Environmentally-realistic concentrations of anthelmintic drugs affect survival and motility in the cosmopolitan earthworm *Lumbricus terrestris* (Linnaeus, 1758)

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Abstract

Anthelmintic drugs are used to control intestinal parasitic worms in animals worldwide. Although generally effective in managing animal health, such treatment can introduce anthelminthic compounds to the wider environment as active ingredients and metabolites are often excreted. This can have detrimental effects on non-target species, especially when drugs are used excessively. Here, we examine the effects that two environmentally-realistic concentrations of four anthelminthics have on the common earthworm *Lumbricus terrestris* (Linnaeus, 1758), a species with a cosmopolitan distribution and that is often vital in maintaining functional edaphic ecosystems. The drugs were ivermectin (0.502 and 2.511 mg kG^{-1} active ingredient), fenbendazole (0.309 and 1.547 mg kG^{-1}), pyrantel (79.480 and 397.400 mg kG^{-1}), and praziquantel (2.299 and 11.499 mg kG^{-1}): these concentrations were typical of soils where pasture grazed by animals treated with anthelminthics. Both lethal effects (mortality) and sub-lethal effects (motility) were considered. Earthworms exposed to fenbendazole and praziquantel over a 12-week period experienced high mortality (55.0% and 32.5%, respectively). Mortality rates among earthworms exposed to pyrantel and ivermectin were much lower (2.5% and 7.5%, respectively). However, earthworms exposed to pyrantel and ivermectin suffered decreased motility (time to burrow into substrate when exposed to heat and light) relative to a control group. Burrowing times were up to 40% longer for pyrantyl-exposed earthworms and 28% longer for ivermectin-exposed earthworms. For both drugs, the magnitude of the effect increased as concentration increased; all differences were statistically significant. There was little effect of fenbendazole and praziquantel on motility. Based on this study, which is seemingly the first to examine effects of ivermectin/fenbendazole on earthworm motility and the first to consider any effects of praziquantel/pyrantel, we conclude that environmentally-realistic concentrations of all four anthelmintics have sub-lethal (pyrantel and ivermectin) or lethal (fenbendazole and praziquantel) effects on a vital keystone species. Methods to reduce carry-over effects in ecologically-important, non-target, organisms should be urgently sought and care should be taken not to use anthelminthics routinely without first testing helminth burden to determine whether there is clinical need.

Keywords

Anecic earthworms, non-target effects, anthelminthic residue, soil ecotoxicology

1. Introduction

Helminths are intestinal parasitic worms with a cosmopolitan distribution throughout which they are both prevalent and abundant. They regularly infect livestock (Wolstenholme et al., 2004) and can have a substantial negative effect on the health and fitness of the host. Helminthiasis can result in poor intestinal health, malnutrition, slow growth, reduced fertility and fecundity, compromised immunocompetence, and, in extreme cases, death (Charlier et al., 2014; Coop and Holmes, 1996; Greter et al., 2017). Infestation can result in substantial economic cost: gastrointestinal nematodes have been estimated to cost cattle *Bos taurus* farmers $445 million in Mexico alone (Rodríguez-Vivas et al., 2017). Such losses are largely due to reduction in milk and meat yields as well as, in some cases, loss of animals (Charlier et al., 2014 and references therein; Fitzpatrick, 2013; Holzhauer et al., 2011). Because of such issues, farmers are often advised to use anthelmintic drugs regularly (or continually via long-acting injection, slow-release boluses, or reticulo-rumen devices) to maintain good parasite control (e.g. Beynon et al., 2012; Svensson et al., 2000; Vandamme, 2014). Such advice has been widely heeded and the annual spend on anthelmintics for ruminants in Europe alone is now in excess of €400 million per annum (c.$470 million) (Morgan et al., 2013). Anthelmintics are also widely prescribed for domestic and companion animals, including dogs *Canis lupus familiaris*, cats *Felis catus* and horses *Equus caballus* (Epe and Kaminsky, 2013).

Although different anthelmintics have different specific mechanisms of action, all drugs act to either cause helminth mortality or to reduce movement through drug-induced paralysis (Köhler, 2001). The benzimidazole family is the largest chemical group of anthelmintic drugs, with commonly-used derivatives including fenbendazole, albendazole and oxfendazole. These drugs compromise the cytoskeleton through a selective interaction with b-tubulin, which effects cellular structure and mitosis (Köhler, 2001). Worldwide, however, the most common anthelmintic drugs are the avermectins: macrocyclic lactone compounds with both nematicidal and insecticidal properties. One popular semi-synthetic derivative of avermectin is ivermectin, which has been used extensively since the 1980s (Shoop et al., 1995). All macrocyclic lactones, including ivermectin, act on both gamma-aminobutyric gated and glutamate-gated chloride channels of nerve and muscle cells, causing chloride permeability, hyperpolarization of the cell membrane, and ultimately paralysis (Cobb and Boeckh, 2009). Two other chemical groups are often used as anthelmintics: (1) tetrahydropyrimidine (which encompasses the single anthelmintic pyrantel); and (2) praziquantel (McKellar and Jackson, 2004). The former is a nicotinic anthelmintic and elicits spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine (nAChR) receptors (Aceves et al., 1970; Aubry et al., 1970;

Köhler, 2001; Unwin, 1995). The mechanism of action of the latter is still unclear despite considerable research, but it appears that ion transport is disrupted resulting in a rapid influx of Ca^{2+}, which accompanied (but not necessarily caused) by intense muscular paralysis (Cupit and Cummingham, 2015; Pica-Mattoccia et al., 2008; Salvador-Recatalà and Greenberg, 2012)

In addition to anthelmintic resistance commonly observed in target animals (Pedreira et al., 2006; Brady and Nichols, 2009; Besier and Love, 2003; Matthews, 2014; Wolstenholme et al., 2004), incorrect or excessive use of anthelmintics can result in chemicals being released to the environment. Environmental residues arise because anthelmintics are commonly excreted in urine and dung with active ingredients and their metabolites still present (Canga et al., 2009; Diao et al., 2007; Floate, 2006; Horvat et al., 2012). Once in dung, anthelminthic chemicals can be leached into soil and affect non-target (soil) invertebrates (Boxall, 2010; Pope et al, 2008; Wall and Strong, 1987). This problem is exacerbated by: (1) the proportion of the initial dose that is excreted – up to ~85% in the case of ivermectin and pyrantel (Gokbulut et al., 2005, 2014); and (2) their persistence in the environment as a result of slow degradation (Chiu et al., 1990; Cook 1993; Horvat et al., 2012; Pope et al, 2008). Anthelmintic drugs can be administered in different ways, including orally, via injection, or via a drench that is poured onto the skin. The worming regime will vary with establishment type and livestock type. For example, some intensive farmers will treat all animals at one time, resulting in large but comparatively infrequent high-intensity “pulses” of anthelmintics entering the environment while others will treat animals in groups but don’t treat the entire heard at one time, resulting in regular pulses of lower amplitude (Boxall, et al 2007). Conversely, use of long-acting or sustained-release methods typically result in low concentrations of residual anthelmintic being excreted continuously (Errouissi and Lumaret, 2010).

Over the past two decades, the effect of environmental anthelmintic residue has been the subject of considerable field- and lab-based research (e.g. Bueno and Freitas, 2004; Bai and Ogbourne, 2016; Jensen et al., 2007; Römbke et al., 2010; Svendsen et al., 2002). Studies have shown that anthelmintics can be highly toxic to aquatic species, with ivermectin affecting zooplankton, copepods and macroinvertebrates (most notably mayflies: Ephemeroptera), and fenbendazole affecting Crustacea (Sanderson et al., 2007; Wagil et al., 2015). Within the terrestrial environment, ecotoxicological effects of avermectins and benzimidazoles have been identified for a number of invertebrates including dung-associated flies in the genera *Haematobia, Musca* and *Stomoxys* (Fincher, 1991; Madsen et al., 1990; Strong et al., 1996) and multiple species of dung beetles in the family Scarabaeidae (Errouissi et al., 2001; Hempel et al., 2006; Madsen et al., 1990; Verdú et al., 2015).

Relative to this, however, there has been comparatively little research into the potential effects of anthelmintics on soil fauna (see reviews by Edwards et al., 2001 and Bai and Ogbourne, 2016). This is despite soil biota being tightly linked to above-ground communities through trophic interactions and feedback mechanisms that affect ecosystem functioning (Bardgett and van der Putten, 2014; Bardgett and Wardle, 2010; van der Putten et al., 2013). Without such information, it is impossible to fully assess the risks associated with anthelminthic use, especially for ecologically- and economically-valuable ecosystem services.

Earthworms (Oligochaeta) are a vital part of edaphic ecosystems (Römbke et al., 2005). As a keystone group, earthworms are vital for managing soil structure, effective nutrient recycling and decomposition of organic matter including animal dung (see reviews by Blouin et al., 2013 and Römbke et al., 2005). Indeed, these processes are so integral to maintaining functional edaphic ecosystems that long-term exclusion of earthworms negatively affects soil bulk density, organic matter, and hydrological properties (Blouin et al., 2013) and limited biodegradation of dung with negative consequences at agroecosystem level (as observed in Australian and New Zealand pastures before the introduction of European lumbricids: Svendsen, 1957). However, despite the ecological importance of earthworms, little attention has been given to potential anthelmintic ecotoxicity. The few studies that have tested anthelmintic chemicals on earthworms have mainly examined short-term effects on population size (e.g. Madsen et al., 1990; Sommer et al., 1992; Wall and Strong, 1987). With the exception of the field study by Yeates et al. (2007), nearly all research has been performed on earthworms to date have used surface-dwelling (epigeic) redworm *Eisenia fetida* or compost worm *Eisenia andrei* exposed to ivermectin (see Bai and Ogbourne (2016) and references therein; Lumaret et al. (2012)). These studies have typically found no adverse anthelminthic effect but as redworm and compost worm are normally not naturally associated with dung (except in vermicomposting contexts, which would be useful to consider) or soils, such studies might not reflect the complexity of anthelmintic-earthworm interactions (Svendsen et al., 2002). Moreover, toxicity research has typically focused on lethal endpoints (Lowe and Butt, 2007a). It is now widely recognised that measuring sub-lethal endpoints, including both motility and avoidance behaviour as two aspects of locomotory behaviour, can provide more sensitive and ecologically-relevant data (Ricketts et al., 2004) and therefore better reflect real-world impacts of drug residues on ecosystem functioning. This is especially important when assessing impacts at agroecosystem level including financial consequences (Beynon et al., 2012; Svendsen, 1957).

In this study, we test whether exposure to four different anthelmintic drugs, each from a different chemical group, affects the mortality (lethal endpoint) or motility (sub-lethal endpoint) of the common

earthworm *Lumbricus terrestris*. Two different dose levels over a three-month period were used to allow for possible cumulative effects and give longer-term insights into potential impacts of anthelmintic drugs. Mortality and motility were selected as response variables because the two factors that ultimately determine earthworm ecosystem services are: (1) population size; and (2) motility behaviour of individuals. This latter is especially true for anecic species, such as the common earthworm, which move vertically through the soil profile rather than other (epigeic) earthworm species that live on the soil surface (Römbke et al., 2005). We discuss our findings in relation to non-target effects of anthelminthics and offer recommendations for anthelmintic use.

2. Material and Methods

2.1 Study species

We used the common earthworm *Lumbricus terrestris* for this study, which is an anecic species (i.e. moves vertically through the soil structure) in contrast to more commonly-studied surface-dwelling epigeic species. The rationale for using common earthworm was four-fold: (1) its widespread distribution and status as a obligatory soil-dwelling (rather than surface dwelling) species (Römbke et al., 2005); (2) its keystone role in many ecosystems in controlling edaphic processes and regulating the biochemical and biological properties of soil (Blouin et al., 2013); (3) its potential exposure and sensitivity to chemical changes in soil, which is greater than in redworm due to its burrowing behaviour and feeding habits (Pelosi et al., 2013; Römbke et al., 2005); and (4) its ecological relevance as an active agent of dung decomposition (Svendsen et al., 2005). Moreover, the species is commercially available, with known age, origin and drug exposure provenance (Lowe and Butt, 2007b) and has a life cycle that is long enough for it to be suitable for chronic toxicity tests (Lowe and Butt, 2007a).

In total, 200 common earthworms were procured from Blades Biological Ltd (Cowden, Kent, UK). These had been laboratory reared in conditions free from drug residues as per Lowe and Butt (2005). All earthworms were adults weighing ~3 g at the start of the study and displaying a swollen clitellum; 18 atypically small earthworms were excluded as were two earthworms with visible damage. The remaining 180 earthworms were divided equally between nine containers (see below) to give eight treatment groups plus one control group, each comprising 20 earthworms. At the end of the experiment, all remaining earthworms (n = 141) were retained in clean soil with no anthelmintics added for a period of two weeks before being released. Earthworm density was 30-35 g/L which was within

the optimum density of 20-40 g L^{-1} recommended by Lowe and Butt (2005).

2.2 Study set-up and earthworm husbandry

This study was undertaken over a 12-week period between October 2016 and January 2017 to allow detection of possible long-term or cumulative effects of anthelmintics in natural environments (Horvat et al., 2012), and long enough to detect detrimental changes to life histories (Lowe and Butt, 2007a). There are no legal restrictions covering common earthworms used in laboratory studies in the UK but we followed the ethical guidelines of Rollin and Kessel (1998).

Both abiotic and biotic factors influence the survival of earthworms. It was therefore essential that the effect of such factors was minimised by maintaining appropriate husbandry conditions in a light- and temperature-controlled environment (Lowe and Butt, 2007b). Nine identical 4L plastic containers (280 x 160 x 90 mm) were used to accommodate the earthworms. To contain the earthworms and reduce desiccation, each container was fitted with a lid in which several small holes were drilled to allow air circulation (Hankard et al., 2005). Although some previous studies have used artificial soil (e.g. Diao et al., 2007; Gunn and Sadd, 1994), natural topsoil was used here since the physical and chemical properties of artificial soils often do not represent the diverse properties of natural soils and thus can be inadequate surrogates of the conditions experienced by soil biota in the field (Kuperman et al., 2006). Accordingly, 1.8 L of natural loam was collected for each treatment container from a private grassland site (centred on 51°54'20"N, 2°4'46"W) that was guaranteed not to have been contaminated with anthelminthics or any other artificially-applied chemical (insecticide, pesticide, herbicide or fertiliser). This soil was placed in each container to a depth of 40 mm. Physiochemical soil parameters were quantified using methods in Radojević and Bashkin (1999) and Suzuki et al. (2006): specifically, pH = 7.52; sand/silt/clay ratio = 3%/55%/42%; organic matter = 14%; water holding capacity = 43% and soil wash conductivity = 142μS/cm. Before soil was added to the containers, it was sieved through 5mm mesh to remove large particles and frozen at -20˚C for 72 hrs to destroy any natural soil fauna. It was then air dried (Zorn et al., 2005) and rehydrated until it was moist but not wet (i.e. no water appeared on the surface following compression: OECD, 1984). Soil temperature remained constant at 15 ˚C ± 3 ˚C and the containers were kept in consistent darkness (Lowe and Butt, 2005; Svendsen et al., 2002).

It is recognised that, as an anecic species, common earthworms housed in laboratory conditions

would ideally have dung regularly added and distributed throughout the soil profile (e.g. Lowe and Butt, 2005). However, it was important to minimise excess stimulation of the earthworms because this could have confounded assessment of earthworm motility (see below) by habituating earthworms to external stimuli. Accordingly, therefore, we instead added 10 g ± 0.1 g of grass cuttings in a thin even layer on the top of the soil in each container at the start of the study. This is supported by research from Valckx et al. (2011), which has shown that common earthworms feed effectively on grass at the top of the soil column. Every two weeks, any unused detritus on the soil surface was removed and 10 g of new plant material was added.

The stocking density and experimental conditions used in this study were validated by: (1) lack of mortality in the control group for the entire 12-week period; and (2) maintenance of consistent mass (~3 g) in the control group for the duration of the study (no significant difference in mass between week 1 and week 12, independent samples t-test $t = 0.739$, d.f. = 38, $P = 0.215$); mass would likely decline if stocking density was too high or husbandry was sub-optimal (Lowe and Butt, 2005).

2.3 Study anthelmintics and concentrations

To give a broad assessment of potential toxic effects of anthelmintics, four different chemical groups were tested (Table 1). Two of the drugs, ivermectin and fenbendazole, have been fairly well-studied for other faunal groups, but pyrantel and praziquantel have not been widely researched in the context of their effects on non-target species. Two concentrations of each drug were used: a 'standard' concentration and 'high' (5x standard) concentration. The concentrations were based on the level of active ingredient that could reasonably be expected to leach into soil from dung in areas of low animal density or low treatment intensity (standard concentration) versus high animal density or pulse treatment of multiple animals (high concentration). The former scenario might, for example, occur in smallholdings, equine livery yards, or where sustained release boluses are used, whereas the latter scenario might occur at intensively-managed farms. As such, all concentrations were environmentally-realistic and were calculated based on information from previous studies as detailed in Table 1. Because the aim was to simulate drug residues in soil, the concentrations used were generally lower than (or at least not in excess of) studies where dung was spiked (Table 1).

Table 1. Anthelminthics used in this study at standard and high concentrations (see Methods).

Chemical group	Specific drug	Concentration of active ingredient (mg kG^{-1}) dry weight		Environmentally realistic concentrations derived from:
		Standard	High	
Macrocylic lactone	Ivermectin (Alomec® 18. 7 mg G^{-1} oral paste)	0.502	2.511	Previous research: Hempel et al. (2006); Römbke et al. (2010) Reported excretion: Sommer et al. (1992) found levels of excreated drug in cattled of up to 9 mg kG^{-1} after drench application and up to 4 mg kG^{-1} after injection; NRA (1998) ~2.8 mg kG^{-1} after oral treatment.
Benzimidazole	Fenbendazole (Panacur oral paste 18.75% w/w)	0.309	1.547	Previous research: Grønvold et al. (2004); Gokbulut et al. (2006)
Tetrahydropyrimidine	Pyrantel (Alonate-P 400 mg G^{-1} oral paste)	79.480	397.400	Previous research: McKellar (1997); Gokbulut et al. (2014)
Praziquantel	Praziquantel (Equitape 90 mg G^{-1}oral gel)	2.299	11.499	Previous research: Hempel et al. (2006)

Each anthelmintic (Table 1) was made into a solution with 100 ml of distilled water to act as a carrier (Blackwell et al, 2007; Pope et al, 2008). For each treatment container, the top layer of soil was sprayed with the homogenised solution as per best practice guidelines (OECD, 1984). As this study was designed to test the effects of continuous or repeated exposure to anthelmintics as would occur in natural conditions, treatments were administered once per week throughout the 12 week study. This not only simulated the use of long-acting drugs but also mimicked the poor practice found in many equine facilities whereby individual horses are often treated at different times by owners rather than *en masse* by instruction of the livery owner. The amount of water used as a carrier was set at 100 ml following pilot study work, which found that adding this amount of once per week counterbalanced the natural evaporation that occurred during the proceeding seven days at the maintenance temperature of 15 ˚C (see above) and maintained soil at field capacity rather than saturation (Ward and Robinson, 2000). A ninth container was a procedural control and was simply spiked weekly with 100 ml of distilled water as per the above protocol. Unfortunately a lack of facilities precluded testing the concentration of each anthelmintic in the soil directly, so the relationship between earthworms and anthelminthics described here is based on input concentrations. This should be noted as a caveat throughout since it remains possible that the laboratory procedure did not fully mimic the way that earthworms would be exposed under natural field conditions.

2.4 Quantifying lethal endpoint: mortality

Each week, every earthworm in each treatment group was located and removed from its test container and mortality was assessed by testing response to gentle mechanical stimulus (Edwards, 1984). As per OECD (1984) guidelines, the anterior end of each earthworm was lightly touched using a glass rod. If no response was elicited, the earthworm was partially immersed in lukewarm water, providing a final form of stimulus, and was then assessed for any movement. All dead earthworms were discarded from test containers. It was decided a priori that the findings from anthelmintic mortality testing would be viewed as robust only if mortality in the control group does not exceed 10% as per the criteria proposed by ASTM (2004) and OECD (1984).

2.5 Quantifying sub-lethal endpoint: motility

We tested for possible effects of anthelmintic exposure on earthworm motility on the basis that: (1) earthworms can only be considered healthy if they readily move through substrates; (2) in a natural environment, predation is likely to increase if ability to burrow is compromised; and (3) motility is needed to find a mate to reproduce through simultaneous hermaphroditic copulation. To test motility, 40 mm of soil was placed in a new container. This soil had been collected and processed as outlined previously. To promote motility, a 40 watt light source was set up 50 mm away and directed at the soil to generate light and heat and thus stimulate movement. Earthworms were removed individually from their treatment container and moved to the motility test container. Each earthworm was held straight in callipers with its anterior end placed on top of the soil in the centre of the container before being released. The time interval between release and when the earthworm had buried its anterior or posterior at least 10 mm into the soil was recorded. The light source was turned off between each test to regulate the temperature; each motility test started when the soil was 20 ˚C ± 1 ˚C. Water was sprayed on the soil surface between motility tests to ensure soil moisture was appropriate and consistent. We only tested one individual at a time and observed it continually during the testing procedure. When it had been tested, it was removed to a temporary holding area to ensure that it was not inadvertently resampled that week. When all earthworms had been tested, they were returned to their treatment container. Because the individuals were not uniquely marked, it was not possible to relate repeated measurements in the different weeks to one another to track temporal change in motility at the level of the individual but measuring multiple individuals gave a robust population-level measurement per treatment group per week.

2.6 Statistical analysis

To examine any lethal effects of anthelmintic exposure, we used a 5 x 2 chi square contingency test to examine the association between treatment type (4 drugs plus the control) and mortality (1 or 0). Then, to quantify the effects of anthelmintic exposure and time (weeks elapsed since the beginning of the study) on motility as a sub-lethal effect, a Generalised Linear Model (GLM) framework was used with a Poisson distribution and a log link function. For each drug, motility was entered as the response variable and two fixed factors – treatment and time – were entered as well as their interaction. Treatment had 3 levels (standard concentration; high concentration; control) while time had 12 levels (12 weeks of study). Burrowing time data were normally distributed and did not require transformation. All GLM assumptions were checked and found to hold. Bonferroni-corrected post-hoc testing was used to establish pairwise contrasts between the three treatment types while reducing the probability of obtaining false-positive results due to family-wise error. All analysis was undertaken using IBM SPSS (v22.0).

3. Results

3.1 Lethal endpoints: mortality

Mortality in the control group was 0%, such that the soil toxicity results from the treatment groups were considered robust based upon ASTM and OECD criteria (see Methods). Mortality across all treatment groups increased over time. However, there was a substantial association between treatment type and mortality, with total mortality rates over the entire 12 week experiment ranging from 2.5% for ivermectin to 55% for fenbendazole (Fig. 1). This association was highly statistically significant (chi square test for association: $\chi^2 = 47.9$, d.f. = 4, $P < 0.001$).

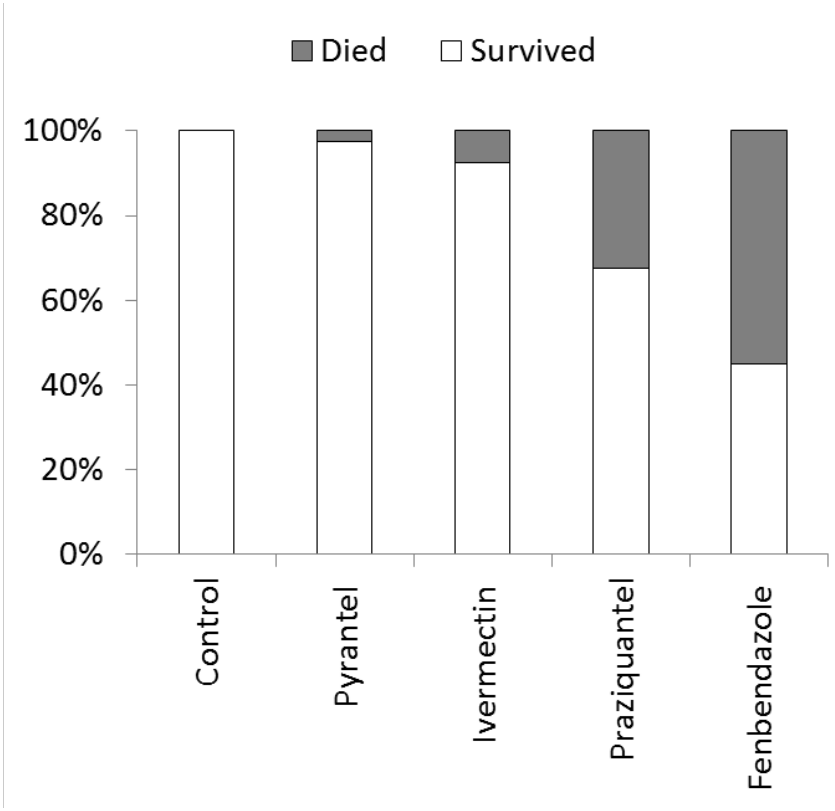

Fig. 1. Treatment-specific mortality rates of earthworms exposed to four different anthelminthic drugs over a twelve week study period (see Methods).

3.2 Sub-lethal endpoints: motility

There was a significant effect of time (weeks since experiment start) on earthworm motility in all treatment groups as well as the control group, such that time was significant in all models (Table 2). This was due to earthworms taking longer to burrow into the substrate as the experiment progressed (Fig. 2). For pyrantel and ivermectin, there were significant differences between standard and high concentrations and relative to the control over the full 12-week period (Table 2). For both drugs, this was due to response times being quickest among earthworms in the control group, slower among earthworms exposed to the standard concentration, and slowest among earthworms exposed to the high concentration (Fig. 3a and 4b). In terms of pairwise comparisons, and again using the data from the entire 12 weeks, standard vs high and control vs high was significant for both drugs; control vs standard was significant for pyrantel but not ivermectin (Fig. 3a and 3b). These were substantial, as well as significant, effects with burrowing times being 40.2% and 28.4% longer relative to control for high-concentration pyrantel and ivermectin, respectively. Primary observations during our study were that the majority of the earthworms from pyrantel treatment groups displayed spastic movements

(writhing and twisting) during motility tests, especially towards the end of the study. For praziquantel, the overall effect of treatment was non-significant (P = 0.067) but there was a significant pairwise comparison for control vs high concentration (Table 2; Fig. 3c). For fenbendazole, there was no effect of treatment overall and all pairwise comparisons were non-significant (Table 2; Fig. 3d). There was no interaction between time and treatment for any of the drugs, suggesting that the effect of time was consistent (uniformly slower response times as the experiment progressed; also suggested by the parallel temporal trends shown in Fig. 2). The overall models, encompassing both time and treatment, explained 18-33% of variability in earthworm mortality (Table 2).

Table 2. General Linear Models of earthworm motility relative to time (weeks since experiment start from 1 to 12), treatment (drug standard concentration, drug high concentration, control) and the interaction between them. Significant values are given in bold text.

Drug	Parameter	Motility			
		F	df	P	Adj. r^2
Pyrantel	Corrected model	3.590	35	**<0.001**	0.329
	Time	6.918	11	**<0.001**	
	Treatment	11.779	2	**<0.001**	
	Time*Treatment	0.826	22	0.689	
Ivermectin	Corrected model	2.159	35	**0.001**	0.180
	Time	4.609	11	**<0.001**	
	Treatment	4.891	2	**0.009**	
	Time*Treatment	0.723	22	0.811	
Praziquantel	Corrected model	3.027	35	**<0.001**	0.277
	Time	7.888	11	**<0.001**	
	Treatment	2.753	2	0.067	
	Time*Treatment	0.498	22	0.971	
Fenbendazole	Corrected model	2.472	35	**<0.001**	0.218
	Time	5.923	11	**<0.001**	
	Treatment	7.186	2	0.171	
	Time*Treatment	0.717	22	0.817	

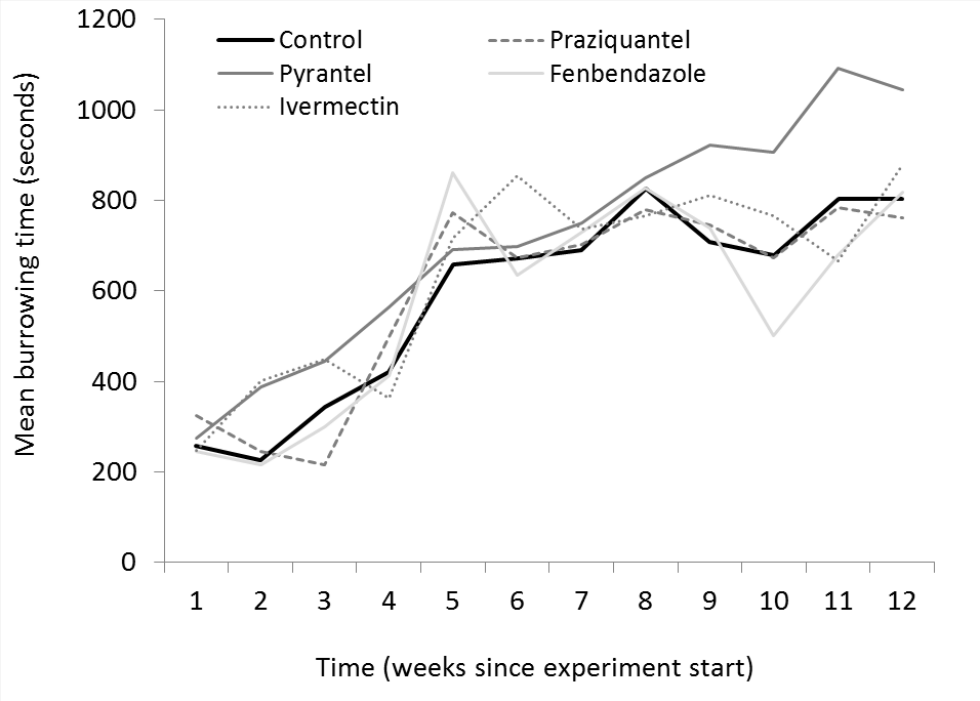

Fig. 2. Temporal change in motility of earthworms either exposed to a specific anthelminthic drug (standard and high concentrations combined) or forming the control group. Motility is defined as the time taken for individual earthworms to burrow into substrate from the surface of the substrate within a test chamber.

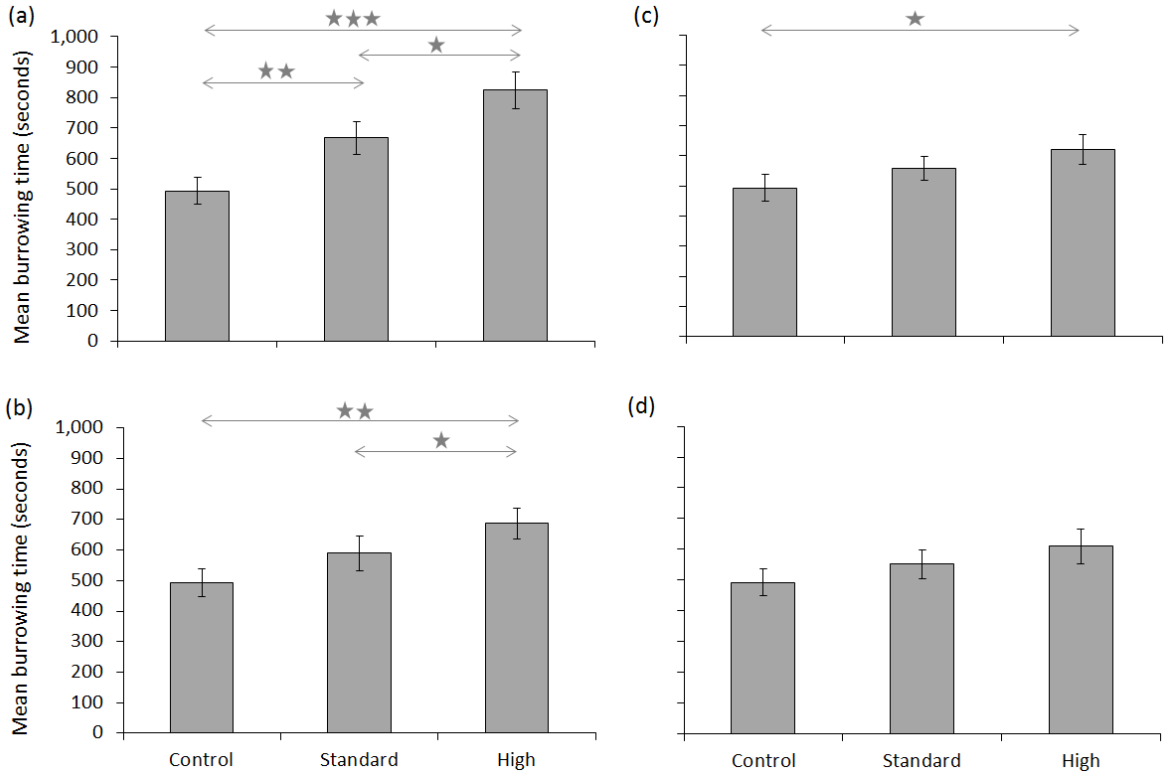

Fig. 3. Differences in mean motility of earthworms (time to burrow into substrate from the surface of soil in a test chamber) between standard and high concentrations of four different anthelminthic drugs relative to one another and a control group: (a) pyrantel; (b) ivermectin; (c) praziquantel; and (d) fenbendazole over a 12-week period. Error bars show standard error; horizontal lines show significant differences based on Bonferroni-adjusted post-hoc tests (* P< 0.05, ** P < 0.01, *** P<0.001).

4. Discussion

Our results show that environmentally-realistic chronic exposure (over 12 weeks) to environmentally-realistic concentrations of all four anthelminthic drugs tested here had substantial and significant negative effects on common earthworm mortality (lethal effects: fenbendazole and praziquantel) or motility (sub-lethal effects: pyrantel and ivermectin). As the effectiveness of the ecosystem services provided by earthworms – managing soil structure, facilitating nutrient recycling, maintaining drainage and aeriation – is determined by the number of individuals and their activity, anything that affects mortality or motility can have an extremely important effect (Blouin et al., 2013; Edwards and Bohlen 1996; Römbke et al., 2005). Interestingly, the results of this study suggest that individual anthelmintic drugs either have sub-lethal effects (reduced motility) or lethal effects (increased mortality). The drugs associated with the lowest mortality levels were pyrantel and ivermectin; these had the largest effect on motility. By contrast, the drugs with the highest mortality were fenbendazole and praziquantel; these had little or no effect on motility. This suggests that drugs either reduce movement or kill earthworms directly; no drug was associated with substantial lethal and sub-lethal effects (indeed, the rank order of the drugs in terms of the magnitude of their lethal and sub-lethal effects was completely reversed).

4.1 Motility effects: pyrantel and ivermectin

The temporal trend in motility in all earthworm groups (including the control), whereby burrowing times increased as the experiment progressed, suggests that there was an underlying process that affected all earthworms. Possibilities include habituation to stimulus or a seasonal effect that was not fully controlled as autumn progressed despite lighting, temperature and humidity remaining constant throughout the study (see Methods). There is also the possibility this was due to competitive interactions between earthworms or oxidative stress that intensified over time, especially as earthworms were kept at a relatively high density (Sanchez-Hernandez et al., 2018) and because individuals that died in the treatment containers could potentially remain *in situ* for up to 6 days given

the weekly nature of the experiment. That said, the density of earthworms was within recommended limits (Lowe and Butt, 2005), there was no loss of mass in control earthworms to indicate oxidative stress or effects of poor husbandry, and the increase in burrowing times occurred in the control group to the same level as in the treatment groups despite there being no mortality in this group.

In addition to the temporal effect discussed above, exposure to pyrantel and ivermectin was associated with substantial and significant reductions in earthworm motility. Very little work has been conducted on the effects of anthelminthics on the motility of soil biota prior to this study. In what appears to be the only previous study on this topic using pyrantel, Singh et al. (2012) showed direct application of solutions containing 20 mg mL^{-1} of active ingredient could cause paralysis in Indian earthworms *Pheretima posthuma* (average time until paralysis = 22 minutes). Such effects are not surprising given that pyrantel elicits spastic muscle paralysis (Köhler, 2001) as noted in this study (see Results).

In terms of ivermectin, earthworms exposed to high concentrations had reduced motility relative to those in the standard-dose ivermectin or control groups. This is seemingly the first time that motility effects have been found in common earthworms exposed to ivermectin, but Sun et al. (2005) observed slowed response to mechanical stimuli in redworms *Eisenia fetida* exposed to abamectin, another derivative of avermectin that is chemically very similar to ivermectin. The likely explanation involves the mechanism of action of macrocyclic lactones, which ultimately cause paralysis (Cobb and Boeckh, 2009). Given that this is a physiological response, the effects on earthworm motility described here could indicate the drug's likely effect on other non-target species (Verdú et al., 2015), although this is not inevitable. We recommend that further work is undertaken to investigate motility effects of anthelminiths on common earthworms and other anecic species, including using the avoidance behaviour response test as is standard for redworm (Hund-Rinke and Wiechering, 2001) and allowing for differences in drug sensitivity linked to phylogenetic relationships (Puniamoorthy et al., 2014).

Theoretically, the chemical pathways used by both pryantel and ivermectin could cause mortality in earthworms if drug-induced paralysis affected the consecutive aortic arches (pseduohearts) that pump blood through earthworms' circulatory system (Edwards and Bohlen, 1996). However, at least at the environmentally-realistic concentrations of these two drugs used here, this effect was not evident. Instead, mortality effects were found following exposure to two other anthelminthic drugs tested here:

fenbendazole and praziquantel.

4.2 Mortality effects: fenbendazole and praziquantel

In our study, fenbendazole was the anthelminthic associated with the highest mortality rate. Unlike pyrantel and ivermectin, the anthelmintic efficacy of benzimidazoles is not based on inducing paralysis but rather on its ability to compromise the cytoskeleton (Unwin, 1995). Very little research has previously been conducted on this class of drugs in relation to earthworms but one previous study (Gao et al., 2007a) on redworm exposed to albendazole, which is also in the benzimidazole chemical group with a similar mechanism of action (Küster et al., 2014), also found mortality effects. Specifically, the authors found that exposure to low concentrations of albendazole (100 and 200 mg kG^{-1}) did not cause mortality in redworm but up to 50% of exposed individuals died at higher concentrations (400 and 600 mg kG^{-1}) within two weeks after which the experiment ceased. Such mortality was ascribed to enzymatic effects (Gao et al., 2007b) or mitochondrial disruption (Gao et al., 2015).

The results of the current study (and those of Gao et al. 2007a, 2007b and 2015 on redworm) contrast with other work that has found no effect of fenbendazole on adult mortality (Svendsen et al. 2005) and little effect on the hatchling mortality (Svendsen et al., 2002). This might arise from differences in the concentrations of drug to which the earthworms were exposed (not measured in Svendsen et al. 2002 or 2005) or because of differences between undertaking the studies in laboratory conditions (which are highly controlled but not necessary totally representative of the real-world) versus field conditions (which are subject to much more variability).

The anthelminthic associated with the second highest mortality in this study was praziquantel. There has seemingly only been one previous study on the biotic effects of residual levels of this drug: Hempel et al. (2006) administered praziquantel to larvae of a dung beetle *Aphodius constans* in a laboratory environment. They found very high concentrations of the drug (1000 mg kG^{-1} of dry dung) caused a significant reduction in survival, but that concentrations $\leq$ 400 mg kG^{-1}did not affect mortality. Given that the concentrations used in this study were much lower at 2.3 and 11.5 mg kG^{-1}, and these were still associated with mortality in earthworms, our research suggests that effects might be species-specific, as has been found previously for anthelminthic sensitivity even for non-target species that are comparatively closely related (Puniamoorthy et al., 2014).

The anthelminthic most widely-studied as regards mortality of soil biota is ivermectin. The lack of mortality among earthworms exposed to this drug at the environmentally-realistic concentrations used in this study is in general agreement with the findings of previous studies (Madsen et al., 1990; Svendsen et al*.,* 2002, 2003, 2005; Wratten et al*.*, 1993). Indeed, the mortality rate (2.5%) in our study of earthworms exposed to ivermectin was almost identical to the mortality rate (3%) found by Svendsen et al. (2002). Vaidya (2016) has suggested that earthworms are capable of reducing toxic effects of chemicals by adjusting their internal biochemical responses, including potentially by excreting macrocyclic lactones (Sun et al., 2005), which might regulate drug accumulation at levels low enough to avoid lethal (but not sub-lethal) effects. Even if this occurs to buffer the effects of anthelminthics on some species, exposure to extremely high concentrations of ivermectin might have a significant effect on mortality. For example, Gunn and Sadd (1994) exposed redworm to ivermectin in soil and found no survival at soil concentrations >20 mg kG^{-1}; this is almost an order of magnitude higher than the high concentration used here and the concentrations used in other studies (e.g. Svendsen et al., 2002, 2003, 2005) and would be unlikely to occur in any natural conditions (NRA, 1998).

It should be noted that there might be an interaction between drug-induced mortality and mortality due to oxidative stress (Sanchez-Hernandez et al., 2018), which would not be possible to disentangle in this experiment, especially if oxidative stress was more likely to occur when individuals were exposed to mortality-causing anthelminthics or in treatment containers where other individuals had died, either as another result of oxidative stress or cascade deaths. Ascertaining the cause of death in drug treatment groups would be a useful area for further study.

5. Conclusions

The effects of anthelminthics on earthworms reported here are important because they include sub-lethal effects, drugs that have not previously been widely tested and focus on an understudied, ecologically-important species. For both pyrantel and praziquantel, this research seemingly constitutes only the second ever study on the effects of these drugs on non-target biota (after Singh et al. (2012) and Hempel et al. (2006), respectively) and the first on common earthworms. For ivermectin, our results are the first to show any negative effects on common earthworms as previous studies have focussed either on lethal effects or effects on growth rate (juveniles) or loss of biomass (adults). For fenbendazole, our results found high mortality rates of common earthworms in contrast to previous

studies (Svendsen et al., 2002, 2005).

We conclude that all four anthelmintics studied here have adverse effects on common earthworms at environmentally-realistic concentrations that can be sub-lethal (pyrantel and ivermectin) or lethal (fenbendazole and praziquantel). Use of these popular anthelmintics should thus be reassessed to avoid potential negative effects to ecologically-important organisms worldwide, and this should involve explicitly linking of soil concentrations of anthelminthics to lethal and sub-lethal effects rather than relying on input concentrations as was the case in the current study. In the meantime, particular care should be taken not to use the drugs excessively where there is little clinical need, as can happen, for example, if drugs are applied according to a regular schedule based on manufacturer recommendations made on the basis of parasite levels averaged across many farms without testing the actual helminth burden of the actual animals being treated. Although it might be more cost-effective to routinely treat rather than paying for regular tests of helminth load and just treatment to the clinical needs of the animals, this is far from ideal from an ecological viewpoint and the mantra of testing-before-treating should be embedded within risk mitigation measure procedures.

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