Universidade de Aveiro Departamento de Biologia
2008

Vanessa de Menezes Oliveira

Fármacos veterinários utilizados como aditivos alimentares na indústria galinácea: avaliação dos efeitos por meio de uma bateria de testes de solo

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, Biodiversidade e Gestão de Ecossistemas, realizada sob a orientação científica do Dr. Amadeu Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro

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o júri

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(Autocarro-bar, 3 de Junho de 2008)

palavras-chave Fármacos veterinários, contaminação do solo, organismos não alvo e ecotoxicologia

resumo

A utilização dos compostos de uso veterinário e seus efeitos nos diferentes compartimentos ambientais têm sido alvo de grande atenção nos anos recentes.

Produtos medicinais veterinários tais como os antibióticos são utilizados no combate aos parasitas e na prevenção de doenças em animais. Esses compostos são largamente utilizados como aditivos alimentares na indústria de criação de galináceos como aceleradores de crescimento. As conseqüências e efeitos ainda não conhecidos desses fármacos no ambiente matéria de estudo de muitos trabalhos.

Os compostos fármacos veterinários podem entrar no ambiente por inúmeras formas, incluído a via direta, onde esses compostos são aplicados nas águas superficiais tal como nos tratamentos de aquacultura através da lixiviação oriunda dos tratamentos e das excretas das excretas dos animais.

O propósito deste estudo era de avaliar o impacto de dois compostos de uso veterinário (nicarbazin e monensin) utilizados como aditivos alimentares na indústria de criação de galináceos em organismos não alvos e duas espécies de plantas. Ensaios ecotoxicológicos foram realizados para avaliar a toxicidade aguda e crônica em minhocas (Eisenia andrei), colêmbolos (Folsomia candida) e as duas espécies de plantas (Brassica rapa e Triticum aestivum). As medidas analíticas dos químicos no solo estiveram de acordo com as concentrações nominais utilizadas. Os resultados obtidos demonstraram que não houve efeito significativo na exposição ao nicarbazin a todos os organismos testados, enquanto que a exposição ao monensin apresentou uma dose-resposta de efeito observado. A sensibilidade das espécies decresceu na seguinte ordem: Brassica rapa < Eisenia andrei < Triticum aestivum < Folsomia candida, com os valores de EC50 variando entre aproximadamente 10 e 100mg/kg. Esse estudo demonstrou a importância da utilização de uma bateria de testes para avaliar os efeitos ecotoxicológicos, principalmente pela utilização de diferentes parâmetros de avaliação de resposta e/ou espécies de diferentes níveis tróficos.

keywords veterinary pharmaceuticals, soil contamination, non-target organisms, ecotoxicology.

abstract

The usage of veterinary pharmaceutical (VP) compounds and their effects in the different environmental compartments have received a special attention in recent years.

Veterinary medicinal products such as antibiotics are used to combat parasites and prevent diseases in the animals. They are widely used as food additives in the poultry industry and also in other farm animals to promote growth. The unknown consequences of these VPs into the environment are a matter of concern and many works have been made to analyze the effects of these compounds.

The VP compounds may enter into the environment via a number of different pathways, this include direct via, when it's applied in the surface waters as in the aquaculture treatments by wash-off from topical treatments and the excreta of animals.

The purpose of this study was to examine the direct impact of two veterinary pharmaceuticals (nicarbazin and monensin) used in the poultry industry on non-target organisms and two plant species. Ecotoxicological tests were used to evaluate the acute and chronic toxicity in earthworms (Eisenia andrei), collembolans (Folsomia candida) and two species of plants (Brassica rapa and Triticum aestivum). Analytical measurements of the chemicals were in good agreement with the nominal concentrations used. The results obtained showed no effect of nicarbazin for all the tested organisms, while in exposures with monensin, there was a dose-response pattern. Species sensitivity decreased in the following order: Brassica rapa > Eisenia andrei > Triticum aestivum > Folsomia candida, with EC50 ranging between approximately 10 and 100mg/kg. This study showed the importance of the test battery when assessing ecotoxicological effects, mainly by using different endpoints and/or species from different trophic levels.

Summary

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Chapter I

Introduction and Objectives

I. Introduction and objectives

1.1. Introduction

In recent years the use of veterinary pharmaceuticals, mainly antibiotics, to prevent diseases and to promote growth has been intensified. Despite this, their effects in the environment are still mostly unknown and as such it has been a matter of concern and scientific research.

Pharmaceutical compounds are designed either to be highly active and interact with receptors in humans and animals or to be toxic for many infectious organisms, including bacteria, fungi and parasites (Boxall et al., 2004). Veterinary pharmaceuticals (VPs) are physiologically highly active substances used in husbandry for combating parasites, prevention and treatment of bacterially transmitted diseases (Tolls, 2001). In addition, veterinary antibiotics are used to promote growth and feed efficiency in a range of animals (Kim and Carlson, 2006), since disease decreases animal performance in livestock production: one of the purposes of the use of antibiotics is to limit progression of disease in a population (Kemper, 2007).

The presence of human and veterinary pharmaceutical compounds in the environment has received increased attention in recent years (Kim and Carlson, 2006). During the past decade, concern has grown about the adverse effects that the use and disposal of pharmaceuticals might potentially have on human and ecological health (Kümmerer 2003), because once in the environment these compounds can affect other living forms and humans. Nicarbazin, lasalocid, monensin, salinomycin and narasin (Fig. 1) are some of the most commonly used anticoccidial drugs in the poultry industry and also in other farm animals. They are authorized for use under the Additives Directive 70/524/EC. In addition to their anticoccidial activity, these compounds enhance the conversion of feed

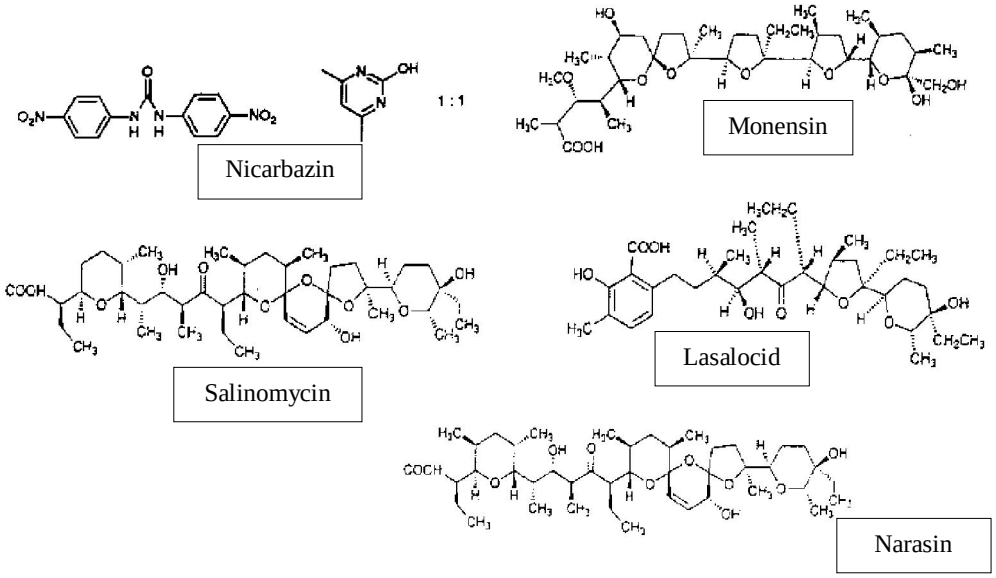

Figure 1 - Chemical structure of common anticoccidial drugs used in the poultry industry (Matabudul et al., 2002, adapted)

Depending on the country and even region, different combinations of veterinary drugs are used in animal feed. Veterinary medicines are used widely in the United Kingdom (UK) and are important aids in safeguarding animal health and welfare, helping to prevent economic loss, and indirectly, to safeguard the food supply and protect public health (Capleton et al., 2006).

There is a high concern that veterinary pharmaceuticals pose a potential risk for consumers and this has resulted in the development of methodologies for the analysis of such compounds in food products (Furtula et al., 2005).

There is a prioritized list for veterinary active ingredients, build according to their potential for indirect exposure and toxicity profile (see table 5 in Capleton et al., 2006). Among these chemicals we can emphasize antimicrobial compounds (e.g. albendazole, amoxicillin) and coccidiostat MFAs (e.g. monensin, salinomycin sodium), whose potential to reach the environment is high or unknown, have a high usage and toxicity profile classification, hence being to a priority for detailed risk assessment.

These compounds are, among others, present in 962 veterinary medicine products that were approved for use in the UK (as of August/September 2004, NOAH, 2004) (Fig. 2).

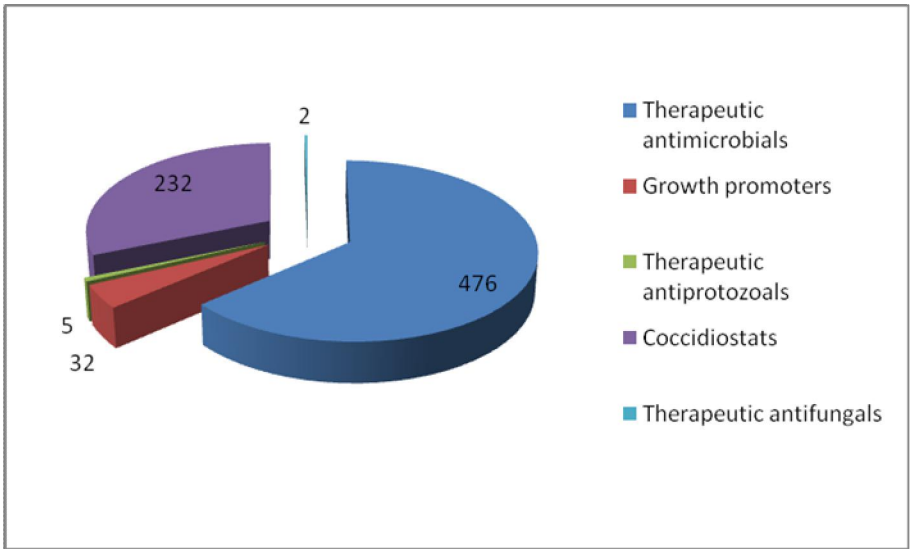

Figure 2 - Quantities of therapeutic antimicrobials, antiprotozoals, antifungals and coccidiostats and antimicrobial growth promoters in tones a.i. sold in the UK in 2004 (VMD, 2004, adapted).

These include antimicrobials, coccidiostats, ecto and endo-parasiticides, hormones and immunological products. Published sales data for antimicrobial products authorised for use as veterinary medicines indicate that during 2003, 456 tones a.i. of therapeutic antimicrobials (87–93% of which was used in food producing animals), 241 tones a.i. of coccidiostats, 36 tones a.i. of antimicrobial growth promoters, and 2 tones a.i. each of therapeutic antiprotozoals and therapeutic antiprotozoals, were sold in the UK (VMD, 2004). Tetracyclines accounted for about half (46%, 212 tonnes) of therapeutic antimicrobials sold, while trimethoprims/sulfonomides, β-lactams, aminoglycosides and macrolides accounted for 20% (89 tones), 14% (62 tones), 4.6% (21 tones), and 13% (60 tones), respectively. Most of the therapeutic antimicrobials sold (57%, 261 tonnes) were authorised specifically for use in pig and poultry species (VMD, 2004).

The amounts of antibiotics used in one year can only be calculated roughly: in 1999, 13.288 tones of antibiotics were used in the EU and Switzerland, of which 29% were used in veterinary medicine, 6% as growth promoters and 65% were used in human medicine (FEDESA, 2001).

All these compounds can enter into both the terrestrial and aquatic ecosystems by several routes.

1.2. Routes of entry

The most important routes of entry into the environment are likely to be the direct discharges of aquaculture products, the excretion of substances in urine and faeces of livestock animals, and the wash-off of topical treatments from livestock animals (Fig. 3).

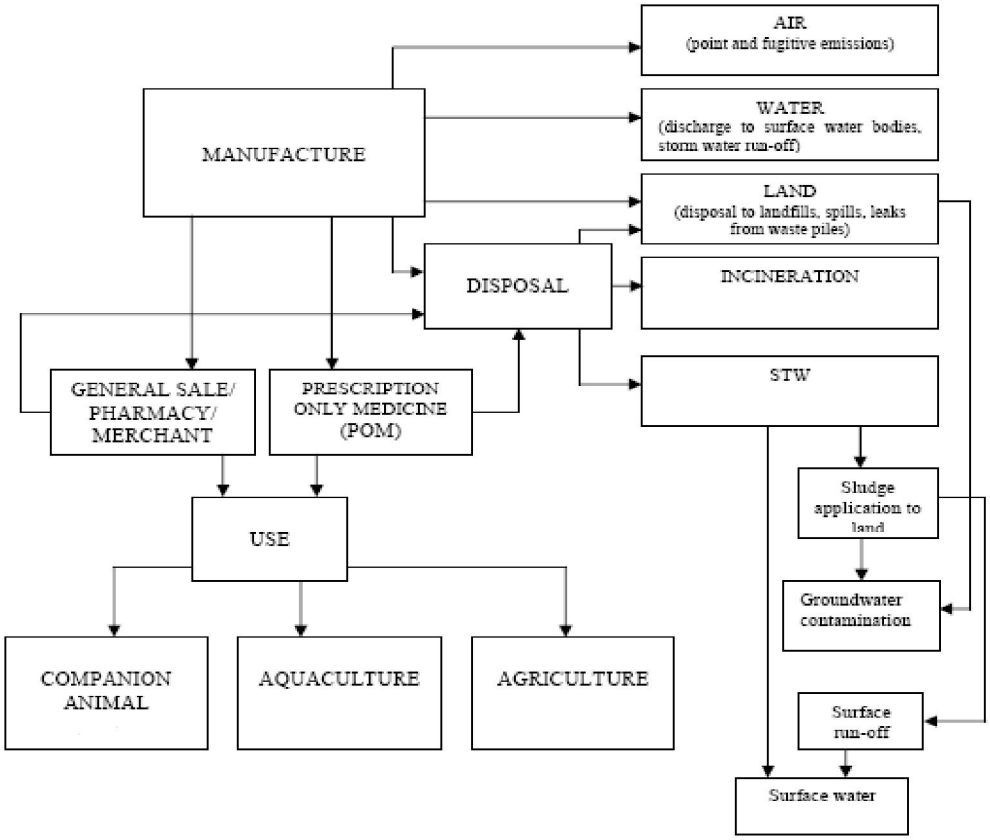

Figure 3 - Pathways of veterinary medicines to the environment (Boxall et al., 2002).

Contributions from the manufacturing and formulation processes are likely low in the United States and European Union, where manufacture and formulation are subject to tight regulatory controls. Generally these routes of entry are considered less relevant

than emissions to surface water and soils from aquaculture and herd treatments, respectively (Boxall, 2003).

Human and Veterinary Pharmaceuticals compounds have been found in groundwater, surface water, waste lagoon water, and effluent water from wastewater treatment plants. Tolls (2001) had shown that ionophore antibiotics are hydrophobic compared to other pharmaceuticals, and one could expect significant concentrations in sediments.

Large amounts of veterinary medicines, such as antibacterials, antifungals and parasiticides from aquaculture and agriculture, may also cause stress in the environment, particularly since they can be directly discharged into soils and surface waters and do not pass through a water treatment plant first, like human medicines (FEDESA, 2001).

Once in the environment, metabolites from parent VPs may be transported and distributed between the major environmental compartments. The concentrations in these compartments depend on numerous factors and processes, including how the parent compound is released into the environment, how fast it degrades, the half-lives of the metabolites, partitioning to sludge, soil and sediment, and subsequent movement to air and water (Boxall et al., 2004).

Examples about the distribution of these compounds in the different environmental compartments, according to previous studies, are given here after.

1.2.1. VPs in the soil compartment

Antibiotics used for veterinary purposes are excreted by animals and end up in soils via grazing livestock or manure used as agricultural fertilizer (Jørgensen and Halling-Sørensen, 2000). Once in the soil, antibiotic efficiency depends on the physical–chemical properties, prevailing climatic conditions, soil types and a variety of other environmental factors (Kemper, 2007).

Measured concentration of VPs (sulfonamides and trimethoprim) in animal waste or manure can be very different, e.g. ranging from 0.11 to 12.4 mg/kg (Haller et al., 2002). Residuals of antibiotics (oxytetracycline, tetracycline, chlortetracycline and tylosin) extensively used in livestock production have been detected in soil previously fertilized

with animal slurry (Hamscher et al., 2002). Kim and Carlson (2006) found an average concentration of 198.7mg/kg of tetracycline and 4.6–7.3mg/kg of chlortetracycline at a soil depth of 10–20 cm and they concluded that when liquid manure is applied repeatedly, antibiotics could enter the environment in significant concentrations and accumulate persistent residues in the soil.

1.2.2. VPs in the water compartment

In recent years, the occurrence and fate of antibiotics in the aquatic environment has been subject to many investigations carried out in several countries. More than 30 antibiotic substances have been found in sewage influent and effluent samples, in surface waters and even ground and drinking water. Antibiotics used in animal husbandry, their metabolites or degradation products can reach the water compartment by leaching and or run-off from the application of manure or slurry to areas used agriculturally, or from pasture-reared animals excreting directly on the land (Kemper, 2007).

Several studies have detected low levels of medicinal active ingredients in the environment. In Germany and the United States, low levels (<1µg/l) of antibiotic residues have been detected in surface water samples taken from sites considered susceptible to contamination (Hirsch et al., 1999; Kolpin et al., 2002), and only four residues were detected in 59 groundwater samples taken from areas with extensive livestock breeding (Hirsch et al., 1999).

Kim and Carlson (2006) performed a study to understand the importance of the sediment matrix and the partitioning to water for ionophore antibiotics. The average pseudo-partitioning coefficients were calculated using sediment concentrations with overlaying water concentrations to help the understanding of ionophore antibiotics partitioning characteristics in the environment. This study also indicates that antibiotics can significantly accumulate in the sediment, potentially impacting the stream benthic biota. Hence, they highlighted that when studying the occurrence of antibiotics in the environment it is imperative to include the sediments in the analyses.

1.3. Humans and Environment: exposure and health risk.

The available studies demonstrate that low levels of active ingredients of veterinary medicine may enter the wider environment and, hence, there is a potential exposure risk for ecosystems and humans to veterinary medicines and residues.

The inherent risk to humans from direct exposure to veterinary medicine residues in food products has been widely assessed (as evidenced by the development and application of Maximum Residues Levels), but their potential risk from indirect exposure via environment has not yet been adequately established.

Indirect human exposure to medicine compounds may however occur through the consumption of (Capleton et al., 2006):

- contaminated groundwater and surface waters;
- crops that have taken up veterinary medicine from soils to which contaminated manure and/or slurry has been applied;
- fish from natural environments (and other edible aquatic fauna) unintentionally exposed to VP from aquaculture discharges;
- non-target animals that have accumulated veterinary medicine through the food chain.

In addition to contamination risks, the extensive use of veterinary pharmaceuticals is supposed to be a daunting public health risk resulting in the emergence and spread of resistant bacteria, and also in other human, animal and environmental impairments.

1.4. Bacteria / antimicrobial resistance

A wide range of antibacterials has been observed in waters and soils and many of these persist for some time. It is possible that such exposure will result in the formation of resistant microbes, which could pose a serious threat to human and animal health (Boxall et al., 2004).

The UK Government has made clear that this problem is taken seriously and has developed a comprehensive strategy to address it so that the effectiveness of

antimicrobial products in both humans and animals can be maintained. A key element of this strategy is the collection and publication of information on the quantities of antimicrobial products sold each year for veterinary use in the UK (VMD, 2004). Generally, the most probable path for the infection of humans with antibiotic resistant bacteria from animal origin is considered to be the consumption of contaminated food products derived from treated animals (Haller et al., 2002).

1.5. Treatments, persistence and biodegradability

Pharmaceuticals can be removed when treated through physical processes, such as sorption or volatilization, biological degradation or chemical reactions (e.g. through ozone treatment). Many of the treatment methods, whilst removing the pharmaceuticals, may also produce transformation products that are more persistent and mobile than the parent compounds, some of which may also have similar or enhanced toxicity. Few studies have been performed to assess the environmental impacts of these transformation products in the environment (Boxall et al., 2004). Antibiotic metabolites can also be transformed back to their parent compound after excretion (Kemper, 2007). Then, if intracorporal degradation takes place, it is often preceded in the feces, but if antibiotics are not metabolized, recalcitrants persist in the environment (Kümmerer et al., 2000).

From above, the question on whether one should worry about transformation products can be raised. Most work so far has focused on the parent compounds. However, it is known that transformation products are produced in the environment and in treatment processes (Boxall et al., 2004). Pharmaceutical substances may also be degraded by biological organisms in treatment systems, water bodies and soils as well as abiotic reactions. Generally, these processes reduce the potency of medicines, however, some breakdown products have similar toxicity to their parent compounds (Halling-Sörensen et al, 2002). Furthermore, degradation varies significantly depending on chemistry, biology and climatic conditions. For example, the half-life of the antiparasitic ivermectin under winter conditions is six times greater than in the summer and the compound degrades faster in sandy soils than in sandy loam soils (Halley et al, 1993). In laboratory studies with manure-amended soils, a half-life of 13.5d was estimated for monensin, and in comparison to field studies shorter half-lives of 3.8 and 3.3 d were

observed in manure-amended and unamended fields, respectively, and no monensin was detected below a depth of 25 cm (Carlson and Mabury, 2006). In addition, recent studies showed that monensin degrades fairly rapidly under aerobic conditions in manure, but degradation is slowed down under anaerobic conditions. Additionally, climate changes can accelerate the degradation process too.

1.6. Regulation of veterinary compounds in the world

The regulation of the veterinary compounds is different according to the country. For instance, Health Canada regulates the sales of drugs through the Food and Drugs Act and Regulations, and the Controlled Drug and Substance Act. For human drugs, these legislations are administered primarily through the Therapeutic Products Directorate (TDD). For veterinary drugs, including antimicrobials for food animals, these legislations are administered primarily through the Veterinary Drugs Directorate (VDD), formerly Bureau of Veterinary Drugs (EIVD). The VDD is responsible for human food safety issues pertaining to veterinary drugs. Each province in Canada has its own regulatory body. Quebec has more stringent regulations than other provinces. The sale of veterinary drugs is restricted to pharmacists and veterinary surgeons. Some drugs may only be sold under veterinary prescription, while others may be sold freely. Permits are required to manufacture, distribute and sell medicated premixes or medicated feeds (VDD 2002).

In the UK, the Veterinary Medicines Directorate (VMD), an Executive Agency of the Department for environment, food and rural affairs (Defra) is responsible for the authorization of veterinary medicines (Haller et al., 2002).

Within the European Union, national and/or EU authorities control the marketing authorisation for new veterinary pharmaceutical product to be released and commercialised, ensuring its efficacy, quality and safety to public health and environment. There are some requirements for ecotoxicity testing that can be found in the European legislation by Directive 81/851/EEC, and Directive 81/852/EEC and have recently been codified in Directive 2001/82/EC (Koschorreck et al., 2002). The European Agency for the Evaluation of Medicinal Products (EMEA) is the entity responsible for the coordination of scientific resources in the Member States of the EU

in order to evaluate and supervise medicinal products for both human and veterinary use.

Environmental Risk Assessment procedures as part of the authorisation routine for VPs or other chemical compounds have identified mitigation measurements that can diminish contamination to acceptable levels.

Several harmonization efforts have been made to improve ERA procedures in terms of scientific and regulatory efficacy (Van den Brink et al, 2005). In April 1996 the VICH programme (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) was officially launched. This is a trilateral (EU-Japan-USA) program aimed at harmonizing technical requirements for veterinary product registration. This programme aims to establish and implement regulatory requirements for veterinary medicinal products by increasing quality, safety and efficacy standards and minimize the use of test animals and costs of product development. Another goal of VICH is to ensure efficient processes for maintaining and monitoring consistent interpretation of data for the implementation of guidelines. The interaction between regulatory authorities and industry can provide technical guidance enabling response to significant emerging global issues and science that impact on regulatory requirements within the VICH regions (http://www.vichsec.org/).

1.7. Objectives and structure

The main goal of this study was to determine the impact of two veterinary pharmaceuticals (nicarbazin and monensin) used in the poultry industry on non-target organisms and two plant species.

Ecotoxicological tests were used to evaluate the acute and chronic toxicity in earthworms (*Eisenia andrei*), collembolans (*Folsomia candida*) and two species of plants (*Brassica rapa* and *Triticum aestivum*).

This thesis is structured as follows:

- **Chapter I**: the actual chapter consists of a general introduction, focusing on general applications of veterinary medicine compounds, their potential to reach the environment, toxicity profile and classification to human and environmental

health, and also regulatory and environmental risk assessment procedures worldwide;

- **Chapter II**: paper entitled: “Veterinary Pharmaceuticals used as food additives in the poultry industry: effect assessment through a soil test battery”

- **Chapter III**: general discussion and conclusion.

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Chapter II

Veterinary pharmaceuticals used as food additives in the poultry industry: effect assessment through a soil test battery

Veterinary pharmaceuticals used as food additives in the poultry industry: effect assessment through a soil test battery

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Abstract

Veterinary pharmaceuticals are widely used as food additives in the poultry industry and the unknown consequences of releasing these compounds into the environment are a matter of concern. The purpose of this study was to determine the direct impact of two veterinary pharmaceuticals (nicarbazin and monensin) used in the poultry industry on non-target organisms and two plant species. Ecotoxicological tests were used to evaluate the acute and chronic toxicity in earthworms (*Eisenia andrei*), collembolans (*Folsomia candida*) and two species of plants (*Brassica rapa* and *Triticum aestivum*). Analytical measurements of the chemicals were in good agreement with the nominal concentrations used. The results obtained showed no effect of nicarbazin for all the tested organisms, while the exposure to monensin caused a dose-response pattern. Species sensitivity decreased in the following rank order: *Brassica rapa* > *Triticum aestivum* > *Eisenia andrei* > *Folsomia candida,* with EC_{50} ranging between approximately 10 and 100mg/kg. This study showed also the importance of using a test battery when assessing ecotoxicological effects, mainly by using different endpoints and species from different trophic levels.

Keywords: nicarbazin, monensin, soil contamination, soil arthropods, ecotoxicology.

Introduction

Veterinary pharmaceuticals (VPs) are physiologically highly active substances used in husbandry to combat parasites, prevent and treat bacterially transmitted diseases (Tolls, 2001). In addition, veterinary antibiotics are used to promote growth and feed efficiency of animals in farm activities (Kim and Carlson, 2006). Veterinary compounds enter the environment through several pathways, e.g. during the manufacture and formulation process and disposal, emissions to surface water and soils from aquaculture and herd treatments. Once in the environment, VP may be transported and distributed between the major environmental compartments. The concentrations in these compartments depends on numerous factors and processes, including how the parent compound is released into the environment, how fast it degrades, the half-lives of the compounds, partitioning to sludge, soil, and sediment and subsequent movement to air and water (Boxall et al., 2004). All these have increased the concern about the adverse and potential effects of pharmaceuticals on human and ecological health (Kümmerer, 2003). It is known that once in the environment, these VP compounds can affect several living forms. Nicarbazin and monensin are two of the most common anticoccidial drugs used in the poultry industry and also in other farm animals, and their use is authorized by the Additives Directive 70/524/EC. In addition to their anticoccidial activity, these compounds enhance the conversion of food to weight gain in the animals raised on medicated feeds (Matabudul, 2002). Nicarbazin is a carbanilide compound also used as a inhibitor of egg hatching in the geese *Branta canadensis* by interfering in the formation of the vitelline membrane, separating the egg yolk and egg white (EPA factsheet for Nicarbazin).

The main goal of this study was to determine the impact of these veterinary pharmaceuticals in the terrestrial environment, using a battery of standardized toxicity tests to assess toxicity in soil, and in which different trophic levels were assessed using plants, earthworms and collembolans.

Materials and Methods

Test species

Earthworms

Eisenia andrei (Oligochaeta) was used as test species in acute toxicity tests. They were maintained in culture laboratory in a moist substrate (50% horse manure, 50% peat) at 20±2ºC, and a 16:8h light:dark cycle (OECD 1984). Organisms between 300-600mg, with well developed clitellum, were selected and acclimatized for 24h in the test soil (with no contamination) prior to the experiment.

Collembolans

Folsomia candida (Collembola), a blind, unpigmented, euedaphic collembolan reproducing parthenogenetically (Hopkin 1997) was used in the reproduction bioassay. Organisms were kept in culture in a moist substrate of plaster of Paris and activated charcoal, at 18ºC, in the dark, being weekly fed with dried baker's yeast (*Saccharomyces cerevisae*) (ISO, 1999). Synchronized cultures were established prior to the start of the test, to use organisms within the same age interval (between 10-12 days old).

Plants

Two plant species, the monocotyledonous *Triticum aestivum* and a dicotyledonous *Brassica rapa*, were used in a seedling and growth test based on the species list available in the ISO guideline 11269-2 (1995). Seeds of *Brassica rapa* were purchased from Carolina Biological Supply Company (US) and *Triticum aestivum* in a local supplier (Aveiro, Portugal).

Test substances

Nicarbazin ($C_{19}H_{18}N_6O_6$, Sigma Aldrich) is a complex of two compounds, 4,4'-dinitrocarbanilide (DNC, M=302.25 g/mol) and 4,6-dimethyl-2-pyrimidinol (HDP, M=124.14 g/mol), being DNC considered the active component, while HDP aids in absorption. Monensin ($C_{36}H_{61}O_{11}Na$, M=692,9 g/mol, 90-95% purity by thin-layer chromatographic (TLC) Sigma Aldrich) is a sodium salt of a polyether monocarboxylic acid produced by *Streptomyces cinnamonensis.*

Test soil

All tests were performed with the natural standard soil LUFA 2.2 from Speyer, Germany (Lokke and Van Gestel, 1998). The main characteristics of the test soil are presented in Table 1.

Table 1 - Main pedological characteristics of the LUFA 2.2 soil, showing values of pH, organic matter content (OM), Carbon/Nitrogen ratio (C/N), grain size distribution, cation exchange capacity (CEC) and water holding capacity (WHC).

Soil	pH ($CaCl_2$)	OM (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (cmol/kg)	WHC (%)
LUFA 2.2	5.8	4.4	14.0	6.0	17.0	77.0	11.2	55.0

The contamination of the test soil with nicarbazin was carried out directly into the soil as powder due to its insolubility. Contamination with monensin was done by mixing solvent solutions of the chemical into the pre-moistened soil, each test concentration into the whole batch of soil for all replicates. After homogeneous mixing, subsamples of the batch of soil were introduced into the test vessels. In addition, a control solvent (acetone) was prepared when testing monensin. Details from the experimental setup and concentration range are presented in table 2.

Table 2 - Summary of tests performed, showing details about chemical application and concentration range used. DW: Dry Weight; a.i.: active ingredient.

Compound	Test specie	Chemical Application	Concentration range (mg a.i./kg soil DW)
Nicarbazin	*Eisenia andrei*	directly	10-100-1000
	Folsomia candida	directly	10-32-100-320-1000
	Triticum aestivum	directly	1- 3.2-10-32-100-320-1000
	Brassica rapa	directly	1- 3.2-10-32-100-320-1000
Monensin	*Eisenia andrei*	solution	3.2-10-32-100-320
	Folsomia candida	solution	10-32-100-320-1000
	Brassica rapa	solution	1- 3.2-10-32-100-320
	Triticum aestivum	solution	1- 3.2-10-32-100-320

Test procedures

Earthworms

Test procedures followed the standard OECD earthworm acute toxicity test (OECD, 1984). Ten adult worms with well-developed *clitellum*, weighting 300-600mg, were introduced in a glass vessel, containing 500g of test soil each, moistened to 40-60% of the maximum WHC. Water was replenished weekly based on weight loss. Four replicates per treatment were used. The test duration was of two weeks. Test run at 20ºC, with a 16h:8h (light:dark) photoperiod. At the end of the test, organisms were counted and weighed. The endpoints were survival (monitored at days 7 and 14) and loss of biomass (day 14). Earthworms growth ratio (GR) was calculated using the following equation:

GR= (final weight - initial weight) / initial weight

Collembolans

Test procedures were as described in the ISO guideline 11267 for *F. candida*. Ten organisms with 10 to 12 days old were used per test container, which already contained the test soil plus food supply. Four replicates per treatment were used. Vessels were covered with a parafilm layer in which a few holes for aeration were made. Food (2 mg of granulated dry yeast) was added at the beginning of the test and at day 14. Water was added weekly based on weight loss. Tests ended after four weeks, the test ended, and each test vessel was filled with distilled water, gently stirred with a spatula, causing floatation of the organisms. Through digital imaging and using special software SigmaScan Pro 5 (SPSS, 1999), organisms were automatically counted. Adults and juveniles were easily distinguished by their size.

Plants

The methodology used to evaluate the toxicity of VPs to plants followed the standard protocol ISO 11269-2 (ISO 1995). The test duration was 14 days after 50% of seeds had emerged in the control soil. Four replicates per treatments were used. Each replicate is a plastic pot with 400 ± 50 g of soil, where 10 seeds were placed at a maximum depth of 1 cm from the soil surface. A fibreglass wick (between 5-10 mm Ø) was located at the pot's bottom, where a hole was previously made (Loureiro et al. 2006). The pot was

then placed on a smaller pot with water so that moisture maintenance could be done by capillary action through the fiberglass wick. Bioassays were carried out at 20 ± 3ºC, with an illumination of 10000 lux, in a 14:10h light:dark photoperiod. In the first 7 days, seeds' germination time was reported. At test end, growth (shoot length), fresh and dry weight were recorded. The hydric content ((DW-FW)/DW*100) was calculated at day 14 and compared within treatments.

Soil analysis

Compounds were analyzed by liquid chromatography coupled with electrospray ionization mass spectrometry (LC-API-ES-MS) and confirmed by LC-MS-MS technique.

Statistics

One way analyses of variance (ANOVA), followed by Dunnett's test, was used to analyse differences between control and treatments (SPSS, 1997). Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on Ranks was performed (Zar, 1996), followed by the Dunn's method when significant differences were found. Differences between control and control solvent were analysed using a t-test (SPSS, 1997). EC_{50} values were calculated using a sigmoidal (logistic, 3 parameter) equation (SPSS, 1997). LC_{50} values were calculated through Probit (SPSS, 2003).

Results

Eisenia andrei

Results of the exposure of *Eisenia andrei* to nicarbazin and monensin can be observed in figure 4.

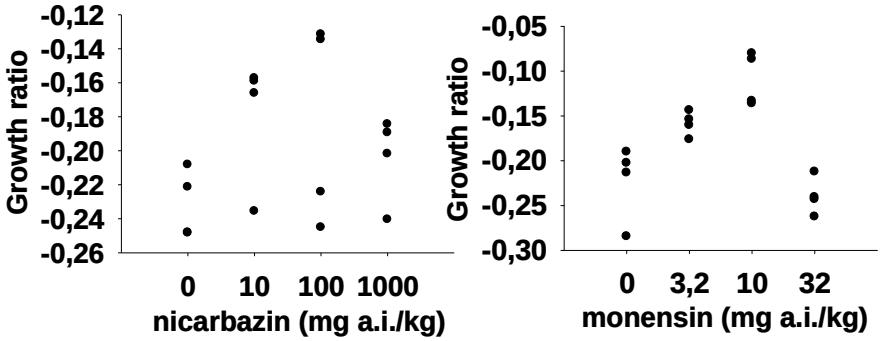

Figure 4 - Effect of nicarbazin and monensin in *Eisenia andrei* acute bioassay, in terms of growth ratio.

Results are shown as growth ratio, calculated using the following equation:

GR=(Wf-Wi)/Wi where GR is the growth ratio, Wi is the initial weight and Wf is the final weight.

For nicarbazin, no effect on survival could be observed (one dead worm in the highest concentration in one single replicate). In terms of biomass loss, there was no significant differences between the control and treatments (ANOVA, $F_{3,15} = 0,074$; $p = 0,973$).

For monensin, there was no effect of the solvent used (t test, $p>0.05$). Monensin caused an effect on the worms' survival (LC_{50}=42.7mg/kg) (Tab. 2) and in terms of biomass, showed as growth rates, there was a stimuli in the organisms exposed to the concentration of 10mg/kg, showing an increase of approximately 20% when compared with the control group. The EC_{50} value was not possible to calculate.

Folsomia candida

Results of the exposure of *Folsomia candida* to nicarbazin and monensin can be observed in the following figure (Fig. 5).

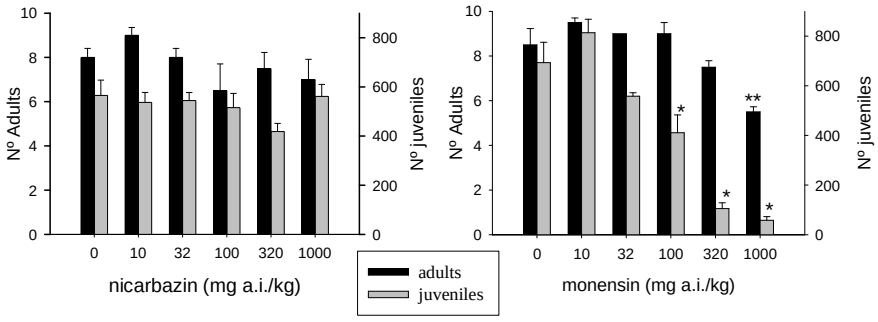

Figure 5 - Effect of nicarbazin and monensin in *Folsomia candida* chronic bioassay, in terms of survival (number of adults) and reproduction (number of juveniles). Results are expressed as average plus standard error. *- Dunnett's test, p≤0.05, for juveniles production; **- Dunnett's test, p≤0.05, for adult's survival.

Nicarbazin caused no effect on survival and reproduction of the organisms (ANOVA, p>0.05).

When testing monensin, there was no effect between the control and the control solvent (t-test, p>0.05). There was a dose-response pattern at the reproduction level, with significant effects between control and the concentrations of 100, 320 and 1000mg/kg (ANOVA, Dunnett's method, p≤0,05) and the EC_{50} value obtained was of 100.6 mg/kg.. For adults, significant difference was observed only between control and the highest concentration (ANOVA, Dunnett, p≤0,05).

Plants

Results of the exposure to nicarbazin and monensin of *Brassica rapa* and *Triticum aestivum* can be observed in figures 6 and 7.

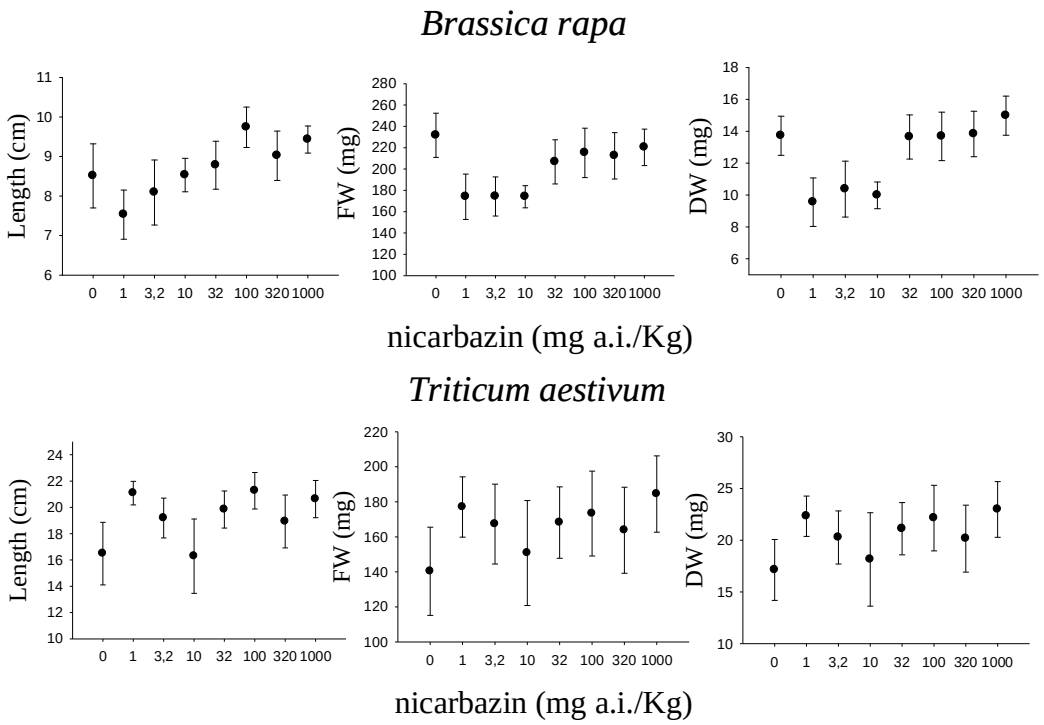

Figure 6 - Effect of nicarbazin on plant length, fresh weight (FW) and dry weight (DW) of *Brassica rapa* and *Triticum aestivum*. Results are expressed as average plus and minus standard error. *- Dunn's Method, p≤0.05.

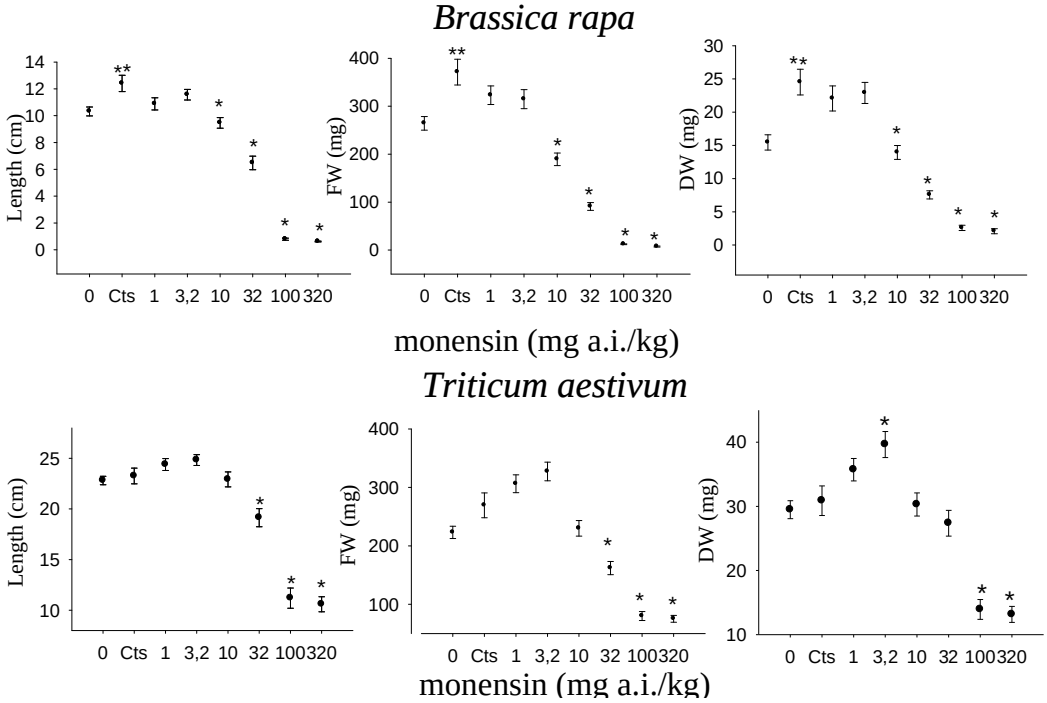

Figure 7 - Effect of monensin on plant length, fresh weight (FW) and dry weight (DW) of *Brassica rapa* and *Triticum aestivum*. Results are expressed as average plus and

minus standard error. *: Dunnett's test, p ≤0.05; Cts- control solvent and **: t-test, p ≤0.05.

For nicarbazin, no dose response effect could be observed in both plant species, (ANOVA, p >0.05).

When testing monensin, there was a significant effect caused by the solvent for *Brassica rapa* (t-test, p ≤0,05) while no effect was observed in *Triticum aestivum* (t-test, p>0.05). A dose-response could be observed for both tested plants. $EC_{50,}$ NOEC and LOEC values are presented in Table 2. For *Brassica rapa*, significant differences were observed for all measured endpoints, between the control and the test concentrations of 10, 32, 100 and 320 mg/kg (ANOVA, Dunn's Method, p≤0.05). For *Triticum aestivum,* significant differences occurred between control and the test concentrations of 32, 100 and 320mg/kg, except for dry weight, where there was a significant stimuli at 3.2mg/kg and a significant decrease at 100 and 320mg/kg (ANOVA, Dunnett's Method, p≤0,05). The percentage of water retained in plants of *T. aestivum* exposed to monensin was significantly higher in the control when compared with the highest treatments (32, 100 and 320 mg/Kg) (Dunn's Method, p≤0.05). In the *B. rapa* experiment, this effect was even more pronounced with all concentrations inducing a significant decrease in the hydric content, with the exception of the lowest one (Dunn's Method, p ≤0.05).

Table 3 - Toxicological values calculated for the different test species and endpoints when exposed to monensin: EC_{50}, LC_{50}, LOEC and NOEC obtained for all tests performed. n.d.: not determined.

Test species	EC_{50} / LC_{50} (mg/kg)	LOEC (mg/kg)	NOEC (mg/kg)
Eisenia andrei			
Biomass	n.d.	32	100
Survival	42.7	32	n.d.
Folsomia candida			
Juveniles	100.6	100	32
Adults	1261.8	1000	320
Brassica rapa			
Plant length	32.8	32	10
Fresh weight	11.8	32	10
Dry weight	15.2	32	10
Triticum aestivum			
Plant length	135.0	32	10
Fresh weight	40.3	32	10
Dry weight	99.9	100	32

Soil

The actual chemical analysis of nicarbazin in soil showed a recovery varying from 63 to 93% of nominal values (Table 4), meaning that this chemical is very stable in soil and that the concentrations were maintained throughout the test periods. So, all calculations were based on nominal concentrations.

Table 4: Analitical soil data resulting from chemistry analysis of nicarbazin in soil, showing the nominal and measured concentrations at the test ends.

Nominal concentration in soil (mg/kg)	Measured concentration at day 0 (mg/kg)	Recovery (%)	Measured concentration at test end (mg/kg)		Average stability of Nicarbazin in soil in the end of the test (%)
***Eisenia andrei* (14 days)**					
10	6.73	67	6.83		101
100	101.7	102	99.3		98
1000	762.3	76	815.8		107
***Folsomia candida* (28 days)**					
10	8.83	88	6.49		73
32	20.1	63	21.5		107
100	95.2	95	95.7		101
320	299.2	94	264.8		89
1000	766	77	743.4		97
***Triticum aestivum* & *Brassica rapa* (14 days)**					
			T. aestivum	***B. rapa***	
1	0.89	89	0.59	1.33	108
3.2	2.77	87	2.36	2.21	82
10	8.83	88	7.93	7.99	90
32	20.1	63	25.1	20.55	114
100	95.2	95	86.8	57.5	76
320	299.2	94	320.5	320.5	99
1000	776	78	808.3	808.3	104

Discussion

The overall results with the VP nicarbazin showed that this compound causes no harmful effect to the tested soil organisms and plants, within a broad range of concentrations. Additionally, the average concentration found in field soil is around 10-20 mg/kg (Furtula et al., 2005), a much lower dose than the ones tested here. Therefore this chemical is unlikely to pose immediate concern. The low toxicity may be related with its low solubility and bioavailability as pure compound. Analyses made in this study to determine the presence of nicarbazin in soil detected that four weeks after

application in the soil the concentrations measured varied from 63% to 95% of the nominal values.

Contrastly, the other VP tested, monensin, caused a much higher effect on the tested species. In *Eisenia andrei* there was a high effect on survival. Nevertheless, biomass changes were not statistically significant at nonlethal concentrations and there was even a growth stimuli at 10mg/kg. Further studies should include the assessment of effects at the reproduction level. In *Folsomia candida* there was a very low effect on adults (LC_{50}>1000mg/kg) but the reproduction was highly affected. In plants, both test species were affected, showing a dose-response pattern, with *B. rapa* being more sensitive than *T. aestivum*. An interesting fact was the stimuli caused by the solvent acetone in both species, which is in accordance with evidences showed in previous studies. In fact, Bhattacharya and coauthors (1985) showed that acetone, among other solvents (e.g. ethanol and methanol), had an enhanced effect on root formation on etiolated hypocotyl cuttings of *Vigna radiate*; In another study with wheat, onion, garlic, and cabbage, low acetone concentrations increased the ability of callusing and meristem formation, as well as the capacity to regenerate (Vnuchkova et al., 1993).

In the exposure to monensin it was observed that this VP induces changes in the osmotic regulation of plants. The hydric content was affected by monensin even at low concentrations. However, monensin seems to be of little concern to the environment: in a study where 5 sites were analyzed (with two in areas of great impact of urban and agricultural activities), the highest concentration found in the soil was 31,5µg monensin/kg (Kim and Carlson, 2006), which is much lower than the concentrations used in this study. Similarly, studies with other veterinary pharmaceutical compounds (e.g. tylosin, oxytetracycline) demonstrate that the effect concentrations for soil fauna are normally higher than the ones found in the environment. Baguer and coauthors (2000) showed that the concentration effect for reproduction of *Folsomia fimetaria* and *Aporrectodea caliginosa* exposed to tylosin was 5000mg/kg and 4000mg/kg respectively, and no effect was observed in the exposure to oxytetracycline for both species in the same concentrations range.

Conclusion

The effect of veterinary pharmaceuticals depends greatly on the chemical compound, varying from a no effect scenario (nicarbazin) to a higher effect (monensin). For monensin, species sensitivity decreased in the following order: *Brassica rapa* > *Triticum aestivum* > *Eisenia andrei* > *Folsomia candida*, with EC_{50}s ranging between approximately 10 and 100mg/kg. Nevertheless, neither nicarbazin nor monensin showed to be causing an ecological risk.

From the EC_{50}, LOEC and NOEC values one can conclude that single species tests by their own are not sufficient to evaluate properly the potential ecotoxicity of chemical compounds. In addition, several endpoints showed also dissimilar sensitivities towards exposures. This might be due to the fact that chemical compounds can act in specific targets that are later traduced in specific changes. If the endpoint studied is not related to the target, it can lead to wrong assumptions. Hence, the use of a test battery and higher effect levels when assessing chemicals in the environment is highly desirable and recommended.

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Chapter III

Discussion and Conclusions

Discussion

Generally, the impact of antimicrobial drugs administered to animals in the different environmental compartments have many dependent variables such as the amount of chemicals used, type of administration, animal husbandry practices, animal metabolism, manure handling and storage and degradation rates (Kemper, 2007).

Many studies are being developed worldwide to study the behaviour and toxicity of these compounds in the environment and their inherent consequences to the soil fauna and flora.

In studies reported by Baguer et al., (2000), two veterinary pharmaceuticals (tylosin and oxytetracycline) showed a low toxicity to soil dwelling fauna, with EC_{10} values around 150mg/kg. The highest concentration of monensin found in the soil in another study carried by Kim and Carlson (2006) was 31,5µg monensin/kg. These results as well as the ones obtained in this work showed that the effect concentrations are higher than the ones usually found in the environment.

The overall results presented in this study with the VP nicarbazin showed that this compound causes no harm to the tested soil organisms and plants, within a broad range of concentrations. Additionally, the average concentration found in the field is around 10-20 mg/kg of soil (Furtula et al., 2005), a much lower dose than the ones tested here. Therefore this chemical poses also no immediate concern. However, this compound is very stable and four weeks after its application in soil the concentrations measured varied from 63 to 95% of the nominal initial values. This confirms that nicarbazin is quite persistent and may have some impact on terrestrial and aquatic ecosystems in longer-term periods.

Dolliver et al., (2008) studied the degradation of three antibiotics (chlortetracycline, monensin and tylosin) during manure composting during a period of 22-35 days. For chlortetracycline it was observed a reduction on its concentration of 99%, whereas for monensin and tylosin the observed reduction ranged from 54 to 76%. The half-lives for chlortetracycline, monensin, and tylosin were 1, 17, and 19 d, respectively. Other studies carried out by Carlson and Mabury (2006) detected half-lives of 4.5, 24, and 3.3 days with the addition of manure, and of 6.1, 21, and 3.8 days without manure for tylosin, chlortetracycline and monensin respectively. All these compounds were not highly mobile with the exception of chlortetracycline that was the only antibiotic founded at 25 to 35 cm depth.

The long-term dispersion of liquid manure on fields may create serious problems of contamination, mainly when these compounds have a potential to accumulate in the soil and be biomagnified throughout food chains (Kemper, 2007).

In addition, another matter of concern is the exposure of bacteria to repeated dosages of these compounds. The long-term and repeated exposure can cause an adaptation to the presence of VP and increase resistance (Gavalchin and Katz, 1994). This might lead in the future to an increase in dosages or to the creation of other compounds with higher toxicity, due to the development of resistance mechanisms in the bacteria, as already reported for other medicines.

Further research should be conducted using these VP compounds as a formulation, for better simulating real scenarios.

Conclusion

The overall results of this study showed that the veterinary pharmaceutical nicarbazin causes no harmful effect to the plants *Brassica rapa* and *Triticium aestivum* as well as to soil organisms (*Eisenia andrei* and *Folsomia candida*), within a wide range of concentrations. Contrastly, for monensin a dose-response pattern was found but the concentrations that impaired the tested organisms are higher than the ones that can be found in real scenarios in soils.

As previously described, in the discussion of the results, other studies carried out with VP compounds have demonstrated similar results concerning the usual concentrations found in the environment, which are much lower than the concentration ranges used in the laboratory to obtain a dose-response of the compounds.

Usually, ecotoxicity studies use VPs as pure compounds for their exposure assessment, and this has been sometimes difficult due to the lower solubility of these compounds. Hence, formulations should also be assessed because they will make easier the adsorption of the compound to the soil particles and organic matter, and will also make possible its incorporation in the soil interstitial water. This will better simulate real scenarios and as such deserves further research.

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