects of antibiotics on single species of the decomposer system represented by soil fauna. On the basis of the obtained results it is not very likely that antibiotics potentially present in manure will pose any direct risk to the soil fauna. However, as soil ecosystems are build up by complex and linked food webs it is not yet possible to exclude that indirect effects on soil fauna driven by changes in the microbial community and alteration of the decomposer system may happen. This may be studied in multispecies mesocosms.

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