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Toxicity of ivermectin residues in aged farmyard manure to terrestrial and freshwater invertebrates

Running title: Toxicity of ivermectin in aged manure

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Abstract

1. Farmyard manure is often stored, aged and spread on fields as fertiliser, however dung may be contaminated with residues of veterinary endectocides used to treat livestock for parasites. The persistence of these chemicals during storage, and impact on invertebrate biodiversity after spreading, are not well understood.
2. This study considered whether residues in aged cattle manure could have impacts on terrestrial and freshwater invertebrate biodiversity and ecosystem function.

3. Fresh cattle dung was spiked with known concentrations of the endectocide ivermectin or an excipient only (control) and aged in the field for four months between February – May 2020. Each month rainwater run-off was collected and used to examine toxicity using the freshwater invertebrate *Daphnia magna*. In June 2020, manure was spread on mesocosms containing topsoil, and above-ground insect emergence, soil fauna feeding rate, earthworm abundance and pasture productivity (perennial ryegrass growth), were measured over 10 weeks.

4. Rainwater-runoff from manure contaminated with ivermectin was highly toxic (60-100% mortality) to *D. magna* for the entire 4 months of storage. Coleoptera and Diptera emergence was lower from mesocosms spread with ivermectin-contaminated manure compared with control manure. Pasture productivity was significantly lower (18-20%) in mesocosms spread with ivermectin-contaminated manure.

5. The results indicate that ivermectin residues aged manure have the potential to retain toxic effects on terrestrial and freshwater invertebrates for at least 4 months of storage,
could reduce pasture productivity, and may have pervasive impacts on invertebrate biodiversity in agricultural systems.

**Keywords:** Veterinary parasiticides, agrochemicals, environmental pollution, *Daphnia magna*, Coleoptera, Diptera, biodiversity, pasture productivity, agriculture

**Introduction**

The close integration of crop and livestock in ‘mixed farming’ systems may play an important role in the development of more sustainable agricultural practices. Organic amendments such as the incorporation of livestock manure or crop residues, can make a cost-effective contribution to degraded soil reclamation, and increase soil nutrients, organic matter content and soil biodiversity (Larney & Angers, 2012). Regenerative agricultural practices involving mixed crop and grazing agroecosystems have the potential to offset livestock greenhouse gas emissions (Janzen, 2010, Liebig et al., 2010a), through increased soil microbial activity enhancing the storage of soil carbon from rhizospheric inputs (Lange et al. 2015), including the oxidation of CH₄ by methane-oxidising bacteria (Sullivan et al., 2013; Brenzinger et al. 2018). Cropping can provide livestock with fodder and bedding, while livestock provide farmyard manure as fertiliser and soil conditioner.
Cattle manure may be stored in temporary field heaps, earthen lagoon structures or slurry tanks depending on solids content, and is often spread on fields in early spring before summer planting, and in late summer following harvest. Livestock manures are a source of nutrients and organic matter, and have lower nitrogen losses via leaching and runoff compared to synthetic fertilizer (Xia et al. 2017). Cattle manure has been estimated to have a financial value of £142 per hectare (AHDB, 2018). However, manures may be contaminated with the residues of veterinary pharmaceuticals. For example, in Australia, commercial beef feedlot manure has been found to contain parasiticides at concentrations toxic to Scarabaeidae dung beetles and dung breeding Diptera and their larvae (Lumaret & Errouissi, 2002; Khan et al., 2008), as well as steroidal hormones and antibiotics.

The impacts of veterinary parasiticide residues in dung on important decomposer insect communities such as dung beetles and flies are well studied (Floate et al., 2005; Finch et al., 2020; Junco et al., 2021). The macrocyclic lactone ivermectin has been shown to be excreted in dung at concentrations that are toxic to insects for 28, 35 and at least 49 days after treatment with pour-on, injectable and sustained release bolus formulations respectively (Herd et al. 1996). Once excreted, persistence in the dung can be 180 days, depending on environmental conditions (Lumaret et al. 1993; Suarez et al., 2003). Parasiticides detected both fresh and aged manure from five feedlots sampled in Australia include abamectin (1-16 μg/kg), doramectin (1-21 μg/kg), ivermectin (2-36 μg/kg) and eprinomectin
These values are of concern because they are close to the reported LC$_{50}$ for various dung invertebrate larvae, including 12 μg/kg (doramectin, *Onthophagus gazella*), 0.88 μg/kg (ivermectin, *Aphodius constans*) and 41 μg/kg (eprinomectin, *Musca autumnalis*) (Pfizer, 1996; Hempel *et al.*, 2009; HPRA, 2018).

Furthermore, aquatic organisms such as the freshwater crustacean *Daphnia magna* are highly sensitive to ivermectin (Halley *et al.*, 1989; Garric *et al.*, 2007). A single direct excretion of cattle dung containing 1.3 mg/kg (dry weight) ivermectin into water-sediment systems resulted in significant mortality of both pelagic (*D. magna*) and benthic (*Chironomus riparius*) freshwater invertebrates (Schweitzer *et al.*, 2010). Re-establishment of new populations were inhibited for up to 4 weeks after the excretion event. In water-sediment mesocosm experiments ivermectin has been shown to persist in the water phase for up to 30 days, dissipating into the sediment over this time where it remained highly stable (Sandserson *et al.*, 2007). Repeated exposure to ivermectin is therefore likely to result in accumulation in freshwater sediments. However, it is not known whether ivermectin from manure aged or stored in the field may be present in rainwater run-off, for example from a manure heap, or for how long it persists.

Rapid photodegradation of ivermectin when exposed as a thin film to sunlight has been observed, with a half-life of 14 days in faeces/soil mixtures placed outdoors in a New Jersey summer
compared to 217 days in winter, and 240 days indoors in darkness at 22°C (Halley et al. 1989). In aquatic environments, a degradation rate of ivermectin of 28.3% was observed in sediment 70 days after introduction into a simulated riverway environment (Wu et al. 2014). However, little is known about the impact of manure aging and storage on chemical contaminant breakdown, particularly in temperate climates where manure stored from winter housed livestock is not exposed to significant levels of UV light. In addition, when this manure is applied to soils, the effects of veterinary pharmaceutical contaminants on beneficial soil fauna are not well understood. Soil organisms including protists, nematodes, enchytraeids, mites, earthworms and a wide range of insects, are essential for soil quality and agricultural ecosystem function (Wurst et al. 2012. Biocidal contaminants in soil amendments such as livestock manure therefore have the potential to impact a wide range of key ecological processes which are fundamental to maintaining the productivity of agricultural land.

The aim of the current study, therefore, was to examine whether residues of ivermectin in aged farmyard manure (stored in the field for 4 months) have negative impacts on terrestrial and freshwater invertebrates, and agricultural ecosystem function in relation to pasture productivity after spreading.

**Methods**

**Manure**
Thirty kg of freshly voided cattle dung was collected in January 2020 from a single beef farm where the animals had not been treated with any veterinary parasiticides for the previous 12 months. A commercial subcutaneous injectable formulation for cattle (Ivomec Classic, Boehringer Ingelheim Animal Health UK Ltd, containing 1% ivermectin in a 40% glycerol formal and 59% propylene glycol excipient base) was mixed into 15 kg of the dung and thoroughly homogenised, to achieve a concentration of 2.5 mg/kg wet weight ivermectin. This is consistent with the upper limit of concentrations found in cattle faeces 48 h after treatment with pour-on ivermectin (Herd et al. 1996). Since 89% of the ivermectin can be found unaltered in the dung after dosing (Sommer et al., 1992), spiked dung was considered appropriate for the purpose of this study. For the control manure, an equivalent volume glycerol formal and propylene glycol was mixed into the remaining 15 kg of dung and the dung was thoroughly homogenised. Two experimental manure heaps were then created at a farm south west of Bristol, UK. Fifteen kg of ivermectin-treated and control dung was placed into each of two 20 L buckets that were left open and exposed to natural weather conditions. Nine evenly spaced 12 mm holes were drilled into the base of the buckets, which were placed on a plastic stand inside a large collecting bowl, to allow rainwater run-off to be collected in the bowl. A tarpaulin was secured around the rim of the bucket, covering the collecting bowl, so that only rainwater which had passed through the manure was collected (Fig. S1). These experimental manure heaps were left in place for four months until
the end of May 2020. Rainwater run-off was collected every two weeks and frozen until used in toxicity assays with *Daphnia magna*. Monthly precipitation was 178.6, 99.3, 54.6 and 10.4 mm respectively for Feb, March, April and May (Wraxall Weather Station, North Somerset, UK).

Two further sources of manure were used in the mesocosm experiments. The first was dung and incorporated untreated straw bedding from beef cattle housed indoors over winter. The cattle had not been treated with veterinary pharmaceuticals for at least 12 months. The dung and straw bedding had been collected as a part of the routine farm management over winter and deposited at regular intervals in an outdoor field heap. While housed, the cattle had been fed organic hay or haylage. The second source was dung and incorporated straw bedding collected from an open concrete lagoon where the bedding had been treated with a probiotic additive. These beef cattle had been housed indoors from December 2019 – March 2020, had been treated with anthelmintics after turn-in and had a rolling 12-month antibiotic level of approximately 5 mg/kg PCU (Population Correction Unit). The animals had been fed silage, crimped maize and cereal rations. Fifteen kg dung from both sources was collected from farms in May 2020, from an area of the lagoon/field heap which was approximately four months old (consistent with the age of the ivermectin-treated and control treatment groups) and was thoroughly homogenised before use in experiments.
Rainwater run-off toxicity assays

To investigate toxicity of rainwater run-off from contaminated manure stored in the field, run-off from the ivermectin-treated and control manure was tested for freshwater invertebrate toxicity using D. magna Straus, 1820 (model organism population Prof. G. Persoone, LETAE, Ghent University) laboratory assays (DAPHTOXKIT F™, MicroBioTests Inc, Gent, Belgium). A positive control was included; this used the commercial 1% ivermectin formulation diluted in standard freshwater (ISO medium, ISO 6341, DAPHTOXKIT F™) to a concentration of 2.5 mg/kg, equal to that found in the manure. Two negative controls were also included, and these consisted of standard freshwater containing the appropriate volume of the excipients propylene glycol and glycerol formal, or standard freshwater alone. The assays were performed according to manufacturer’s instructions (DAPHTOXKIT F™, 1996). Ephippia of D. magna were hatched in standard freshwater 72 h prior to the start of the toxicity test in an incubator at 21°C and under 6000 lux continuous illumination (MLR-351H; Sanyo, Panasonic Biomedical Sales Europe BV, Loughborough, UK). Rainwater run-off was thawed at room temperature and combined for each month (Feb, Mar, April, May), and 100 ml samples were used for toxicity testing. Serial dilutions (100%, 50%, 25%, 12.5% and 6.25%) of the rainwater run-off collected each month and the positive and excipient-only negative control were used to fill 30-well test plates with six rows of
five wells, one row for each dilution and one row for the standard freshwater negative control. For the positive control, this resulted in ivermectin concentrations of 2.5, 1.25, 0.63, 0.31 and 0.16 mg/kg. Once hatched, *D. magna* neonates were fed with spirulina powder for 2 h to prevent starvation, and then five neonates were added to each of the 30 wells. These were incubated at 20 °C in darkness, and then removed and the number of dead neonates was counted after 24 and 48 h. Immobilized neonates that showed no locomotory movement after gentle agitation of the liquid for 15 s were classed as dead.

*Mesocosm study*

To investigate the impact of manure contamination on terrestrial invertebrates, including above-ground insects, soil fauna and earthworms, a mesocosm field experiment was carried out in June 2020. Mesocosms consisted of 14 L plastic buckets filled with 10 L of topsoil, which had had no known exposure to chemical pesticides or fertilizers for at least 12 months. Nine 6 mm diameter holes were drilled in the bottom of the buckets to allow drainage and free movement of earthworms. Fifty mesocosms were placed on a meadow paddock at a farm south west of Bristol, UK, which was adjacent to a field of grazing cattle, and spaced 10 m apart in a 5 x 10 grid. Buckets were numbered and 10 were randomly assigned to each of the five treatment groups (control manure, ivermectin-treated manure, untreated farm field heap, conventionally managed
farm lagoon, and a no-manure control). Manure was applied in a thin layer to the surface of the topsoil at a standard spreading rate of 50 t/ha (Defra, 2010), which was 355 g per mesocosm, except for the no-manure control which was left as bare topsoil.

The mesocosms were left in place for seven days to allow insect colonisation, after which time emergence traps were fitted by covering the top of the buckets with blackout material, and attaching 200 mm lengths of 40 mm diameter clear plastic tubing in to 40 mm diameter holes which had been made in the sides of the buckets above the level of the soil and manure. Clear plastic 2 L bottles were then attached to the tubes, and ¼ filled with water containing 0.5 mL detergent. Insects emerging from the soil or manure flew towards the light, through the tube and were collected in the bottle. These were left in place for nine weeks; insects were removed every week and stored in ethanol. In the laboratory, insects were counted and identified by BS, MN and Professor Richard Wall (University of Bristol, UK) using morphological keys (Skidmore, 1991) to species level wherever possible. However, deterioration of small Diptera samples prevented identification in 54% of cases and subsequent statistical analysis was therefore based on order.

After 6 weeks in the field, soil fauna feeding activity was measured in the soil below the manure (or the no-manure control) using bait lamina (Terra Protecta GMBH, Berlin, Germany). These are 1 mm x 6 mm x 120 mm PVC strips which have sixteen 1.5 mm holes spaced 5
mm apart along their length. The holes are filled with a standard bait of cellulose powder, wheat bran and activated charcoal (70:27:3). Five bait lamina strips were inserted vertically into the soil in each of the 14 L mesocosms, evenly spaced with one in the centre and one in each quarter. When removed, the proportion of bait eaten was assessed by classifying each hole as ‘consumed’ or ‘intact’. The exposure time is strongly related to location and soil moisture (Kratz, 1998) and is normally between 10 – 20 days. A separate test bucket was set up containing topsoil only, with five bait lamina strips which were removed daily from 7 days after insertion, to identify the appropriate exposure period (30-70% bait eaten). After 10 days, all bait lamina strips were removed and analysed for feeding activity.

At the end of the 10-week experiment, the 14 L mesocosms were each tipped out on to a 2 m x 5 m tarpaulin and the soil was hand-searched for earthworms (Lumbricidae). Earthworms were taken back to the laboratory and counted and weighed to the nearest 0.001 g.

Pasture productivity

To measure pasture productivity between treatment groups, a further 50 mesocosms were set up as described above, using 2 L plant pots instead of 14 L buckets. Six weeks after spreading manure from each treatment group on to the topsoil, 10 mL of perennial ryegrass seed (Cotswold Seeds Ltd, Gloucestershire, UK) was sewn
evenly over the surface of each of the 2 L mesocosms (Manning et al. 2017). Mesocosms were brought into an unheated glasshouse to prevent the seed being eaten, and were watered daily. After 2 weeks, all above-ground biomass was cut at the soil surface, dried in an oven at 75 °C for 48 h, and weighed to the nearest 0.01 g.

**Statistical analysis**

All analyses were undertaken using the R statistical package (R Core Team, 2019). For the rainwater toxicity assays, binomial logistic regression was performed on *D. magna* mortality at 24 and 48 h, across the run-off dilution gradient for each treatment group (control or ivermectin-treated manure) over the four months. The run-off *serial dilution* concentration resulting in 50% mortality (LC$_{50}$) was calculated using a binomial general linear model (logit). The LC$_{50}$ reported here refers to the concentration of rainwater run-off in the *serial dilution*, and not to actual ivermectin concentrations. A general linear model was then used to compare the LC$_{50}$ between the treatment groups, with month as a covariate.

In the mesocosm analysis of insect emergence, insect orders with fewer than 25 individuals (<1% of total abundance) were excluded and a Pearson’s Chi-squared test of independence was performed to identify associations between manure type (control, ivermectin-treated, untreated farm field heap, conventionally managed farm lagoon and no-manure control) and insect abundance. For analysis of the soil fauna feeding activity, analysis of variance was performed
with the arcsin square-root transformed proportion of bait consumed from bait lamina strips as the dependent variable and manure type as a factor. For analysis of earthworm abundance, analysis of variance was performed with the log10 transformed count of earthworms recovered from the mesocosms as the dependent variable and manure type as a factor. In the analysis of pasture productivity, a linear model was used to compare the log10 transformed ryegrass dry weights between manure types. All post hoc comparisons between groups used Tukey HSD tests, except for the Pearson’s Chi-squared test which used Bonferroni adjustments.

Results

Rainwater run-off toxicity assays

The run-off serial dilution concentration resulting in 50% *D. magna* mortality (LC50) was significantly lower for exposure to the ivermectin-treated than the control manure run-off (t68=-18.8, P<0.001) for the entire four months (Fig. 1). This indicates that the run-off from the ivermectin-treated manure was significantly more toxic to *D. magna* than the run-off from the control manure.

For the ivermectin-treated manure, there was a significant interaction between *D. magna* exposure time and experiment month (t28=-8.05, P<0.001) so data for 24 and 48 h were analysed separately. After 24 h exposure, LC50 significantly increased between April and May (Z14=14.31, P<0.001; Fig. 1), indicating toxicity
reduced between months 3-4. After 48 h exposure there was also significant increase in LC₅₀ between February to April (Z₁₄= -2.6, P<0.05) and May (Z₁₄= 4.96, P<0.001; Fig. 1). The LC₅₀ after exposure to run-off from April (Z₁₄= -2.9, P<0.05) and May (Z₁₄= 17.2, P<0.001) were significantly higher than the positive control (ivermectin formulation in standard freshwater), also indicating that mortality resulting from ivermectin contamination in the manure run-off decreased in toxicity after three to four months (Fig. 1).

For the control manure, there was no significant difference in LC₅₀ between D. magna exposure time (24 – 48 h), and the LC₅₀ significantly increased over the four months between February – May (Z₆₆= 4.95, P<0.001; Fig. 1). No mortality was observed after 24 h exposure to control run-off from April, hence the LC₅₀ could not be calculated. Exposure to the negative control (excipients in standard freshwater) resulted in significantly higher LC₅₀ than the February (Z₆₆= -6.15, P<0.001) and March (Z₆₆= -6.15, P<0.001) run-off, indicating that the manure initially leached some freshwater pollutants into run-off irrespective of ivermectin contamination.

Mesocosm study

Above-ground insect emergence

A total of 2479 invertebrates were collected from the emergence traps (Table S2) representing the orders Coleoptera (64.54%), Diptera (28.80%), Hymenoptera (1.21%), Thysanoptera (0.97%),
Hemiptera (0.56%), Dermaptera (0.04%), Collembola (Entognatha) (0.21%), Chilopoda (0.12%), Arachnida (0.89%) and disintegrated fragments of individuals which could not be identified (2.66%).

There was a significant association between manure type and insect abundance ($\chi^2_{12}=1116, P<0.001$), and Bonferroni adjusted post-hoc analysis of residuals revealed that significant associations were present within the orders Coleoptera and Diptera.

Significantly fewer Coleoptera ($P<0.05$) and Diptera ($P<0.001$; Fig. 2) emerged from mesocosms spread with ivermectin-treated manure than control manure. Significantly fewer Coleoptera ($P<0.001$) and Diptera ($P<0.05$) also emerged from the no-manure controls than the control manure mesocosms. However, while significantly fewer Coleoptera emerged from the no-manure controls than the ivermectin-treated ($P<0.001$) mesocosms, the converse was true for the Diptera, where significantly fewer emerged in the ivermectin-treated mesocosms compared to the no-manure controls ($P<0.001$; Fig. 2).

For the farm manure, significantly fewer Diptera emerged from the mesocosms spread with conventionally managed farm lagoon manure than untreated farm field heap manure ($P<0.001$; Fig. 2). Significantly more Coleoptera emerged from the mesocosms spread with conventionally managed farm lagoon manure than untreated farm field heap manure ($P<0.001$), however this should be interpreted with caution because the lagoon manure appeared to be colonised by a disproportionate amount of Staphylinids which
dominated the community. Of the 1402 Coleoptera which emerged from the lagoon manure mesocosms 99% were Staphylinids, whereas 44% of the 25 Coleoptera which emerged from the farm field heap mesocosms were Staphylinids.

Soil fauna feeding activity

There were significant differences in the proportion of bait eaten between manure treatment groups (F = 7.42, df = 4, P<0.001). Significantly less bait was consumed by soil fauna in the mesocosms spread with conventionally managed farm lagoon manure than all the other treatment groups; untreated farm field heap (P<0.001), control manure (P<0.001) and ivermectin-treated manure (P<0.001), and the no-manure control (P<0.05) (Fig. 3).

Earthworms

There were significant differences in the number of earthworms (Lumbricidae) recovered from mesocosms (F=4.25, P<0.05). More earthworms were recovered from the mesocosms spread with control manure (P<0.05), ivermectin-treated manure (P<0.05) and the untreated field heap manure (approaching significance P=0.059) than the no-manure control (Fig. 4). There was no significant difference in earthworm abundance between the no-manure control and the mesocosms spread with conventionally managed
farm lagoon manure. There were no significant differences in earthworm weights between any of the treatment groups.

Pasture productivity

There were significant differences in ryegrass biomass between mesocosms ($F_4=4.12, P<0.05$). Mesocosms spread with ivermectin-treated manure had significantly reduced ryegrass biomass compared to mesocosms spread with control manure ($P=0.05$), untreated farm field heap manure ($P<0.05$), conventionally managed farm lagoon manure ($P<0.05$) or the no-manure control ($P<0.05$) (Fig. 5).

Discussion

Rainwater run-off collected over four months from a manure heap experimentally contaminated with the livestock veterinary parasiticide ivermectin was highly toxic to D. magna for the first two months (February and March), resulting in 100% mortality after 24 h exposure to all dilutions tested. When exposed for 48 h, D. magna toxicity was higher still in rainwater run-off collected over the entire four months (February-May) with 60-100% mortality at all serial dilutions. This was significantly greater than the control manure or negative control treatments. The LC$_{50}$ (serial dilution concentration required to kill 50% of D. magna) for the positive control (across the dilution gradient 2.5 - 0.16 mg/kg) was equivalent to the LC$_{50}$ for
ivermectin-treated run-off in February, March (24 h exposure) and April (48 h exposure) (Fig. 1). Because the actual concentrations of ivermectin in the run-off were not measured, these results must be considered preliminary and interpreted with caution. For example, while *D. magna* mortality in the ivermectin-treated run-off was similar to the positive control for the first three months, indicating sustained toxicity of rainwater run-off from the ivermectin-treated manure over time, it is not known whether the ivermectin degraded over this period.

The lowest concentration of ivermectin in the positive control serial dilution was 0.16 mg/kg, which is four orders of magnitude greater than the predicted environmental concentration in a 100x1 m waterbody with ivermectin-treated animals grazing 1 ha (2.5x10^{-5} – 6x10^{-5} mg/kg) (Sanderson *et al*., 2007). The ivermectin concentration in the dung in this study was consistent with the upper limit of concentrations found in cattle faeces 48 h after treatment with pour-on ivermectin (*Herd et al. 1996*). The direct run-off from manure in the present study naturally contained much higher concentrations of ivermectin than would be found after leaching into a waterbody, but resulted in near total elimination of *D. magna*. Previous laboratory assays have reported the 48 h LC$_{50}$ for *D. magna* between 1.2x10^{-6} – 1.07x10^{-5} mg/kg and long-term effects on reproduction and growth at concentrations as low as 1.0x10^{-9} mg/kg (*Garric et al., 2007*). Halley *et al.* (1989) reported the 48 h LC$_{50}$ as 2.5x10^{-5} mg/kg which is an order of magnitude higher than Garric *et al.* (2007) and variation between actual and original ivermectin
concentrations could be due to adsorption and photodegradation and may result in significant changes in \textit{D. magna} mortality due to the steep concentration-effect relationship. Nevertheless, these LC$_{50}$ values are equivalent to, or lower than, the predicted environmental concentrations of $2.5 \times 10^{-5} – 6 \times 10^{-5}$ mg/kg (Sanderson \textit{et al.}, 2007) suggesting that negative impacts on freshwater invertebrates are possible in aquatic ecosystems. However, the preliminary evidence presented here must be examined under more realistic field conditions, such as analysing run-off, groundwater and sediment samples from hydrologically isolated catchments with full-size manure heaps directly on the ground, which would considerably increase the dilution factor. Mortality observed from exposure to run-off from the control manure in the first two months may be attributed to other pollutants such as high particulate cellulose, low oxygen, NH$_4$, excess N and P or bacterial pathogens. However these toxic effects were short-lived compared to those observed from the manure contaminated with ivermectin.

Because of the high affinity of ivermectin with particulate matter, aqueous leaching is not likely to be a major source of freshwater contamination unless manure from treated animals is stored in proximity to water bodies, or treated cattle defecate into standing water (Liebig \textit{et al.}, 2010b). However, ivermectin accumulates in sediments where it remains highly stable (Sanderson \textit{et al.}, 2007). Repeated exposure from regular manure spreading, and the transport of sorbed ivermectin with eroded soil, particularly when manure is spread on bare ground in autumn (postharvest) and
spring (preseeding), may have important impacts on freshwater invertebrates – particularly benthic species (Liebig et al., 2010). For example, long-term effects of ivermectin contamination on sediment-active organisms (e.g., *Chydrorinae and Ephemeroptera*) have been identified and populations did not recover by 270 d (Sandserson et al., 2007).

Current UK legislation regarding storage of animal manures in Nitrate Vulnerable Zones (NVZ) considers the risk of leaching and run-off from areas of agricultural land which drain into waters that could be polluted by nitrates. Manure should not be stored within 10 m of any surface water or 30 m if the land slopes (Defra, 2015). It is not clear whether this would be sufficient to prevent ivermectin residues in run-off or sediment from reaching waterbodies.

Considering the potential for sustained toxicity of rainwater run-off from manure heaps over several months reported in the present study, it would be beneficial to investigate this further in the context of manure storage placement, as well as run-off from recently spread fields contaminated with veterinary pharmaceuticals reaching proximal waterbodies. Recent research investigating widespread fipronil and imidacloprid contamination of English rivers at levels above their chronic toxicity limits, suggests that these pesticides are entering waterways via household drains in the form of veterinary flea-treatment products applied to companion animals (Perkins et al., 2021). Cypermethrin (a pyrethroid parasiticide used in both livestock and pet treatments) has been found in Danish freshwater sediment samples at concentrations sufficient to affect
zooplankton populations (Jensen et al., 2012). Clearly there is a need to re-evaluate the environmental risks posed from the ubiquitous use of parasiticide products associated with both domestic and agricultural use.

Above-ground insect emergence was negatively affected by ivermectin residues in the manure that had been stored for four-months and then spread on topsoil at a standard spreading rate of 50 t/ha (Defra, 2010). The emergence of both Coleoptera and Diptera was significantly lower from mesocosms spread with the ivermectin-treated manure than control manure. This suggests that the negative effects of ivermectin residues in cattle dung from animals treated with veterinary parasiticides (reviewed by Floate et al. 2005; Junco et al. 2021) remain concerning even in manure aged in the field. However, the concentrations of ivermectin used in this study were at the upper limit of those reported in the literature for naturally excreted cattle faeces, and further studies with a range of concentrations would be useful to examine impacts across the excretion profile (Herd et al., 1996; Sommer et al., 1992; Lumaret et al., 2007). The mesocosms spread with manure from conventionally treated animals which had been stored in a farm lagoon had a disproportionately high abundance of Staphylinids which dominated the community (99% of Coleoptera compared to 44% in the manure from untreated animals stored in a farm field heap). It has previously been reported that Coleopteran decomposer communities colonising cattle dung from conventionally managed farms which regularly use veterinary parasiticides can become
dominated by a small number of resilient species (Sands & Wall, 2018). Of the 9,606 Staphylinids collected in their study, 41% were from farms regularly using macrocyclic lactones such as ivermectin, 32% were from farms using synthetic pyrethroids and 27% were from farms which had not treated their cattle (Sands & Wall, 2018). While the farms which used macrocyclic lactones had higher proportions of Staphylinids, they had significantly lower species richness and diversity of Coleoptera overall, particularly of functionally important paracoprid dung beetles. In addition to insecticidal impacts, endectocide residues have been shown to affect the abundance of insects which colonise and oviposit in dung by attractive or repellent properties (Floate, 2007). Emergence from mesocosms in the present study should therefore be interpreted as a measure of insect activity rather than absolute residue toxicity, until laboratory tests can be performed using standardised insect numbers on manure samples. Further replication with manure samples from a wider range of farms and different farm management systems, with known residue concentrations, would be required to allow a complete understanding of this issue, but the current data highlight the potential problems arising from the use of contaminated manure spread on fields.

As is reported in other studies, the concentrations of ivermectin in manure excreted by cattle following treatment appeared to have no impact on earthworm abundance (Svendson et al., 2003), or soil faunal feeding activity (Manning et al., 2017). Halley et al. (1989) found the NOEC (no observed effect concentration) to be 12 mg/kg.
ivermectin for *the earthworm Eisenia fetida*, which is at least five times higher than expected concentrations found in cattle dung after treatment with pour-on formulations. In the present study, spreading manure on soil in the mesocosms increased the abundance of earthworms significantly regardless of ivermectin contamination. Earthworm spatial distribution is strongly affected by cattle dung pats on pastures (Bacher *et al.*, 2018), with up to four times more earthworms aggregating beneath pats than in control locations away from them. Here, using livestock manure as a soil amendment also appeared to attract earthworms, which may be beneficial for vital ecosystem services such as soil formation, structure maintenance, water infiltration, nutrient cycling, primary production and pollution remediation (Blouin *et al.*, 2013). However, the mesocosms spread with farm lagoon manure from the conventionally managed animals did not have a significantly higher number of earthworms than the no-manure control. Additionally, soil faunal feeding rate was significantly reduced by 16-20% after spreading with this manure compared to all the other treatments groups. It is unclear whether the impact on soil fauna was a result of differences in manure quality due to the type of manure storage, the presence of antibiotics, other veterinary treatments, livestock feed type and quality, bedding additives or unknown factors, and chemical residue analysis is required in further studies to contextualise these results. Given the importance of soil organisms to soil quality, ecosystem function, decomposition, nutrient cycling and greenhouse gas regulation including carbon sequestration and
CH₄ oxidation (Powlson et al., 2001; Wurst et al., 2012; Schnitzer et al., 2011; Jacoby et al., 2017), further investigation involving a large sample of farms with varying manure storage and livestock management practices is indicated.

Mesocosms spread with ivermectin-contaminated manure had significantly reduced perennial ryegrass growth after two weeks by 18-22% compared with all the other treatment groups. Previous work investigating the effect of ivermectin on white mustard Sinapsis alba seed germination and early root growth suggested that there was significant inhibition of root growth by up to 24% at concentrations of 0.044 – 0.44 µg/ml ivermectin (Vokřál et al., 2019). Ivermectin has been shown to move from cattle faeces to the underlying soil as well as to nearby plants (Iglesias et al., 2017). After watering with ivermectin in solution, uptake into soybean Glycine max leaves, and reduced number and weight of soybeans, has also been observed (Navrátilova et al., 2020). These results are unexpected given that abamectin, which differs from ivermectin by a double bond on C-22-25 (Lespine, 2013), is a widely used crop pesticide (Lasota & Dybas, 1990). The potential phytotoxicity of ivermectin at concentrations likely to be found in livestock manure after treating with veterinary parasiticides may be of considerable economic concern to farmers, yet appears currently underacknowledged. The results indicate that spreading contaminated manure in spring or autumn before reseeding or drilling could reduce pasture growth and damage productivity, however field-scale experimental studies are required to investigate
this thoroughly. Given that atmospheric carbon sequestration and recalcitrance into the rhizosphere by plant roots is a function of root biomass and depth (Kell, 2012), these risks should also be evaluated in the context of agriculture’s role in greenhouse gas regulation. It must be noted that the application of farm lagoon manure in this study resulted in marginally higher ryegrass growth than the no-manure control (Fig. 5). Although this was not significantly significant, the ryegrass was cut after 2 weeks and beneficial impacts on productivity may have been observed over a longer growing period. In this case the advantage of spreading manure on pasture productivity may be beneficial regardless, however a limitation of this work was that the actual concentrations of residues in field collected dung were not known.

**Manure contaminated with ivermectin and stored for 4 months in the field appeared to retain toxic impacts on terrestrial invertebrates and pasture productivity after spreading, and aquatic invertebrates exposed to rainwater run-off collected over this time.** Rapid photodegradation of ivermectin has been demonstrated, for example when placed outdoors in faeces/soil mixtures in the summer it may have a half-life of 14 days or less (Halley et al., 1989). In winter however, the half-life can be as much as 217 days, and when stored as a field heap or in an outdoor lagoon the manure is only in contact with sunlight at the surface, limiting the extent of photodegradation (Pope, 2009). Conditions including temperature, oxygen and pH vary depending on storage facility, for example slurry stored in tanks will not be exposed to sunlight but will be under
anaerobic conditions. Anaerobic digestion of manure has been shown to reduce levels of contamination with veterinary antibiotics (Gurmessa et al. 2020) although they are not completely removed. However, data is lacking regarding the effects of anaerobic digestion on ivermectin or other macrocyclic lactones in manure, and aerobic degradation via soil microbial activity is thought to be important (Halley et al., 1993). Composting manure has also been shown to accelerate the degradation of veterinary steroidal hormone contamination (Khan et al., 2008), but data is again lacking on the effects of this on macrocyclic lactones.

In conclusion the data suggest that it should not be assumed that the environmental impacts of parasiticide residues in dung will be eliminated after storing the manure or slurry to be spread at a later date. The preliminary evidence reported here indicates that ivermectin residues in aged manure have the potential to retain toxic impacts on terrestrial and freshwater invertebrates, as well as reducing pasture productivity. Given the importance of conserving a functioning pastureland ecosystem for environmental processes including soil formation and structure, nutrient cycling, greenhouse gas regulation and agricultural productivity, further data regarding the impacts of manure storage conditions on parasiticide residue degradation are needed to inform future practice.

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Conflicts of interest

The authors declare no conflicts of interest.

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larvae of the dung beetle *Aphodius constans* in the laboratory.

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Figure Legends

Fig. 1 Mean (±SE) run-off serial dilution concentration resulting in 50% *Daphnia magna* mortality (LC$_{50}$) after 24 and 48 h exposure to a dilution gradient of rainwater run-off. Over the months Feb-May, grey points show run-off from control manure containing excipients only (propylene glycol and glycerol formal), and black points show run-off from ivermectin-treated manure containing a commercial subcutaneous injectable formulation for cattle at a concentration of 2.5 mg/kg wet weight ivermectin (Ivomec Classic, Boehringer Ingelheim Animal Health UK Ltd). The laboratory controls represent the negative (grey points) and positive (black points) control comprising standard freshwater (DAPHTOXKIT™) containing excipients only or the ivermectin treatment respectively (2.5 mg/kg). Points labelled with the same letters are not significantly different.

Fig. 2 Total number of Diptera emerged between June – August 2020 from mesocosms containing topsoil spread with four-month aged control or ivermectin-treated manure, manure from untreated cattle stored in a farm field heap, manure from conventionally
managed cattle stored in a farm lagoon, and a no-manure control of bare topsoil. Boxes represent the interquartile range (IQ) with central median lines, and whiskers extending to 1.5 IQ.

Fig. 3 Mean (±SE) proportion of bait (%) consumed by soil fauna over a 10 day period, from bait lamina inserted into mesocosms containing topsoil that were spread with four-month aged control or ivermectin-treated manure, manure from untreated cattle stored in a farm field heap, manure from conventionally managed cattle stored in a farm lagoon, and a no-manure control of bare topsoil. Manure was spread 6 weeks prior to insertion of bait lamina.

Fig. 4 Log10 number of earthworms recovered by hand-searching soil from mesocosms containing topsoil that were spread with four-month aged control or ivermectin-treated manure, manure from untreated cattle stored in a farm field heap, manure from conventionally managed cattle stored in a farm lagoon, and a no-manure control of bare topsoil. Manure was spread 10 weeks prior to searching. Boxes represent the interquartile range (IQ) with central median lines, and whiskers extending to 1.5 IQ.

Fig 5 Dry weight (g) biomass of perennial ryegrass after two weeks growth on mesocosms containing topsoil that were spread with four-month aged control or ivermectin-treated manure, manure from untreated cattle stored in a farm field heap, manure from conventionally managed cattle stored in a farm lagoon, and a no-manure control of bare topsoil. Manure was spread 6 weeks prior to
seed sewing. Boxes represent the interquartile range (IQ) with central median lines, and whiskers extending to 1.5 IQ.