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THE USE OF TERRESTRIAL AND AQUATIC MICROCOSMS AND MESOCOSMS FOR THE ECOLOGICAL RISK ASSESSMENT OF VETERINARY MEDICINAL PRODUCTS

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Abstract—In this paper, we investigate the applicability of experimental model ecosystems (microcosms and mesocosms) for the ecological risk assessment of veterinary medicinal products (VMPs). VMPs are used in large quantities, but the assessment of associated risks to the environment is limited, although they are continually infused into the environment via a number of routes. It is argued that the experience obtained by pesticide research largely can be used when evaluating VMPs, although there are several major differences between pesticides and pharmaceuticals (e.g., knowledge of their mechanisms of action on nontarget organisms). Also, because microorganisms are often the target organisms of VMPs, risk assessment should focus more on endpoints describing functional processes. This paper provides a review of the current risk assessment schemes of Europe and North America along with examples of experiments already performed with veterinary medicinal products in aquatic and terrestrial ecosystem models. We suggest that some of the approaches developed for pesticide risk assessment can be used for VMPs and offer suggestions for the development of a framework for ecological risk assessment of VMPs.

Keywords—Microcosm Mesocosm Environmental risk assessment Veterinary medicines Pharmaceuticals

INTRODUCTION

Background

Data from a few European countries indicate that, among veterinary medicinal products (VMPs), antimicrobial substances are sold in the highest amounts, followed by coccidiostats, sheep dip chemicals, growth promoters, endoparasitic dewormers, anti-inflammatory preparations, and enteric preparations. In general, however, information on the usage of individual VMPs is difficult to obtain [1]. In some estimates of antimicrobial use of VMPs, farm animals consumed 4,700 tons (35%) of all antibiotics administered in the European Union, largely for therapeutic purposes (29%), whereas humans consumed 8,500 tons (65%) [2]. In the United States, it is estimated that farm animals consume 70% of all antibiotics administered, approximately 11,200 tons [3].

Although many VMPs have relatively short half-lives, they can take on a pseudo-persistence or chronic exposure nature because they are continually infused into the environment via a number of routes. It is thought that the most important routes of entry into the environment are direct discharge of aquaculture products (e.g., water used for culture of aquatic organisms), treatment of pasture animals, application of manure and slurry to land, and associated runoff to the aquatic ecosystem [4,5]. Once in the environment, VMPs and their transformation products can be degraded, transported between the different environmental compartments, or both [1,6]. Pharmaceutical compounds from human and agricultural sources have been detected in soils, surface waters (ng/L to μg/L range), and ground waters of many countries [5–9].

Measured concentrations of individual VMPs are generally low; however, the combined concentrations of VMPs of similar modes of action (concentration addition) and the combined effects of differing modes of action (response addition) could prove toxicologically significant in the environment. Interactions such as synergism and antagonism have been reported in humans and animals at therapeutic doses, but interactions in nontarget organisms under actual environmental exposure levels are unknown. Because over 4,500 parent active ingredients are in use [10] and because these occur in mixtures that are known to be biologically active in some organisms, there is a clear need to consider relevant combined toxicities in assessing ecological risks of these products.

A first essential step in risk assessment of VMPs is the problem formulation or prerisk assessment analysis phase, with particular emphasis on which ecosystem components are at risk (i.e., the conceptual model). For risk assessment, it is very important if the potential risk is, for example, related to amended soils or runoff from concentrated feedlot operations. This risk assessment scenario also determines whether mixtures should be addressed and, if so, which mixtures are relevant.

Microcosms and mesocosms

Single-species toxicity tests are the most frequent source for effect data in hazard and risk assessments. Standardization and reproducibility are considered key advantages for these assays. However, this simplicity is associated with several limitations that might be inherent to the conceptual design; for example, single-species toxicity tests cannot detect indirect

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effects. In addition, single-species tests are designed to achieve homogenous test conditions, whereas in real systems, including agricultural land and aquatic ecosystems, physicochemical and biological conditions vary significantly in the horizontal and vertical dimension [11,12]. To overcome these drawbacks, microcosm and mesocosm studies were proposed. Without differentiating between aquatic and terrestrial compartments, Giesy and Odum [13, p 4] defined microcosms as: ''Replicable, artificially bounded subsets of naturally occurring environments with several trophic levels.'' We are convinced that this definition for microcosms can be extended to mesocosms when taking into consideration the distinction discussed in the next paragraph. Hence, the general definition has been continued by introducing the term ''cosm,'' a term that includes both microcosms and mesocosms. Cosms are limited in size, time, and mass of both biotic and abiotic components, and they have boundaries that restrict interaction with the rest of the ecosystem. Cosms do not exactly mimic natural systems at all levels of organization, and they are incapable of self-perpetuation. To be a useful tool, cosms should to a large extent minimize variability without reducing realism [14]. However, gaining replicability in the behavior of cosms in space and time usually results in decreased ecological realism. The size and complexity of cosms used depend on the nature of the question to be answered or the hypothesis to be tested.

There are roughly two ways to distinguish between microcosms and mesocosms: one based on size and one based on the ecological representativeness of the system. In the first definition, an aquatic mesocosm has a volume of 15 m^3 or a length of 15 m or more, whereas a microcosm is defined as a model ecosystem of <15 m^3 or <15 m length [15]. In the second definition, microcosms are generic systems (i.e., generic and defined test systems in which species composition and abiotic characteristics are enforced by the operator), whereas mesocosms are more natural, semirealistic systems with an indigenous (natural) combination of organisms and abiotic conditions.

Cosms can be used to evaluate the fate of chemicals and their effects on structural and functional aspects of ecosystems and to develop conceptual models of chemical transport, fate, and effects [16,17]. Additionally, they allow prognostic approaches for environmental risk assessment of, for example, plant protection products, biocides, and genetically modified microorganisms (e.g., [13,18–21]). The use of cosms is limited in case of assessment of effects of compounds on, for instance, long-lived, higher organisms with a complex life cycle and/or a behavior that requires the presence of several habitats and/or more complex community structure to survive (e.g., birds, mammals, larger fish, and amphibians). Because of their complexity and the costs associated with conducting model ecosystem experiments, microcosms and mesocosms are not appropriate for routine screening of hazardous materials. Hence, it appears that cosms might have their greatest utility for assessing environmental risks when used in a tiered approach, which has been shown to be successful in cases of pesticide registration [22]. Cosm experiments are also valuable tools to validate the lower tiers of risk assessment.

Endpoints in cosm studies

Microcosms and mesocosms have a long history of use in pesticide risk assessment in evaluating the fate and effect of pesticides in soils and surface waters [14,22–27]. In this field, considerable groundbreaking work has been done that could be applicable in the regulation of VMPs. Pesticides in general are designed to minimize the effects of nontarget species, and, as such, their mode of action is often selective to specific target species (e.g., weeds and insects). In the assessment of pesticide risks to nontarget organisms, the majority of lower tier studies focus on mortality and inhibition of growth as the main endpoint [28]. In addition, in higher tier risk assessments, endpoints often focus on the dynamics of populations and the structure and functioning of communities and ecosystems. Community structure is related to the abundance and biomass of all populations and their spatial, taxonomic, and trophic organization, wherease function relates to the processes and the changes in time and flows (e.g., organic matter production and degradation, nitrification, mineralization, soil respiration). An overview of structural and functional endpoints that are frequently studied in aquatic experimental ecosystems is provided by Brock and Budde [29] and Kersting [30], respectively.

Because of their properties and use, it is expected that the primary effect of pesticides is on the mortality of sensitive nontarget organisms and, from these direct toxic effects and resulting shifts in species interactions on community structure, are followed by changes in ecosystem processes (indirect effects). In contrast, VMPs are often not directly designed to eradicate organisms in field ecosystems; rather, they are used to cure animals of infections by microbes and parasites. As such, VMPs usually do not have broad effects on nontarget organisms frequently studied when assessing risks of pesticides (exceptions are VMPs with antiparasitic activity that might also be used as pesticide). Most VMPs are distinctively different from pesticides when mode of action is considered, which should have consequences in the choice of endpoints when assessing their risks. In this study, it is hypothesized that in the risk assessment of VMPs, the order of importance of endpoints is an ecosystem function (mainly driven by microorganisms and aquatic plants), followed by endpoints related to population and community structure. Although microorganisms are present and functionally important in all ecosystems, they are seldom identified and enumerated on the basis of taxonomic keys. It is usually easier to study their functional responses. Consequently, guidance documents on testing procedures for pesticides in freshwater model ecosystems do not mention recommendations to perform measurements on structural aspects of microbial populations [29], although it can easily be hypothesized that effects on microbial populations can lead to indirect effects such as inhibition of decomposition and to a decreased availability of suitable food for macroinvertebrates [29,30].

Although it is recognized that the different types of endpoints (structural, functional) cannot be addressed separately, it is important to acknowledge the fact that differences in compound design should also be reflected in the design of risk assessment procedures. In this study, the use of cosms in the risk assessment of VMPs will be addressed in relation to the experience with cosms in pesticide regulatory studies. We will present several case studies and try to extract a general overview on the similarities and differences between the use of cosms in the regulation of pesticides and VMPs. This will be focused on the selection of endpoints.

REGULATORY BACKGROUND

Legislation, dossier requirements, and guidance documents in the European Union

In the European Union, Council Directive 81/852/European Economic Council (EEC) laid down the framework for the

overall risk–benefit analysis leading to marketing authorizations for VMPs [31]. It lasted to 1992 before the potential harmful effects on the environment caused by the use of VMPs was recognized (Commission Directive 92/18/EEC [32]). Today, both directives have been replaced by Directive 2001/82/EC on the community code relating to VMPs [33]. These directives have been implemented in the national legislation of the Member States but will also be applicable to the decentralized (more than one member state) and centralized (all member states) procedures for obtaining marketing authorizations. In the dossier accompanying an application for a marketing authorization, the potential harmful effects of the product to the environment must be assessed, and any precautionary measures that might be necessary to reduce the risks of harmful effects need to be identified. It must be noted that for feed additives (growth promoters, coccidiostats), a different risk assessment scheme is used than for veterinary medicines, which will not be dealt with in this paper. The risk assessment scheme of feed additives is described in a report of the European Medicines Agency [34].

Practical guidance for both applicants and assessors was laid down by the Committee for Veterinary Medicinal Products [35]. In this document, both Phase I (estimating exposure) and Phase II (assessing effects) of the risk assessment were described. The International Cooperation on Harmonisation (VICH) issued a Phase I guideline in 2000 for technical requirements for registration of Veterinary Medicinal Products [36]; this VICH Phase I guideline now has superseded the Phase I part of the Committee for Veterinary Medicinal Products document, and the VICH Phase II document was published for consultation at the beginning of 2004.

The VICH is a trilateral program, aimed at harmonizing technical requirements for veterinary product registration. Members are regulatory bodies and representative industry associations from the European Union, Japan, and the United States; government and industry representatives from Canada, Australia, and New Zealand are participating in the working process as observers.

The VICH Phase I document is a straightforward decision tree to identify products that can be exempted from further testing because they are unlikely to result in significant exposure of the environment and will consequently be of low environmental risk. The use of pet products or products based on natural substances, for example, usually will not lead to a harmful concentration or distribution in the environment and, as a result, will be identified in Phase I as products of low risk. Examples of products that are likely to be advanced to Phase II are VMPs for fish, unless such products are indicated for use in confined facilities only and the aquatic environmental introduction concentration ($EIC_{aquatic}$, i.e., the concentration of the product in the effluent) is <1 μg/L on entry into the environment. Also, products for which the predicted environmental concentration in soil (PEC_{soil}) exceeds 100 μg/kg and products with ecto- or endoparasiticide activity are expected to be advanced to Phase II. For the initial calculation of $EIC_{aquatic}$ and PEC_{soil}, a total residue approach is used, assuming that 100% of the administered dose is excreted as parent compound.

It is important to realize that for some VMPs, which in Phase I are considered of low risk, additional environmental information that addresses particular concerns could be required if there is a specific reason for that request (e.g., metabolites showing a different pattern of activity compared with the parent, or when it is expected that the mentioned trigger values are not safe for a specific substance). These situations, however, are expected to be the exception rather than the rule, and some evidence (e.g., in the form of results of experiments or monitoring programs in support of the concern) should be available.

For Phase II, at present, the old Committee for Veterinary Medicinal Products document [35] is still in use, but this will in time be superseded by the VICH Phase II document that is currently in preparation. Phase II is divided in two parts: Tier A and Tier B. Tier A makes use of simpler, less expensive studies, mostly described in the guidelines of the Organization for Economic Cooperation and Development (OECD), and focuses on evaluation of the possible fate and acute effects of drugs and their major metabolites [37]. For instance, the determination of degradation half-life of the active substance or relevant metabolites could find a place here. If within Tier A no hazard is detected or the risk management strategy proposed by the applicant takes care of eliminating any potential hazard, thus avoiding harmful effects of the product on the environment, there would be no need to proceed to Tier B. Tier B involves studies of chronic effects on flora and fauna within the environmental compartments of interest. In particular, for these chronic studies, recommended tests are not always available.

It is likely that in the upcoming VICH Phase II guideline an option might be to go beyond Tier B because more complex studies, specific to issues raised or relevant to a specific region, are necessary to complete the risk assessment. It is at this stage that experiments with microcosm and mesocosms can be of use to enable a realistic evaluation of the fate and (prolonged) effects of VMPs on the terrestrial and aquatic environment.

Legislation, dossier requirements, and guidance documents in North America

The United States is in the process of integrating methods used to conduct environmental assessments of VMPs [36,38] with several other countries and the European Union (the European Union is discussed in the previous section). These guidelines are not formally proscribed and are not yet binding. They are similar to environmental assessment guidelines applied to human drugs and biologics [39]. The guidelines for human drugs require data on the chemical identity; physical and chemical characteristics; environmental fate; and estimated environmental concentrations in soil, water, and air. The assessment of effects is tiered, with Tier 1 requiring only one acute toxicity test; Tier 2, a base acute data set with a fish, invertebrate, and alga for aquatic organisms or a plant, earthworm, and microbiological test for terrestrial organisms; and Tier 3, chronic testing, but only if the substance bioaccumulates. Assessment of risk is by hazard quotient (ratio of estimated environmental concentrations to effect concentration), and assessment factors of 1,000, 100, and 10 are applied to Tiers 1–3, respectively.

Canada is not party to the VICH process, and guidelines for assessment of pharmaceuticals have not been published. However, testing of products new to the market since July 1, 1994 (including pharmaceuticals), might be required under the New Substances Notification Regulations of the Canadian Environmental Protection Act [40]. A large number of substances used in commerce (>23,000) before 1994 in Canada are in the process of review and screening assessment, including some pharmaceuticals and VMPs.

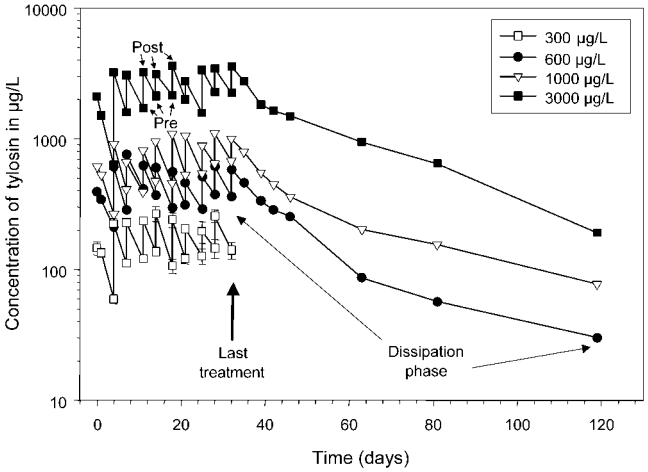

Fig. 1. Concentration of tylosin during and after treatment in three microcosms (mean + SD in 300-µg/L microcosms) and in unreplicated microcosms treated to maintain nominal concentrations of 600, 1,000, and 3,000 µg/L. Drawn from data by R. Brain (University of Guelph, Guelph, ON, Canada; see also [43]).

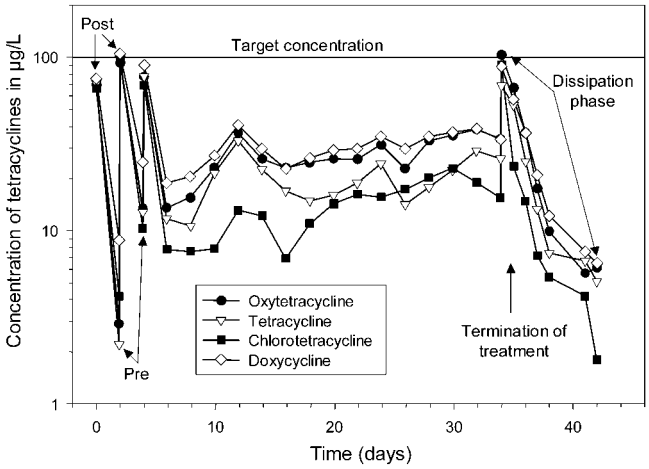

Fig. 3. Mean measured concentrations of tetracyclines in three microcosms treated at a target concentration of 100 µg/L. Initially, immediately posttreatment and pretreatment concentrations were measured. After day 6, only pretreatment concentrations were measured until the last day of treatment when the posttreatment concentration was measured and the dissipation followed over time (R. Brain, University of Guelph, Guelph, ON, Canada, personal communication).

AQUATIC MODEL ECOSYSTEM STUDIES PERFORMED WITH VMPS

Several microcosm studies have been conducted at the University of Guelph Microcosm Facility (Guelph, ON, Canada) on veterinary pharmaceuticals, either singly or as mixtures with other pharmaceuticals for both human and veterinary use. The microcosms used have a volume of ~12,000 L and have been described in detail elsewhere [41]. Water is circulated between the microcosms and the irrigation pond at a rate of ~12,000 L/d for at least two weeks before treatment to ensure consistent initial assemblages of zooplankton, algae, and water chemistry parameters in each microcosm. The microcosms are commonly stocked with caged fish (*Pimephales promelas* L., *Lepomis gibbosus*), or both, to reduce their predation activity in the microcosms. Potted macrophytes (*Mirophyllum sibricum* and *Myriophyllum spicatum*), obtained from a nearby reservoir, are normally placed in each microcosm to provide habitat for zooplankton and, along with the floating macrophyte *Lemna gibba*, is used for effect assessment. Before and after treatment, physical (temperature), chemical (concentration of pharmaceuticals, pH, oxygen concentration, etc.), and biological parameters are measured on a regular basis. Biological parameters include survival of fish, numbers and diversity of zooplankton and macroinvertebrates, algal pigments, survival and growth of macrophytes, and functional microbiological activity [42].

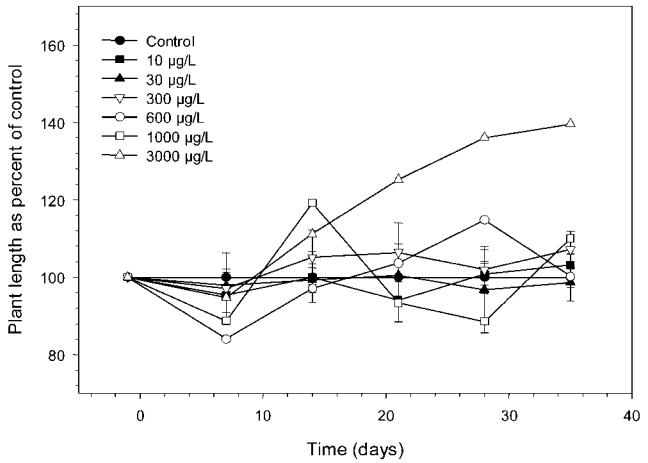

Fig. 2. Response of *Myriophyllum spicatum* length to various tylosin in aquatic microcosms. The data for 10 to 300 µg/L represent the mean (±SE) for three replicates; treatments of 600 to 3,000 µg/L were unreplicated. Redrawn with permission from data of R. Brain (University of Guelph, Guelph, ON, Canada [43]).

Aquatic microcosms have been used in this way to assess dissipation of veterinary pharmaceuticals under more realistic field conditions. In assessing effects in aquatic systems, the initial focus has been on VMPs that are used routinely or continuously, such as feed additives and growth-promoting products. To emulate constant inputs into the environment, VMPs were added to microcosms at regular intervals to maintain a constant concentration. This required rapid analysis to allow adjustment of the treatment amounts to maintain the target concentration. However, at the end of the study, dissipation was observed and rate constants were calculated.

For the study of veterinary pharmaceutical tylosin, three replicated microcosms were each treated with 0, 10, 30, and 300 µg/L and one replicate each with 600, 1,000, and 3,000 µg/L [43]. Tylosin showed dissipation profiles that approximated first-order kinetics at all three concentrations in which dissipation was followed (Fig. 1). Biological responses to tylosin treatments in the microcosms were few. Fish were unaffected. Total zooplankton numbers and chlorophyll concentrations showed no consistent response to concentrations of tylosin. Macrophytes (*Myriophyllum sibericum*) showed no adverse effects in response to tylosin, but, at the greatest concentration tested, wet and dry mass decreased and internode length showed a stimulatory effect (Fig. 2). This was also observed in *L. gibba* in the laboratory [44].

Similar rapid dissipation of tetracyclines was observed from the water column in microcosms treated with a mixture of tetracycline, chlortetracycline, oxytetracycline, and doxycycline (Fig. 3). For this study, three replicate microcosms each were treated with 10, 30, 100, and 300 µg/L of the mixture (R. Brain, University of Guelph, Guelph, ON, Canada, personal communication). Results of these microcosm studies

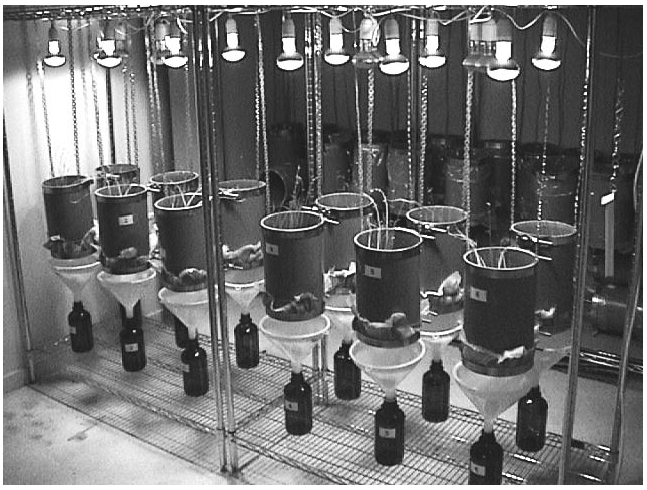

Fig. 4. The Multispecies Soil System (MS·3) arable land, a soil microcosm designed for assessing the effects of veterinary medicines on agricultural soils.

confirm laboratory studies that have suggested rapid dissipation of these classes of pharmaceuticals from waters under field conditions [5,45].

MULTISPECIES SOIL SYSTEM MICROCOSM STUDIES PERFORMED WITH VMPS

Terrestrial microcosms can be constructed from intact soil cores collected in the field or as artificial assemblages built on sieved soil. Morgan and Knacker [46] introduced the term Terrestrial Model Ecosystem, which has been used in several cases for naming these systems. In 1996, the U.S. Environmental Protection Agency published guideline 850.2450 of the Office of Prevention, Pesticides, and Toxic Substances for conducting the Terrestrial (Soil-Core) Microcosm Test [47]. The system is based on intact 60-cm soil cores collected in the field, and the recommended duration is 12 weeks. The use of this method for agricultural soils requires significant modifications. The U.S. Environmental Protection Agency guideline recommends moving aside the ploughed soil, collecting the subsoil core, and backfilling the upper part of the microcosm with the ploughed soil in the laboratory. However, some results suggest that the lower biomass of agricultural soils produces difficulties when interpreting results from these studies because of the higher variability and the low numbers of individuals from some species or taxa observed in some replicates [48].

The alternative design is based on artificial assemblages. Natural soils are collected, sieved, and used for building up soil cores into which soil macroorganisms are introduced. Some designs are relatively large, such as those proposed by Weyer and Schuphan [49], whereas the Multispecies Soil System (MS·3) developed at the Spanish National Institute for Agriculture and Food Research and Technology (INIA; Madrid, Spain) are constructed with soil cylinders of just 2 to 6 kg [50,51]. Within the European Union research project Environmental Risk Assessment of Veterinary Medicines in Slurry, a specific MS·3 was designed specifically for testing VMPs. The system, the MS·3 arable land (Fig. 4), reproduces the conditions of arable agricultural soil in which manure is applied as fertilizer [52]. The system can also be applied for assessing other substances reaching agricultural soils through the application of fertilizers or manure. In this paper, no recommendation is made on whether intact soil cores or artificial assemblages should be used, but some examples of studies evaluating VMPs in soil microcosms are presented.

Table 1. Description of the proposed modified Tier 1 Multispecies Soil System arable land for assessing veterinary medicines

Matrix	Column of sieved (2 mm) agricultural soil (20 cm depth × 20 cm diameter), biologically active and maintained in a plastic cylinder connected to a leachate collecting system
Chemical	The veterinary medicine is homogeneously mixed with the soil at concentrations of 0.01, 1, and 100 mg/kg wet weight
Test organisms	The agricultural soil provides the microbial population. Seeds of three plant species and adult earthworms are added within 24 h
Climatic simulations	Columns are maintained at 20°C, light (daylight lamps; 8,000 lux; 10:14 h light: dark) provides a temperature gradient of soil surface. Irrigation (three times a week) simulates an annual rainfall of 1,000 mm

The list of toxicity endpoints to be evaluated with MS·3 microcosms depends on the introduced species and the testing needs. It is suggested that the following endpoints be included.

Fate properties. Soil degradation, leaching potential for parent and metabolites, bioaccumulation in plants, and soil-dwelling invertebrates.

Soil microorganisms. Respiration and one enzymatic activity selected from modified Tier 1 tests (e.g., phosphatase and dehydrogenase activity). Effects must be assessed on at least two soil layers and at different times (assessing potential for recovery).

Antimicrobials. Indirect effects on nitrification (analysis of inorganic nitrogen in leachate) and acclimatization (dose–response curves for freshly added drug at −1, 7, and 21 d postapplication).

Plants. Seed germination and growth expressed as biomass production or length, or both, for *Triticum aestivum*, *Brassica napus*, and *Vicia sativa*.

Earthworms. Lethality of *Eisenia foetida* after exposure period of 14 to 21 d.

Fish cell lines, daphnids, and algae. Toxicity of leachate after the first and last irrigation/rain simulation event (representing nonaged and aged soil leaching processes, respectively).

The MS·3 assay can be used at two different levels: as a modified Tier 1 test and as a higher tier test. A modified Tier 1 test can replace the standardized single species tests, producing information on the sensitivity of the three taxonomic groups usually employed in soil testing (plants, earthworms, and microorganisms). The system not only combines three tests in one, but produces information on soil dissipation, soil mobility, and toxicity of leachate. The hazard identification and characterization profile obtained with MS·3, in combination with some conceptual models [53,54], can be enough for the screening risk assessment and for targeting the risk refinement if required.

Settings for the modified Tier 1 test are presented in Table 1. Higher tier testing requires specific protocols especially designed to fulfill the assessment needs. The protocol is designed on the basis of the available information on the fate and effect profile, agricultural application conditions, etc. Some of the

Table 2. Studies with veterinary medicinal products conducted on Multispecies Soil System arable land assay. Some experiments were conducted to set the optimal conditions or test different options; therefore, the experimental approach differs from the suggested protocol described in the paper

Tested pharmaceutical	Tested condition	Main results
Doxycycline	Higher tier: effect of spiked and aged pig manure	Manure affects fate in soil and effects on microbial populations
Eprinomectrin	Higher tier: effect of formulated product	Toxicity of leachate on daphnids
Oxytetracycline	Modified Tier 1 test	Agreement with Organization for Economic Cooperation and Development tests
Oxytetracycline	Higher tier: effect of manure coaddition	Effects and recovery of microbial populations are accelerated
Oxytetracycline	Higher tier: effect of additional microbial endpoints	Acclimatization of microbial population
Sulfachlorpyridazine	Modified Tier 1 test	Rapid dissipation and phytotoxicity
Sulfachlorpyridazine	Higher tier: effect of additional microbial endpoints	Acclimatization of microbial population
Tetracycline	Higher tier: testing additional microbial endpoints	Feasibility for including pollution-induced community tolerance as endpoint
Actual mixtures	Higher tier: effect of actual pig manure	Dose–response with hormesis effects for some endpoints

possible variables to be considered are types of soil macroorganims to be included and endpoints, toxicity endpoints for soil microorganisms (including acclimatization, biodiversity), local and climatic conditions (soil, temperature, soil, light, rainfall), soil column depth (arable only, arable plus additional soil layer), application of the tested chemical (on soil surface, homogeneous distribution on soil top layer or soil arable layer, resembling liquid manure application), and coapplication of manure (including application of spiked and aged manure).

Table 2 summarizes the studies conducted with MS·3 soil microcosms on veterinary pharmaceuticals. Both modified Tier 1 and higher tier assays have been conducted. The modified Tier 1 tests conducted on several antimicrobial agents showed consistent results when compared with standard single species tests. The antimicrobials were mixed with the soil before the addition of soil macroorganisms. Initial exposure levels are, therefore, similar to those observed in the standardized single species tests, although the fate of the pharmaceutical can differ significantly. For example, leaching of sulfachlorpyridazine represented a significant contribution to the dissipation of this drug from MS·3 (C. Alonso, Madrid, Spain, personal communication). This phenomenon does not play a role in the standard single species assays. Nevertheless, the MS·3 results were comparable to those obtained in the standardized OECD tests (OECD 207 on earthworms; OECD 208 on terrestrial plants; OECD 217 on soil microbial respiration [37]). As expected, both approaches showed microbial organisms as the most sensitive soil taxonomic group for antimicrobials. Effects on soil respiration were mostly observed at concentrations around 10 mg/kg soil.

The MS·3 allowed study of some additional endpoints that showed higher sensitivity than was observed for the traditional OECD endpoints. Biomass or length, or both, of the aerial part of some plant species showed clear negative effects at 100 mg/kg soil. In fact, the sensitivity of vascular plants to some antimicrobials has been recently described [55]. The effects observed might be a result of irrigation in MS·3, which could enhance the exposure of water soluble chemicals to plants. Effects on soil microbial enzymatic activities were mostly observed at concentrations one order of magnitude lower than those producing effects on soil respiration. Nevertheless, the studied enzymatic activities (phosphatase and dehydrogenase) showed different sensitivities depending on the tested VMP.

The higher tier MS·3 demonstrated the capability of this tool for studying effects under more realistic conditions. Manure addition provoked significant differences in both the fate and the effects observed for VMPs [52]. Some modifications of the testing protocol allowed the inclusion of additional endpoints, such as effects on the structure of microbial populations analyzed through the pollution-induced community tolerance approach [56] or studies on acclimation of microbial populations, although comparison on dose–response curves were conducted at different time points throughout the experiment.

The experiments conducted with eprinomectrin showed the ability to combine MS·3 and aquatic toxicity tests. This antiparasitic drug is highly toxic for several arthropods, including cladoceran crustaceans. Leachates from soils containing 0.01 mg/kg soil or more showed acute toxicity on *Daphnia magna*.

The MS·3 arable land seems to be an effective tool for testing the effects of veterinary pharmaceuticals and other agrochemicals reaching agricultural soils [52]. The assay, based on an artificial assemblage, can reproduce the key conditions of an agricultural system. The ecological relevance is obviously much lower than that observed for intact soil cores. Nevertheless, the use of intact soil cores for arable land requires protocol modifications [47] and creates several problems from the effects of tillage on the soil structure and on the biological community [48] because the ploughed soil cannot be collected in the intact core and must be moved aside and added later and because the systems required the addition of plants and, in some cases, other organisms.

DISCUSSION AND GUIDANCE

Pesticide experience

Cosm studies have been used widely in the assessment of the potential effects of pesticides in aquatic systems [25–27,57] and others. However, these studies have been conducted at higher tiers in the assessment process and certainly after laboratory tests have been conducted on standard test organisms under standard protocols. In this context, microcosms and mesocosms are used to test specific hypotheses related to ecologically more realistic exposure, indirect effects, resiliency, and recovery, which are useful parameters in assessing ecological risks at the field, community, and ecosystem levels. In the last 15 years, many workshops and associated guidance

documents on the experimental setup and conducting, analyzing, and interpreting microcosm and mesocosm experiments to evaluate the risks of pesticides have been organized and published [15,20–24], mainly for the aquatic environment. Much of this guidance and experience can be used in the ecological risk assessment of VMPs in cosms, although there are several major differences between pesticides and pharmaceuticals. In particular, the fast dissipating, contemporary pesticides usually enter the environment in short pulses according to their use pattern and thus have a distinct acute phase. Because pesticides are designed to have effects on pests, their mechanisms of action and specificity are reasonably well known. In these areas, experience with pesticides is not easily applied to pharmaceuticals. Because of the use pattern, many pharmaceuticals enter the environment constantly, and exposures are chronic in nature. Discharge of water used in indoor aquaculture, however, might cause acute pulsed exposure regimes in surface waters. Pharmaceuticals are developed to control diseases in humans and domestic animals, and, although their effects in vertebrates are reasonably well understood, they are not subjected to the same type of routine Tier 1 testing that is legislated for pesticides. With the exception of some parasiticides (which could also be used as pesticides), we have little direct or mechanistic knowledge of action in nontarget (in)vertebrates and plants. Because of the more frequent chronic nature of exposure, less obvious effects than mortality are likely to be important in assessing potential risks to the environment.

Other experience gained in the field of pesticides can be of great value for VMP risk assessment. Suggestions for the composition of cosms, application of the chemical, statistical design of the experiments, and endpoints to be sampled, as developed for experiments evaluating pesticides and applicable to VMPs, are: (1) During the establishing period, necessary action must be taken to ensure that, at the time of treatment, cosms are similar in biological and physicochemical characteristics. (2) The cosm should develop or have a flora and fauna consistent with the study objectives and, where appropriate, should be representative of natural field environments. (3) Aquatic cosms must contain a sediment layer and preferably macrophytes. (4) Vertebrates such as fish in aquatic cosms can present difficulties that need to be carefully considered, such as reducing the effects of fish by caging them. (5) Application of the test substance should usually be made in the period between spring and early summer. For short-term studies (up to one month), a single application is recommended. (6) An exposure–response experimental design with replication is preferred. (7) Cosms must be randomly assigned to treatments. (8) Univariate statistical methods are recommended for investigating effects at the population level and multivariate methods for describing community-level effects. (9) Structural and functional endpoints are generally equal in importance [22–24].

Endpoints to be studied in VMP experiments

As a higher tier tool, cosms offer the possibility for testing the effects of pharmaceuticals under realistic conditions, considering recovery of the initial effects and even the possibility for evaluating indirect effects. Food chain indirect effects can be tested by a proper selection of the assembled species [49]. For example, effects on soil microbial activity can affect the amount of nutrients available for plant growth, and earthworms can contribute to microbial acclimation and resistance by increasing the establishment of bacterial plasmids [58]. These indirect effects can be covered in the experimental design proposed for the higher tier cosms.

In this paper, we demonstrated that including functional measurement endpoints are important in the risk assessment of VMPs by means of micro/mesocosms because VMPs can directly affect microorganisms and assessing their functionality is much easier than determining their structure. That functional endpoints related to microbial activity indeed are sensitive is confirmed by the terrestrial work done by INIA [48,50–52]. Although effects on plants were observed at high concentrations (100 mg/kg), respiration proved to be a more sensitive endpoint (10 mg/kg), and effects on soil microbial enzymatic activities were even observed at concentrations as low as 1 mg/kg. This example clearly indicates the importance of including functional measurement endpoints related to microbial activity in the risk assessment of VMPs and therefore in the execution of cosm experiments.

The work done at INIA showed a potential high toxicity of VMPs (like eprinomectrin) to arthropods, whereas studies performed in Guelph showed few effects of tylosin on structural parameters. The specific effects of VMPs in surface waters is uncertain. Pharmaceuticals are biologically active, and, given a diverse array of chemical classes and pharmacological modes of action, which are often poorly understood in nontarget organisms, the number of potential nontarget drug–receptor interactions are difficult to estimate [59]. The use of cosms enables the testing of a number of species simultaneously and can be used to test hypotheses related to extrapolation from laboratory studies as well as to assess recovery and resiliency of populations and communities. In aquatic experiments already performed with VMPs, functional endpoints are not yet evaluated. The findings in the field of terrestrial ecotoxicology indicate a need in future experiments for inclusion of functional measurement endpoints indicative of microbial activity.

Framework for ecological risk assessment of VMPs

Frameworks for assessing risks of industrial chemicals and pesticides in the environment have been developed in a number of jurisdictions in which these substances are intensely regulated. Although pharmaceuticals are well regulated with respect to human health, regulations specifically addressing their potential effects in the environment are recent and scarce. A recent comparison of the European protocols for environmental risk assessment [60] balances the possibility of integration with specific needs. The situation is improving rapidly as a result of scientific (e.g., [61]) and regulatory (e.g., the VICH harmonization process) efforts. The methodologies developed for the exposure assessment of pesticides and other chemicals are, in principle, applicable to pharmaceuticals (e.g., [5]). The main differences focus on metabolism in animals and manure, requiring the assessment of a mixture of chemically related molecules, and the abundance of ionic forms. Extrapolation of the methods of assessing effects is much more complex because the pharmacological and pesticidal activities are not comparable. A formal framework for risk assessment of pharmaceuticals would encompass a problem formulation step that would define the type of exposure and effect data needed. It is here that use patterns, exposure routes, and exposure circumstances (continuous, episodic, etc.) will define the types of toxicity data needed and the testing conditions. The tests used need to address the possibility that some pharmaceuticals

might have subtle effects that are different from mortality. For example, an increase in growth or reproductive rate for one class of organisms is not necessarily ''good'' and could have significant ecological consequences [62].

Risk assessment for the soil compartment is particularly complex [63], and coapplication with manure must be considered when assessing the effects, as observed for doxycycline [52] and reported for other chemicals [64]. This coapplication should be also considered in the risk assessment framework.

So far, the European regulation deals only with single products and will, at present in most Member States of the European Union, only be applied to new products. Existing pharmaceuticals are, therefore, often not taken into account, the risk of mixtures is not considered, and even the risk of the same active ingredient present in several pharmaceutical products is not aggregated. This approach deviates from the generic European policy on chemicals (e.g., White Paper and draft directives of the European Union related to the new chemicals policy [65]; europa.eu.int/comm/environment/chemicals). The use of exposure triggers within the legislation procedure also limits the number of veterinary pharmaceuticals covered by environmental risk assessment.

This situation also affects discussions on the role of microcosms and mesocosms. Scientifically, the complexity in exposure patterns and the specificity of mechanisms of action suggest that micro- and mesocosms might be a useful tool for assessing the ecological impact of these molecules. Pragmatically, we should recognize that the likelihood for a fast implementation of these tools within the regulatory framework is low, at a time when most veterinary pharmaceuticals are still marketed without any environmental risk assessment.

From an environmental perspective, the combined risk of mixtures is most relevant, but this is based on science and is not currently considered in product registration, although there are some indications that it could occur in the future. For veterinary medicines, the mixture issue should be considered at three different levels. First, the drug reaches the environment after excretion by the target animal and application of manure. For each single drug, we should expect a combined release of a mixture of the parent compound, the metabolites formed during animal metabolisms, and some degradation products produced in manure. All these chemicals appear as a consequence of the administration of the parent and should be considered in its risk assessment. Second, several veterinary medicines can be used on the same animal or herd; therefore, a mixture of several drugs (plus their metabolites and degradation products) is expected in manure. Pharmaceuticals tend to be highly selective, and the combined risk can be critical (e.g., pharmacology demonstrates that a cocktail of selective antibiotics can be much more effective than the sum of their independent effects). Finally, veterinary medicines will reach the agricultural soil and adjacent waterbodies in the matrix of an organic fertilizer containing other toxic substances. Veterinary medicines can be integrated in manure risk assessment models such as EGPE [66], allowing both a holistic risk estimation for manure and a comparative perspective of veterinary medicines versus metals, phenols, nitrogen compounds, and other substances present in manure.

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