ANTHELMINTIC DRUG ALBENDAZOLE TREATED SHEEP URINATION INFLUENCES SOIL NITROGEN TRANSFORMATIONS AND NITROUS OXIDE EMISSIONS FROM GRASSLANDS

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Abstract. This study investigated the deposition of the anthelmintic drug albendazole through sheep urine and how it affects soil nitrogen transformations and nitrous oxide (N_2O) emissions in grazed grasslands. Three one-year-old Tibetan rams were dosed with fast-acting albendazole capsules at 11 mg per kg body weight to measure the concentration of albendazole and its metabolites in each sheep's urine. Three rams were used as a control group. Albendazole, albendazole sulfoxide and albendazole sulfone were all excreted with urine, but albendazole sulfoxide was the main metabolite. The effect of albendazole or its metabolites' excretion in urine on soil nitrogen transformations and nitrous oxide emissions were investigated at an alpine grassland site. Greater nitrification activity was observed in soils where urine from albendazole-dosed rams had been applied. The mean total N_2O emissions over the six-week measurement period for albendazole-dosed sheep urine, control urine and no urine were 0.94 ± 0.34, 0.18 ± 0.11 and 0.02 ± 0.05 g N_2O-N m^{-2}, respectively. These results suggest that N_2O pollution from animal urine patches can be potentially exacerbated through anthelmintic drug administration to grazed animals.
Keywords: *ammonium, nitrate, sheep urine, urine patches, greenhouse gas*

Introduction

Gastrointestinal parasite infection is a major issue in livestock farming throughout the world. Recently, this issue has become a serious concern in the global livestock industry as the number of animals raised on livestock farms has substantially increased due to the increase in demand for meat and milk products. Since the use of anthelmintic drugs is a common practice for controlling internal parasites, there has been a significant worldwide effort into the research and development (Zhang et al., 2019), and applications (Han et al., 2017) of anthelmintics. While anthelmintic drugs are beneficial for overcoming animal health issues, studies have increasingly identified some unintended consequences, as anthelmintics given to ruminants can enter the environment through faeces or urine (Beynon, 2012). Anthelmintics may enter the environment through excreta as the parent active ingredient, metabolites or a combination of both, and the proportions of different anthelmintics excreted in faeces or urine can vary (Wu et al., 2009). Potential environmental impacts of some of these anthelmintic products or their breakdown metabolites on non-target organisms are well documented (Oh et al., 2009; Beynon, 2012; Wagil et al., 2015; Prchal et al., 2016). Among the existing literature, substantial research papers report the potential environmental impacts of the anthelmintic products on aquatic systems (Oh et al., 2006; Belew et al., 2021; Mooney et al., 2021) and contamination

through faeces (McKellar, 1997). Surprisingly, information on how animal-grazed grasslands are ecologically impacted through animal urine excretion is lacking.

This study aimed to investigate the impacts of urine from sheep fed the anthelmintic drug albendazole on soil microbial processes, with a particular focus on the soil nitrogen (N) cycle in grazed grasslands. The microbial-driven soil N transformations in animal urine patches are important in animal-grazed grasslands as they influence plant productivity as well as environmental pollution via nitrate leaching and nitrous oxide (N_2O) emissions to the atmosphere (Haynes and Williams, 1993). Grazing animals harvest N from across the grasslands, and then deposit it through urine in small patches with a high N load. The high N load deposited to soil exceeds plant uptake ability, so it becomes subject to soil microbial-driven N transformations, including N_2O production. The production of N_2O in soils occurs primarily via the biological pathways of nitrification and denitrification. Nitrification in soils produces nitrate (NO_3^-) from ammonium (NH_4^+), while also producing some N_2O. The NO_3^--N produced by nitrification can be taken up by plants, leached or transformed to N_2O production by denitrification (Giles et al., 2012).

Albendazole is a broad-spectrum anthelmintic drug that is widely used to control gastrointestinal parasite infections in sheep and cattle. Single-dose fast-acting albendazole tablets are commonly used in countries including China, while long-lasting slow-release albendazole capsules are generally used in countries such as Australia and New Zealand (Fisher and Van Sittert, 2013). After oral ingestion by animals, albendazole is metabolised into anthelmintically active albendazole sulfoxide, which is then oxidized to the less anthelmintically active albendazole sulphone (Prchal et al., 2016). Pope (2009) reported that albendazole and its metabolites are mainly excreted with animal urine. Several previous studies have indicated that anthelmintics excreted with animal faeces have significant effects on soil organisms such as earthworms, soil bacteria (Sun et al., 2005), soil fungi (Wang et al., 2021) and soil fauna (Madsen et al., 1990). In this study, we hypothesize that the composition of the urine of albendazole-dosed animals would be different from non-dosed animals, and the albendazole and its metabolites excreted with sheep urine would impact soil microbial activity related to the N cycle. We conducted two separate experiments to answer the following specific science questions: 1) What quantities of albendazole and its metabolites are excreted to grazed grasslands through sheep urine? 2) What are the effects of albendazole-treated sheep urine on soil N transformations and soil N_2O emissions?

Materials and methods

Ethics statement

The animal management were in accordance with the rules and regulations of experimental field management protocols (file No: 2010-1 and 2010-2), which were approved by Lanzhou University.

Detection of albendazole and its metabolites' excretion in sheep urine

Experimental setup

This experiment was carried out at Linze Grassland Agriculture Research Station of Lanzhou University in Linze County, Gansu Province, China (latitude 39.24°N, longitude 100.06°E, altitude 1390 m), in June 2018. Six one-year-old Hu sheep × thin-tail Han

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4223 -

crossbred rams with an average body weight of 39.04 ± 4.67 kg were used in the experiment. Three sheep received the anthelmintic dose and three sheep were used as a control group. The sheep were placed in individual metabolic cages (*Fig. 1*). The cages were made of steel with a wooden floor that had a gap for faeces and urine drainage, which was collected in a nylon mesh and tilted plastic cloth beneath the wooden floor. Three sheep received albendazole tablets (Jixing Animal Pharmaceutical Co., Ltd., Sichuan, China) at a dose recommended by the manufacturer of 11 mg per kg body weight, the animals were treated with the albendazole only once. During the experiment period, the sheep were fed oat hay purchased from a local forage feed supplier and alfalfa hay produced at a university farm, mixed at the ratio of 9:1, compositions of mixed forage see *Table 1*. All the sheep had free access to sufficient water.

Figure 1. *Individual metabolic cages used for urine collection at Linze research station*

Table 1. *Chemical composition of forage fed to sheep during urine collection*

Nutrient indexes*	Linze forage	Maqu forage
OM, g/kg DM	906	897
CP, g/kg DM	148	79
NDF, g/kg DM	463	599
ADF, g/kg DM	348	326
Ether extract, g/kg DM	21	32

*OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; DM, dry matter

Urine collection

The sheep were managed in metabolic cages for one week to adapt to the conditions before the albendazole tablets were fed to them. Urine from dosed sheep was collected at 6, 11, 24 h and 3, 4, 5, 6 and 14 days after feeding the albendazole tablets. Urine from control sheep was collected only at 6 and 72 h and 14 days. The urine samples collected up to 72 h were the total urine excretions between sampling times. For example, the

sample at 6 h included urine excreted during the 0 to 6 h period and the sample at 11 h included urine excreted during the 6 to 11 h period. The samples collected at day 6 and day 14 were urine excreted just on that day. Urine collected in the bucket was thoroughly mixed and filtered through a clean gauze. A sub-sample of 50 ml urine was stored in a 50 ml centrifuge tube at −20 °C until further analysis.

Analysis of albendazole and its metabolites

Albendazole and its metabolites, albendazole sulfoxide and albendazole sulfone, were analysed at a commercial laboratory at Sci-tech Innovation Quality Testing Co., Ltd., Qingdao, China, using a liquid chromatograph mass spectrometer (Thermo SHISEIDO SP-TSQ Quantum Ultra, USA). The protocol adapted by the laboratory was briefly: weigh approximately 2 g of urine sample into a 50 ml centrifuge tube, add 15 ml of ethyl acetate solution and 100 μl of 2 M NaOH solution, vortex for 15 min, centrifuge at 6000 rpm for 5 min, collect the supernatant and transfer it to a 100 ml chicken heart bottle, rotary-evaporate at 40 °C to dryness, add 1 ml methanol-aqueous solution (4:6, V/V) to ultrasonic for 1 min to dissolve the residue, transfer the solution from the chicken heart bottle to a 10 ml centrifuge tube, add 2 ml of N-hexane and vortex for 2 min to centrifuge at 6000 rpm for 5 min, take the water phase solution through a 0.22 μm filter membrane and test it on the liquid chromatograph mass spectrometer.

Field experiment to investigate the effects of albendazole-fed sheep urination on soil nitrogen transformations and N₂O emissions

Experimental site

The experiment was conducted at Maqu Grassland Agriculture Research Station of Lanzhou University in Maqu County, Gansu province, China (latitude 35.97°N, longitude 101.88°E, altitude, 3750 m), in August 2018 (*Fig. 2*). The research site is located in the northeast portion of the Qinghai Tibetan Plateau. The climate is continentally cold/humid. The average annual temperature at the site is about 1.2 °C and the average annual rainfall is about 620 mm. The vegetation at the site is botanically diverse, consisting of typical alpine meadow plant species such as *Kobresia* (*K. graminifolia*, *K. capillifolia*, *K. humilis*, *K. Tibetica*), *Elymus* (*E. nutans*), *Potentilla* (*P. anserina*), *Stipa* (*S. aliena*), *Festuca* (*F. ovina*) and various other species. The alpine meadows at the site were subjected to year-round free-grazing by yak. The soil at the site is classified as alpine meadow soil. Site soil properties at 0–10 cm depth were: total C, 70.33 ± 3.66 g kg^{-1}; total N, 4.16 ± 0.15 g kg^{-1}; total P, 492.55 ± 32.4 mg kg^{-1}; and pH 6.23 (1:2.5, soil: water).

Urine collection

Six one-year-old Tibetan rams of the Oula breed with body weight 25.98 ± 3.34 kg were used to collect the urine for this experiment. Sheep were randomly allocated to two groups: the albendazole-dose group and the control group. Sheep were fed with forage cut from the mixed pasture at the site. The chemical composition of forage is shown in *Table 1*. The dose group sheep received albendazole capsules at a dose of 11 mg per kg body weight (Jixing Animal Pharmaceutical Co., Ltd., Sichuan, China), the animals were treated with the albendazole only once. All sheep were supplied with free access to sufficient water. The sheep were placed in individual metabolic cages. The cages and urine collection process were as described earlier. We used cumulative urine collected up to 48 h for the field experiment, as the previous experiment identified that the maximum

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4225 -

amount of albendazole and its metabolites' residues would excrete within 48 h. The dose group urine and control group urine were separately stored at −20 °C before use.

Figure 2. Nitrous oxide emissions measurement using static chamber method at Maqu Grassland Agriculture Research Station of Lanzhou University, China

The experimental design and urine application

The experimental plots were established within a 6 × 8 m area that was fenced to exclude grazing. There were three treatments – albendazole-dosed sheep urine, control sheep urine and no urine control treatment – with five replicates. The treatment plots (gas chamber bases) were arranged according to row and column design. The urine treatments were applied at a rate of 3 L m^{-2}, within a standard sheep urine patch as reported by Haynes and Williams (1993). The N concentration of the applied urine was unknown at the time of application. The N concentration of the urine stored at 4 °C for eight weeks was later detected by the direct distillation method (Hoogendoorn et al., 2010). Urine was slowly poured from a height of 30 cm to inside the chamber base area (0.4 x 0.4 m). No treatments were added to the control plots.

Gas sampling

Nitrous oxide emissions after urine treatment applications were measured by the static chamber method (de Klein et al., 2003). The cubic chambers (0.4 × 0.4 × 0.4 m) were constructed from stainless steel. The stainless-steel chamber was covered with a silver adiabatic material to reduce the impact of direct radiative heating during sampling. The top of the chamber had two holes plugged with rubber septa, which were used to insert a temperature probe and a sampling port connected to a three-way stopcock. For a gas seal at the soil surface, the bottom edge of each chamber was seated into a Y-shaped, water-filled stainless-steel channel, with the lower arm of the 'Y' penetrating the soil to approximately 0.1 m depth. The Y-shaped chamber bases were installed one week before treatment application. The day before the treatment application, herbage was cut to 5 cm above the ground. Gas samples were taken two days before urine application, four days

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4226 -

during the first week, two days during weeks two to four, and then one sample per week until emissions reached the baseline by six weeks.

On each occasion, gas samples were taken between 10 am and 12 pm local time. The chamber was placed on the chamber base channel, which was filled with water to give an airtight seal. Two headspace gas samples were taken during a cover period of 30 min at times 0 and 30 min. Headspace gas samples were taken using a 60 ml polypropylene syringe and injected into pre-evacuated 500 ml aluminum foil gas-collecting bags (China Dalian Gas Packing Co., Ltd). The N_2O concentration of the gas samples was analysed by gas chromatography (GC-2014 Shimadzu corporation, Japan). The hourly N_2O emission fluxes were calculated for each chamber from the linear increase in headspace N_2O concentrations over the sampling time (de Klein et al., 2003). Hourly N_2O emissions (mg N m^{-2} h^{-1}) were calculated as follows:

$$N_2O flux = \frac{\delta N_2O}{\delta t} \cdot \frac{M}{v_m} \cdot \frac{v}{A}$$

(Eq.1)

where N_2O flux is hourly N_2O emission (mg N_2O-N m^{-2} hr^{-1}), δN_2O is the increase in headspace N_2O during the enclosure period (µl L^{-1}), δt is the enclosure period (h), M is the molar weight of N in N_2O (g mol^{-1}), Vm is the molar volume of gas at the sampling temperature (L mol^{-1}), V is the headspace volume (m^3) and A is the area covered (m^2). Hourly emissions were integrated over time for each chamber to estimate total emissions. The emission factor (EF3) was calculated as the cumulative total amount of N_2O-N emitted as a percentage of urine N applied.

Soil mineral nitrogen measurements

Soil mineral nitrogen changes were measured using the ion exchange resin membranes (IEM) method (Bowatte et al., 2008). Membrane sheets (50 x 10 mm; VWR International Ltd, Poole, England) were fixed to plastic plant labels (100 x 15 mm) and inserted in the soil in the centre of the chamber base to leave the top of the sheet at the soil surface so that the effective depth sampled was 50 mm; the IEM probes were changed weekly. After removal, the IEM were washed with distilled water, extracted with 25 ml of 2 M KCl, filtered with Whatman #42 filter paper, and the nitrate (NO_3^--N) and ammonium (NH_4^+-N) contents were analysed using a FIAstar 5000 flow injection analyser (Foss Tecator, Hoganas, Sweden).

Statistical analysis

The amounts of albendazole and its metabolites excreted in urine over time for both dosed and control animals are presented for individual sheep. The mean and the standard error of the mean is presented to indicate the variability of the values among individual sheep.

The effect of urine treatments, time and their interactions on IEM-absorbed N and N_2O flux were tested by analysis of variance (ANOVA) using SPSS 20.0 statistical analysis software. All data were checked for assumptions of normality by the Kolmogorov–Smirnov test and log-transformed where necessary. The statistical significance of treatment means was tested by the Duncan method at the $P<0.05$ level.

Results

Albendazole and its metabolites' excretion in sheep urine

The amounts of albendazole, albendazole sulfoxide and albendazole sulfone detected in urine samples of six sheep are shown in *Table 2*. Albendazole and its metabolites were detected in both treated and untreated sheep urine samples, albeit at mostly trace amounts in untreated sheep urine. The highest amount of albendazole and its metabolite excretion, and the time when the highest amount appeared, varied between individual sheep. The maximum amount of albendazole was detected by the 24–36 h period from sheep 1 and 3, whereas it was by 6 h from sheep 2. The maximum amount of albendazole sulfoxide was detected from sheep 1 by the 24–36 h period, sheep 2 by 6 h and sheep 3 by 6–11 h. The maximum amount of albendazole sulfone was detected from sheep 1 by the 24–36 h period, sheep 2 by the 36–48 h period and sheep 3 by 6–11 h. The amount of albendazole sulfoxide excretion was substantially higher than albendazole or albendazole sulfone. By 72 h after the sheep were dosed with albendazole, the average total amounts of albendazole, albendazole sulfone and albendazole sulfoxide excreted with urine were 458.71 ± 106.12, 679.49 ± 575.98, 2341.31 ± 879.59 µg, respectively. After 48 h of dosing, low levels of albendazole and its metabolites were observed.

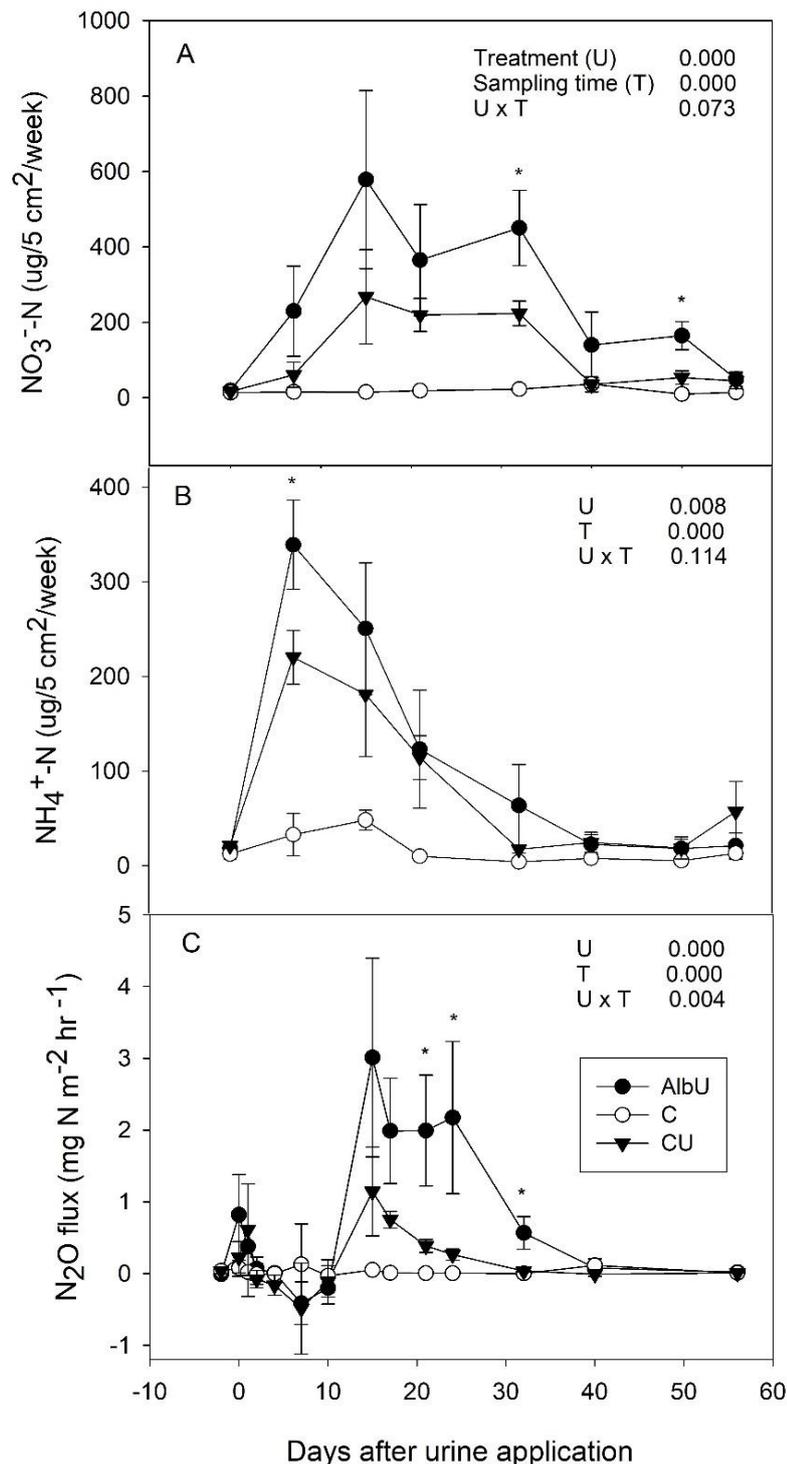### *Effects of albendazole-fed sheep urination on soil nitrogen transformations and N_2O emissions*

The changes in soil NO_3^- content after urine application are shown in *Fig. 3A*. The NO_3^- content in soil increased after the urine application in both urine treatment plots, indicating nitrification was occurring. Higher soil NO_3^- content was found in urine treatment soils compared with no urine control plots up to four weeks after urine application. The soil NO_3^- content was generally higher in albendazole-fed sheep urine treatment plots, where significant differences ($P<0.05$) in soil NO_3^- content between urine treatments were observed at 32 and 50 days after urine application.

The changes in soil NH_4^+ content after urine application are shown in *Fig. 3B*. The NH_4^+ content in soil increased after the urine application in both urine treatment plots compared with the no urine control plots, and the increase was significantly greater ($P<0.05$) in albendazole-fed sheep urine-treated plots at 7 days. The NH_4^+ content in soil then decreased with time in both urine treatment plots and by 40 days reached the level found in no urine control plots. Significant differences in soil NH_4^+ content between urine treatments were found only in the first week after urine application.

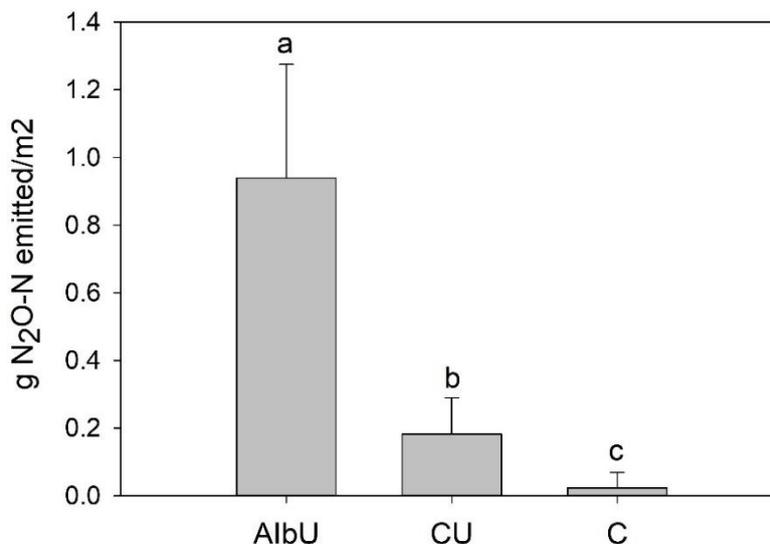The changes in soil N_2O fluxes after urine application are shown in *Fig. 3C*. Two weeks after urine application, higher N_2O emissions were observed from urine treatment plots compared with no urine control plots. The N_2O emissions were significantly higher ($P<0.05$) from the albendazole-fed sheep urine-treated plots than the control urine plots at 21, 24 and 32 days. The mean total N_2O emissions over the six-week measurement period were significantly different ($P<0.05$) among the treatments: 0.94 ± 0.34, 0.18 ± 0.11 and 0.02 ± 0.05 g N_2O-N m^{-2} for albendazole-dosed sheep urine, control urine and no urine, respectively (*Fig. 4*). The N_2O-N emitted as a percentage of urine N applied (emission factor, EF3) was significantly different between urine treatments ($P<0.05$): 1.88 ± 0.72 in albendazole-fed sheep urine-treated plots compared with 0.41 ± 0.27 in control urine-treated plots (*Fig. 5*).

Table 2. *Amount of albendazole and its metabolites (µg) in sheep urine at different times, after a single dose of albendazole was administered*

Metabolite	Treatment	Sheep ID		Urine collection period (hours after dosing)								
				0-6	6-11	11-24	24-36	36-48	48-60	60-72	6 d	14d
albendazole	Dosed		1	8.79	11.09	0.23	627.95	13.27	0.19	0.4	0.14	0.44
			2	240.59	9.56	0.13	0.08	52.71	0.78	0.19	0.44	0.45
			3	60.99	7.52	3.44	321.43	16.69	0.02	0.07	0.37	0.37
		mean		**103.46**	**9.39**	**1.27**	**316.49**	**27.56**	**0.33**	**0.22**	**0.32**	**0.42**
		sem		**70.2**	**1.03**	**1.09**	**181.27**	**12.62**	**0.23**	**0.1**	**0.09**	**0.03**
	No dose		4	0.09							0.12	0.07
			5	0.07							0.13	0.27
			6	0.06							0.74	0.38
		mean		**0.07**							**0.33**	**0.24**
		sem		**0.02**							**0.36**	**0.16**
albendazole sulfone	Dosed		1	1.44	1.43	1.96	70.37	5.08	0.83	2.32	1.35	6.42
			2	43.1	3.49	3.44	0.35	66.22	3.09	4.15	3.11	3.15
			3	10.85	1739.91	1.03	76.01	3.34	0.07	0	2.21	2.33
		mean		**18.46**	**581.61**	**2.14**	**48.91**	**24.88**	**1.33**	**2.16**	**2.22**	**3.97**
		sem		**12.61**	**579.15**	**0.7**	**24.33**	**20.68**	**0.91**	**1.2**	**0.51**	**1.25**
	No dose		4	0							0	0
			5	0.55							0	0
			6	0							5.04	0
		mean		**0.18**							**1.68**	**0**
		sem		**0.18**							**1.68**	**0**
albendazole sulfoxide	Dosed		1	11.11	14.39	11.53	1094.93	22.36	2.13	8.8	2.58	11.3
			2	1014.81	15.63	10.68	0.51	702.2	25.83	26.66	12.19	3.75
			3	228.92	3358.27	9.12	452.12	13.28	0.08	0.56	11.08	7.74
		mean		**418.28**	**1129.43**	**10.44**	**515.85**	**245.95**	**9.35**	**12.01**	**8.62**	**7.6**
		sem		**304.82**	**1114.42**	**0.71**	**317.53**	**228.14**	**8.26**	**7.7**	**3.04**	**2.18**
	No dose		4	1.17							0.95	0.43
			5	2.07							0.58	1.84
			6	0.71							13.73	2.51
		mean		**1.32**							**5.09**	**1.59**
		sem		**0.4**							**4.32**	**0.61**

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4229 -

*Figure 3. Resin adsorbed soil NO₃⁻-N (A), NH₄⁺-N (B) and nitrous oxide fluxes (C) after the application of urine from sheep treated with an albendazole (AlbU) or untreated (CU) and a non-urine control (C) to soil. Values are means (n=5) and error bars indicate standard error of mean. The statistical significances (P value) of treatment (U), sampling time (T) and their interaction (U×T) are shown on the top right of each figure. * indicate significant differences (P<0.05) between the AlbU treatment and CU treatment*

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4230 -

Figure 4. *The total N_2O emissions over the six-week measurement period from sheep treated with an albendazole (AlbU) or untreated (CU) and a non-urine control (C). Values are means ± SE, n=5. Different lowercase letters indicate significant difference at P<0.05*

Figure 5. *N_2O emissions as a percentage of applied urine N (EF3). Values are means ± SE, n=5. Different lowercase letters indicate significant difference at P<0.05*

Discussion

We observed rapid and substantial excretion of albendazole and its metabolites in urine, indicating that animal urine deposition to grasslands is an important pathway for anthelmintic drugs to enter the environment. We found that albendazole sulfoxide excretion in urine was markedly greater than albendazole or albendazole sulfone. Gyurik et al. (1981) similarly found most metabolites in the urine of albendazole-fed cattle, sheep, rats and mice were sulfoxide and sulfone. In their study, unchanged albendazole was found in minor amounts, but we observed reasonable amounts of unchanged albendazole

Ren et al.: Anthelmintic drug albendazole treated sheep urination influences soil nitrogen transformations and nitrous oxide
emissions from grasslands
- 4231 -

in urine by 72 h (mean 458.71 ± 106.12 µg, n=3). The amount of albendazole and its metabolites excreted after oral administration with time, as well as the time of peak excretion, appeared to vary between individual sheep (*Table 2*), indicating the significant influence of differences in animal physiology on anthelmintic metabolism and excretion with urine. Interestingly, albendazole and its metabolites were also detected in the untreated sheep group, indicating some albendazole and its metabolites may be already present in the environment.

Our study showed that, once these chemicals enter the grassland environment, they can influence important ecosystem processes. Several previous studies have discussed anthelmintic drug resistance as a potential consequence of anthelmintic drug entry into the environment (Han et al., 2017), while others have focused on the impacts on non-target soil organisms (Beynon, 2012). Our study demonstrated a previously unrecognised important consequence of anthelmintic drug release to grazed grasslands, and the influence on soil microbial processes that drive the soil nitrogen cycle. Our observation of higher N_2O emissions from albendazole-dosed sheep urination has a greater significance. Nitrous oxide is the third most important greenhouse gas, after methane and carbon dioxide (Blunden and Arndt, 2013). Atmospheric N_2O has risen steadily since the mid-twentieth century, from approximately 290 ppb in 1940 to 330 ppb in 2017 (Thompson et al., 2019) and grazed grasslands contribute a significant proportion of this increase (Oenema et al., 2010). In grazed grasslands, the biggest source of N_2O is animal urine (Selbie et al., 2015). Our results suggest that N_2O pollution from animal urine patches can be potentially exacerbated through anthelmintic drug administration to grazed animals.

One reason for the greater N_2O emissions observed in albendazole-dosed sheep urine-deposited soils compared with control urine-deposited soils in our study may be due to N loading differences. We were unable to test the N concentration of urine before the treatment application as the laboratory facilities were not available at the remote field site. However, we analysed the N concentration of the urine after the field experiment and found the N concentration of albendazole-dosed sheep urine (15 g L^{-1}) was greater than control sheep urine (10 g L^{-1}). There have been no previous studies in the literature reporting N excretion differences in urine after feeding albendazole or other anthelmintics, but Zhong et al. (2016) have reported enhanced feed intake by Small-tailed Han and Ujumqin ewes after anthelmintic drug administration. Greater feed intake can result in higher urinary N (Jardstedt et al., 2017). We did not monitor feed or water intake in our study, but higher feed intake or lower water intake could also potentially increase the N concentration in urine. Future studies investigating feed and water intake differences after anthelmintic drug administration and potential consequences for N excretion in urine are clearly warranted.

The urine treatments in our experiment maintained the same urine volume per unit area within the range of standard sheep urinations (Haynes and Williams, 1993). The N concentration difference between two urine treatments resulted in a higher N load in albendazole-dosed sheep urine treatment plots. We found greater ion exchange resin adsorption of NH_4^+, indicating higher NH_4^+ in soils of albendazole-dosed sheep urine treatment plots. This difference could possibly be due to the higher N load deposited in the dosed sheep urine treatment plots. The ion exchange resin adsorption of NO_3^- increased with time in both urine treatment plots, indicating that transformation of NH_4^+ to NO_3^- by nitrification was occurring. There was greater ion exchange resin adsorption of NO_3^- in albendazole-dosed sheep urine plots. This observation indicates that soil

nitrification activity was greater in albendazole-dosed sheep urine plots and therefore higher NO_3^- content was available for soil denitrification. These differences in soil N transformations in albendazole-dosed sheep urine treatment plots may have contributed to greater N_2O emissions (*Fig. 3C*) as both nitrification and denitrification contribute to N_2O production. Greater N_2O emissions from albendazole-dosed sheep urine treatment may be due to higher N load deposition (de Klein et al., 2014) but the estimation of the emissions from the same amount of N application (N_2O emissions as a percentage of applied urine N; EF3) was still higher in the albendazole-dosed sheep urine treatment compared with the control urine treatment (*Fig. 5*). This may suggest that the higher emissions in dosed sheep urine treatment plots could have resulted from factors other than greater N deposition to the soil. This conclusion is in agreement with van Groenigen et al. (2005), who did not observe a significant effect of the amount of urine N on emission percentage and highlighted that the most important controlling factors in their study that affected the emission factor were urine volume and C availability rather than the amount of N.

The other possible reason that stimulatory soil nitrogen transformations, and thereby N_2O emissions, were observed from albendazole-dosed sheep urine-treated plots could potentially be due to the direct effects of urine composition changes by albendazole or its metabolites on microbes responsible for soil nitrification and denitrification. Nevertheless, to the best of our knowledge, no such studies have been reported in the literature. However, there is evidence that N_2O can be formed through a process called co-denitrification (Spott and Florian Stange, 2011) where an N–N-linkage can occur between the amino group of an amine species and an N compound of the denitrification pathway (e.g., NO_2^-), which then results in excess N_2O or N_2 gas production. Recently, several studies have shown evidence for N_2O production via co-denitrification under simulated ruminant urine patch conditions (Clough et al., 2017; Rex et al., 2019). Albendazole undergoes rapid metabolism inside the animal gastrointestinal tract and transforms into multiple metabolites. Gyurik et al. (1981) identified nine metabolites of albendazole in the urine of cattle, sheep, rats and mice that had been orally fed albendazole. Stuchlíková et al. (2020) reported that albendazole can metabolise into 21 metabolites in alfalfa plants. Several of these metabolites consist of amine ($-NH_2$) and nitroso ($-N=O$) groups in their chemical structure, so there is the potential for these metabolites to take part in co-denitrification and produce excess N_2O. Future studies are therefore necessary to confirm the role of albendazole metabolites in co-denitrification.

This study was carried out using single-dose administration of fast-acting albendazole to sheep and N_2O measurements at a single grassland site during one season, hence similar studies with other forms of anthelmintics, including long-lasting slow-releasing capsule feeding in wider grassland environmental settings, are required to confirm its findings.

Conclusion

This study provided evidence of the rapid metabolism of orally ingested fast-acting albendazole capsules by sheep and the deposition of a substantial amount of albendazole, albendazole sulfoxide and albendazole sulfone to grazed grasslands through urine. Furthermore, the albendazole-fed sheep urine depositions altered the N transformations occurring in soil, resulting in greater N_2O emissions. The results of this study suggest that N_2O pollution from animal urine patches can be potentially exacerbated through anthelmintic drug administration to grazed animals. Further studies in multiple anthelmintic forms in wider environmental settings are warranted.

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