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Impact of fertilization with pig or calf slurry on antibiotic residues and resistance genes in the soil

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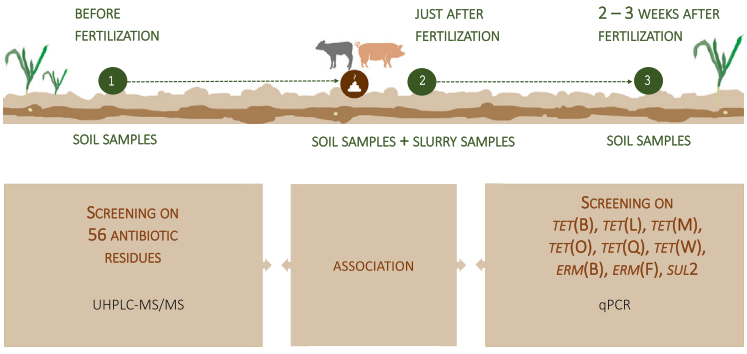
HIGHLIGHTS

- Tetracyclines, flumequine, lincomycin and sulfadiazine frequently present in soil.
- The genes *tet*(M), *sul*2, *erm*(B) and *erm* (F) were most abundant in fertilized soil.
- Resistance selection happens mainly in animal and slurry rather than in soil.
- Soil fertilization increases antibiotic resistance in the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic residues and antibiotic resistance genes can enter the environment via fertilization with calf and pig manure. In a longitudinal study, nine antibiotic resistance genes (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(F) and *sul*2) and 56 antibiotic residues were investigated in 288 soil samples and 8 corresponding slurry samples from 6 pig farms and 2 veal farms using qPCR and LC-MS/MS, respectively. A significant increase in gene copy number of *tet*(M), *erm*(B), *erm*(F) and *sul*2 was observed in all the soil layers between sampling times prior to (T1) and 2–3 weeks after fertilization (T3). *Tet*(B), *tet*(Q) and *tet*(L) were least abundant in the soil among the genes tested. From 7 classes of antibiotics, 20 residues were detected in soil and slurry using an optimized and validated extraction method. Flumequine was detected in all soil samples in concentrations below 100 μg/kg despite being detected in only half of the corresponding slurry samples. Doxycycline, oxytetracycline, lincomycin and sulfadiazine were also frequently detected in concentrations ranging from 0.1 μg/kg to 500 μg/kg and from 2 μg/kg and 9480 μg/kg in soil and slurry, respectively. Furthermore a positive association between the presence of antibiotic residues (total antibiotic load) and antibiotic resistance genes in soil was found. One possible explanation for this is a simultaneous introduction of antibiotic residues and resistance genes upon application of animal slurry.

1. Introduction

Antibiotic resistance, referred to as one of the most important threats to public health, has been receiving increasing attention in recent years (FAO et al., online). Antibiotic use in animal production can result in a selection

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of antibiotic resistant bacteria in these animals. Transfer of antibiotic resistant bacteria from animals to humans can occur either directly, through contact with animals and consumption of animal products, or indirectly via the environment (Kuppusamy et al., 2018; Kumar et al., 2005; Heuer et al., 2011a). All types of exposure lead to an increased risk of antimicrobial therapy failure in case of a bacterial infection (Bell et al., 2014; Chantziaras et al., 2014).

Flanders is responsible for nearly 95% of both pig and veal calf farming in Belgium (Statbel, het Belgische statistiekbureau Available, online; Cijfergegevens Departement Landbouw en Visserij (Regio Vlaanderen) Available, online). Antibiotic use in livestock production in Belgium is still high, even after a 40% reduction since 2011. Measurement of antibiotic use in livestock is based on the percentage of the animal's lifetime that it is treated with antimicrobials (BD_{100}). Flemish veal calves consume the most antibiotics per animal with an average BD_{100} of 22 (BelVetSac Belgian Veterinary Surveillance of Antibacterial Consumption - National consumption report, 2018). Although pigs have a far lower BD_{100} of 7, the sheer number of pigs produced in Flanders easily explains this sector's leading position in antibiotic sales data (BelVetSac Belgian Veterinary Surveillance of Antibacterial Consumption - National consumption report, 2018). Many studies relate the presence of antibiotic residues (ABRs), antibiotic resistance genes (ARGs) and antibiotic resistant bacteria in manure to the intensive use of antibiotics in animals (Heuer et al., 2011b; Hölzel et al., 2010; Peak et al., 2007; Heuer and Smalla, 2007).

Manure is often applied to fields as fertilization, with pig and cattle manure being the most commonly used. In 2019, 22% and 72% of the total pig and cattle manure, respectively (as expressed in N), was applied on agricultural fields (VLM Mestbank Mestrapport_2020.pdf Available, online). Manure is the main source of the introduction of ABRs in soil, groundwater and surface water (Chee-Sanford et al., 2009). The persistence of ABRs in soil depends on several characteristics including the soil type and the physicochemical properties of the antibiotic (Berendsen et al., 2021). Tetracyclines, sulfonamides, macrolides and fluoroquinolones as well as multiresistant *E. coli* and several ARGs (*tet*, *erm* and *sul* genes) have frequently been detected in manure of pigs and calves (Huygens et al., 2021; Rasschaert et al., 2020; Filippitzi et al., 2019; Van den Meersche et al., 2020; Lahr et al., 2018; Patterson et al., 2007). Application of antibiotic-contaminated manure on the field results in a risk that crops and vegetables cultivated on fields fertilized with raw manure can be exposed to ABRs, ARGs and antibiotic resistant bacteria present in the soil (Boxall et al., 2006). Van den Meersche et al. (2020) noted that the number of 9 ARGs (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(F) and *sul*2) significantly increased in soil soon after fertilization with pig slurry, but at harvest (5 to 7 months after fertilization) the number of genes had dropped to background levels (Van den Meersche et al., 2020). The human health risks associated with antibiotic resistance, intensive farming and its associated use of antibiotics and application of raw livestock manure as fertilizer all indicate the need to investigate the spread of ABRs and ARGs in the wider Belgian environment.

The current study adds the following new information to the current body of research: 1) screening for a wide range of antibiotic residues, 2) inclusion of veal calf farms in Flanders, 3) soil sampling within a shorter time frame and 4) the association between total antibiotic load and ARGs. In a previous study, we investigated the presence and fate of 7 ABRs from 6 antibiotic classes on 5 fields fertilized with pig manure (Van den Meersche et al., 2020). Recent studies have found many other antibiotic residues in pig and calf slurry (Huygens et al., 2021; Rasschaert et al., 2020). The present study therefore added several ABRs to the antibiotic screening in order to detect up to 56 ABRs from 10 antibiotic classes in soil. Nearly all antibiotics registered for food-producing animals in Belgium are thus included in the method, with the exception of aminoglycosides (vetcompendium BCFIvet Available, online). In contrast to our previous study (Van den Meersche et al., 2020), where only pig manure was used to fertilize the fields, the present study also includes calf slurry, as calf slurry is often used as fertilizer and normally contains a high load of antibiotic residues. This indicates that calf manure presents a high risk for the spread of antibiotic resistance in the environment (Huygens et al., 2021). The current study also performed measurements in a shorter timeframe in comparison to the previous study. The prior study was based on a longitudinal screening where samples were taken before fertilization, after fertilization, after 1 month, after 2 months and after harvest (approximately 5–7 months). In that study, after 1 month antibiotic residues and antibiotic resistance genes had already declined to background levels. The present study focuses on the sowing and planting period to gain insight into the exposure of sprouting seeds and young plants to antibiotic residues and resistance genes. Samples were taken before fertilization (T1), immediately after fertilization (T2), and 2 to 3 weeks after fertilization (T3). Previous studies describe an increased number of resistance genes in soils due to fertilization with animal manure but the relationship between the presence of ARGs and ABRs in soil has not yet been well clarified (Heuer et al., 2011a; Cycoń et al., 2019). In the present study we screened a large spectrum of antibiotic residues and different antibiotic resistance genes carrying resistance to antibiotics from different classes within the same soil samples. This made it possible to study their interrelationships, specifically the association between the total antibiotic load and 9 ARGs in soil fertilized with pig and calf slurry. To the best of our knowledge this is the first study to detect 56 ABRs in a soil matrix using one extraction method as well as being the first study to associate the total antibiotic load with ARGs in soil.

2. Materials and methods

2.1. Experimental design and sample collection

To investigate the fate of ABRs, ARGs and their relationship in soil, a broad longitudinal study was conducted. Fifty-six ABRs from 10 antibiotic classes and 9 ARGs were investigated in slurry and soil at 8 farm fields (1–8). Different soil types were included (3 fields had a clay soil type, 3 sand, 1 loamy sand and 1 sandy loam) and different crops were grown on the fields (5 fields of maize, 2 potato and 1 sugar beet; Table 1). Calf slurry (locations 2 and 3) and pig slurry (locations 1, 4, 5, 6, 7 and 8) were used to fertilize the fields. At each farm field, the same 4 square plots of 100 m^2 were sampled at 3 depths: 0–10 cm, 10–30 cm and 30–60 cm, and at 3 time points: before (T1), just after (T2) and 2 to 3 weeks after fertilization (T3). This resulted in $8 \times 4 \times 3 \times 3 = 288$ soil samples. Moreover, slurry was collected from the manure tank directly before application on the investigated field, resulting in 8 slurry samples. Soil sampling was performed according to the protocol of Van den Meersche et al. (2020) (Berendsen et al., 2021). Briefly, 15 samples were taken with a gouge auger at three depths within each plot at T1, T2 and T3. For practical reasons the time between T1 and T2 varied between 12 and 42 days (Table 1). T3 was an approximation for the sowing and planting period. At T3 only the fields of locations 1 and 2 had not yet been planted or sowed. All farms were located in Flanders (northern Belgium) and sampled in 2018. Farms were required to meet the following requirements: 1) at least one field fertilized with manure from the farm's own animals, 2) at least one other field had not been fertilized with fresh animal manure for at least 5 months before the start of the first sampling and 3) the animals had been treated with antibiotics within the last year.

2.2. Physicochemical characterization of soil

A physicochemical analysis was performed on 32 soil samples, more specifically on the upper layer (0 cm–10 cm) of the soil samples taken before fertilization. The analyses were carried out at Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Merelbeke, Belgium). Organic carbon was determined according to BAM part 1/10, BOC and ISO 10694 (Compendium voor bemonsterings- en analysemethodes voor mest, bodem en veevoeder (BAM) Available, online; Compendium voor de monsterneming, 2010; ISO 10694:1995, 1995). The pH-KCl was analyzed according to BOC and ISO 10390 and total nitrogen was analyzed according to Dumas by ISO 13878 (Compendium voor de monsterneming, 2010; ISO 10390:2021, 2021; Dumas, 1831). Iron, potassium, magnesium, calcium, manganese, sodium and phosphorus were determined according

Table 1
The soil type, time (days) between T1 and T2, time (days) between T2 and T3, crops grown on the fields and antibiotic use at each farm.

Farm	Animal slurry	Soil type	Time between T1 and T2 (days)	Time between T2 and T3 (days)	Crop grown on the field	Antibiotic use[a]
1	Pig	Clay	12	14	Sugar beets	Chlortetracycline, gentamicin, amoxicillin, colistin, doxycycline, florfenicol, lincomycin, spectinomycin
2	Calf	Loamy sand	21	16	Maize	Doxycycline, oxytetracycline, tilmicosin, trimethoprim, sulfadiazine, paromomycin, amoxicillin, lincomycin, spectinomycin, neomycin, florfenicol, benzylpenicillin
3	Calf	Sand	42	14	Maize	Amoxicillin, doxycycline, florfenicol, paromomycin, tylosin, tilmicosin, phenoxymethylpenicillin, benzylpenicillin, lincomycin, spectinomycin
4	Pig	Sandy loam	36	22	Potatoes	Amoxicillin, sulfadiazine, trimethoprim, florfenicol, doxycycline
5	Pig	Clay	40	22	Potatoes	Amoxicillin, colistin, tiamulin, lincomycin, spectinomycin
6	Pig	Clay	39	18	Maize	Amoxicillin, doxycycline, benzylpenicillin, tulathromycin
7	Pig	Sand	42	19	Maize	Tilmicosin, amoxicillin
8	Pig	Sand	29	19	Maize	Colistin

[a] The antibiotic use from the last year according to the farmer. Data may be incomplete.

to the guidelines from BAM part 1/11 (Compendium voor bemonsterings- en analysemethodes voor mest, bodem en veevoeder (BAM) Available, online). The physicochemical characterization parameters of the soils from the different locations are shown as supplementary data in Table S6.

2.3. Detection and quantification of antibiotic residues by UHPLC-MS/MS

2.3.1. Reagents and materials

A detailed description can be found in the Materials and Methods section of the Supplementary data.

2.3.2. UHPLC-MS/MS

A new extraction method was developed to quantify 56 ABRs from 10 classes (Table 2) in soil. The extraction method was modified from the method in Jansen et al. that was developed to detect ABRs that were difficult to extract from manure (Jansen et al., 2019). Briefly, 2 g of each soil sample was placed in a polypropylene (PP) tube and extracted using 4 mL of a fresh 0.125% (v/v) trifluoroacetic acid (TFA) in acetonitrile (MeCN) solution. The PP tube was firmly shaken for 10 min at 225–250 rotations per minute (rpm) after which 4 mL of McIlvaine – ethylenediaminotetraacetic acid (EDTA) buffer was added. The buffer was made as described by Jansen et al. (Jansen et al., 2019). After shaking for 15 min at 225–250 rpm and centrifugation for 10 min at 3500g, the supernatant was evaporated in a warm water bath (40 °C, under N_2) until 4 mL retained. The obtained extracts were purified using a tandem solid phase extraction (SPE) where a hydrophilic-lipophilic balance (HLB) column is placed upon a mixed-mode cation exchange (MCX) column (tandem HLB/MCX set-up). The columns were separately eluted and the obtained eluates were poured together and evaporated in a warm water bath (40 °C,N_2) until dryness. The extract was re-dissolved in 1 mL mobile phase (H_2O/MeCN/MeOH (50/25/25) + 0.05% AA) and consecutively vortexed, placed in an ultrasonic bath for 5 min, filtered through a 0.22 μm filter, transferred to a vial with insert and injected into the UHPLC-MS/MS system (Acquity UHPLC, column: BEH C18 (100 mm × 2.1 mm i.d.,1.7 μm, solvent A: water + 0.05% AA, solvent B: ACN/MeOH (50/50) + 0.05% AA), Xevo TQ-S mass spectrometer (Waters Corporation)).

In addition, 56 ABRs were quantified in the calf and pig slurry samples using the multi-residue extraction procedures and UHPLC-MS/MS methods described in previous studies (Huygens et al., 2021; Rasschaert et al., 2020).

ABRs in samples below the limit of detection (LOD) but with a signal/noise >3 as confirmed with 2 or more product ions and a corresponding relative retention time were considered to be detected as traces. For simplification, no distinction was made between traces and detections above the LOD for the further interpretation of the results and for the association with ARGs in this article.

2.3.3. Method for validation in soil (UHPLC-MS/MS)

The LOD, limit of quantification (LOQ), and linearity of the method were determined using three sets of a matrix-matched calibration curve of

Table 2
Antibiotics (n = 56) from 10 classes screened in soil samples.

β-Lactamantibiotics	***Fluoroquinolones***	***Sulfonamides and trimethoprim***	***Macrolides***
Amoxicillin[b]	Norfloxacin[a]	Sulfachloropyridazine[a]	Erythromycin[c]
Ampicillin[b]	Enoxacin[a]	Sulfaclozine[a]	Spiramycin[c]
Benzylpenicillin[d]	Difloxacin[a]	Sulfadiazine[a]	Tilmicosin[b]
Cefalexin[b]	Ciprofloxacin[a]	Sulfadimethoxine[a]	Tylosin[c]
Cefalonium[b]	Enrofloxacin	Sulfadoxine	Tylvalosin[b]
Cefazolin[b]	Marbofloxacin[a]	Sulfamerazine[a]	***Tetracyclines***
Cefoperazone[b]	Danofloxacin[a]	Sulfamethazine[a]	Oxytetracycline[c]
Cefquinome[b]	Sarafloxacin[a]	Sulfamethoxazole[a]	Chlortetracycline[c]
Cloxacillin[b]	Flumequin[a]	Sulfamethoxypyridazine	Doxycycline[b]
Dicloxacillin[b]	***Quinolones***	Sulfapyridine[a]	Tetracycline[b]
Nafcillin[b]	Cinoxacin[a]	Sulfaquinoxaline[a]	***Amphenicols***
Oxacillin[b]	Nalidixic acid[a]	Sulfathiazole[a]	Chloramphenicol[c]
Penicillin V[d]	Oxolinic acid[a]	Trimethoprim[a]	Florfenicol[b]
Cefapirin[b]	***Lincosamides***	***Pleuromutilins***	Thiamfenicol[b]
Ceftiofur[b]	Lincomycin[c]	Tiamulin[b]	***Diaminopyrimidinederivates***
	Pirlimycin[b]	Valnemulin[b]	Dapsone[a]

[a] Synthethically produced.
[b] Semi-synthetically produced.
[c] Naturally produced by *Streptomyces* spp.
[d] Naturally produced by *Penicillium chrysogenum*.

6 points in a soil matrix (sandloam) free of antibiotics, with the response plotted as function of the concentration (see Tables S1, S2 and S3). The LOD was calculated as 3 times the standard error of the y-intercept of the regression line divided by the slope. The LOQ was calculated as 10 times the standard error of the y-intercept of the regression line divided by the slope. The recovery, repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) were measured using 3 series of 6 repetition points at 3 concentration levels depending on the antibiotic residue.

The repeatability, reproducibility and recovery at three validation levels for 25 ABRs including the sulfonamides, and for 11 ABRs including the tetracyclines, lincomycin and flumequine, are listed in Tables S1 and S2, respectively. Based on the validation parameters, it can be concluded that only the ABRs in Table S1 can be accurately quantified; the validation parameters of the ABRs in Table S2 and S3 show too much variation. The concentrations of the ABRs listed in Table S2 should therefore be considered as semi-quantitative. The ABRs listed in Table S3 could only be detected in a certain order of magnitude. This is also reflected in a less acceptable linearity (R). The coefficient of correlation (R) between the response (peak area corrected by the corresponding internal standard) and added concentration for all the ABRs ranged between 0.85 and 1.

2.4. Quantification of antibiotic resistance genes in slurry and soil samples using qPCR

2.4.1. DNA extraction

A DNA extraction was performed on 8 slurry samples and 288 soil samples using the PowerSoil DNA Isolation Kit (Qiagen). From each sample, 0.25 g was extracted according to the manufacturer's protocol. The DNA yield was measured using a NanoPhotometer (Implen, München, Germany) and Quantus™ fluorometer (Promega, Madison, WI, USA) in order to determine the DNA quality and quantity of the obtained extract.

2.4.2. Quantitative real-time polymerase chain reaction (qPCR)

In total, 9 ARGs were quantified in 288 soil samples and 8 slurry samples. Six tetracycline resistance genes (*tet*(B), *tet*(M), *tet*(L), *tet*(O), *tet*(Q) and *tet*(W)), two macrolide-lincosamide-streptogramin (MLS) resistance genes (*erm*(B) and *erm*(F)) and one sulfonamide resistance gene (*sul*2) were quantified using the primers, probes and qPCR methods that were optimized and described in detail by Van den Meersche et al. (2020). The abundance of the ARGs in each sample was normalized by the quantification of the 16S rRNA gene, which represents the total amount of bacteria in the sample. As quantification standard for the 16S rRNA gene, a gBlock® gene fragment was developed for primers Bac338-F (5′ ACTCCTACGGGAGGCAGCAG 3′) and Bac518-R (5′ ATTACCGCGGCTGCTGG 3′) (De Mulder et al., 2019). Between those primer binding sites, a 16S rDNA sequence of an *E. coli* strain with a length of 163 base pairs (accession number GenBank: MK506978) was located. Further, two gBlock® gene fragments, one for the tetracycline resistance genes and one for the *sul*2 and *erm*-genes (as described by Van den Meersche et al. (2020)) were used for a standard curve for quantification of the ARGs. The six tetracycline resistance genes were quantified with TaqMan® assays using ZEN™ Double-Quenched Probes and the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene, *erm*(B), *erm*(F) and *sul*2 genes were quantified with qPCR assays based on the intercalating SYBR® Green dye. Specifically, for the 16S rRNA gene the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Temse, Belgium) was used and for the *erm*- and *sul*2 genes the SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad Laboratories, Temse, Belgium) was used. The PCR reactions were performed in a LightCycler® 480 System (Roche) using the same PCR conditions described by Van den Meersche et al. (2020). The prepared DNA extracts were diluted 10 to 1000 fold before qPCR analysis, depending on the investigated resistance genes and whether it was a soil sample or slurry sample (Van den Meersche et al., 2020). If the samples did not contain a gene copy number within the range of the standard curve, the respective gene was considered as not detected in the samples.

2.5. Statistical analysis

2.5.1. Fate over time of the antibiotic resistance genes in soil

To analyse the fate of the ARGs in the soil at several depths over time, a linear mixed model regression (Zuur et al., 2009) was built for each resistance gene with the log of the resistance gene copy number (log(Rgene)) as response variable and time (Time) and depth (Depth) of sampling and their interaction (Time:Depth) as explanatory variables, resulting in the following fixed effect part of the regression model: log(Rgene) ~ Time + Depth + Time:Depth. This longitudinal study includes repeated measurement of the same locations and plots; therefore the observations are not independent. To incorporate the dependency structure, the mixed model included the location and plots nested within locations as random intercepts: (1|Location + 1|Location:Plot). With a log transformation of the gene copy numbers, zero values (i.e. values below the range of the standard curve) cannot be represented directly. For these samples, for all resistance genes, a common small value (−6.5) was assigned, i.e., a value lower than any other copy number within the range of the standard curve for all genes.

In essence, the mixed effect regression is a two-way ANOVA with factors Time and Depth. This makes it possible to represent the results of the analysis in the same way as a classical ANOVA, i.e., a table with the F-tests testing for the effects of Time, Depth and Time:Depth, and post-hoc tests for statistical differences between the means (Table S4). An additional table in comparison to the classical ANOVA contains the variance components, i.e., the decomposition of the variance in a term related to the location level (variability between the farms), the plot level (variability of the plots within the farms) and the residual noise (variability within the plot) (Table S5). Expressing these variances as percentages gives insight into the main sources of variation. These summary statistics were obtained using the R software packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017).

Based on the mixed effect regression models, the mean concentrations (and confidence intervals) of ARGs in soil at 3 time points and 3 soil depths were estimated as predicted means. These so-called estimated marginal means were calculated with version 1.6.3 of the emmeans-package of the R-software (Lenth et al., 2021). The output of the emmeans-package is visualized for each combination of time and depth with the confidence limits around the estimates and a compact letter display (CLD) to synthesize the hypothesis testing between the means taking multiple testing into account. Equal letters in a CLD indicate that the group means do not differ statistically and different letters refer to group means that are statistically different (Piepho, 2000; Piepho, 2018). Statistical significance was considered for P values below 0.05 and 95% confidence intervals were calculated.

2.5.2. Exploratory analysis of the association between the antibiotic load and resistance genes in soil

To explore the association between the ABRs and the ARGs, we first defined an overall indicator of the total antibiotic load to synthesize the information of the 56 antibiotics tested. Several alternative definitions were screened (Fig. S1), but for the current exercise the total antibiotic load was defined as the log of the sum of the square roots of the concentrations of all antibiotics detected at a plot. The square root mitigates the effects of outliers and the sum is log-transformed to compare antibiotic load and resistance genes (log counts) in the same log-scale. For the same reason as for the resistance genes, a value −1 log (0.1 μg/kg) was chosen for samples with no antibiotic load. The slope of a linear regression was used as a descriptive summary statistic to describe the strength of the association.

Statistical analysis was performed in R-4.0.4 (R Core Team, 2021) in combination with Rstudio (2021).

3. Results

3.1. Detection and quantification of antibiotic residues in soil and slurry samples

A total of 20 ABRs from 7 classes (tetracyclines, fluoroquinolones, β-lactam antibiotics, macrolides, lincosamides, sulfonamides & trimethoprim

and pleuromutilins) were detected in the samples from the longitudinal study. Of these, 13 and 17 ABRs were detected in slurry and soil samples, respectively. Fig. 1 shows an overview of all the detected ABRs and Table 3 the detection frequency of ABRs in the soil and slurry samples. For purposes of this article, in Table 3 a distinction was made between traces (detections below LOD as described in Section 2.3.2) and detections above the LOD of these ABRs in the soil. Flumequine, doxycycline, oxytetracycline, lincomycin and sulfadiazine were most frequently detected in the slurry and soil samples, namely in 50% to 100% of the slurry samples and in 36% to 100% of the soil samples (Fig. 1 and Table 3). A few β-lactam antibiotics (ampicillin, benzylpenicillin, cefapirin and cefalonium) were found at T1 and T2 in the different soil types and different soil layers in low concentrations, despite having not been detected in the slurry. These were found in concentrations below 10 μg/kg (mean value of 4 plots) in soil. Similarly, danofloxacin, tiamulin and sulfamerazine were only detected in a few soil samples and not in the corresponding slurry (Fig. 1).

Fig. 2 shows the 9 most frequently detected ABRs in the soil according to the different sampling locations, which are explained in more detail below.

For flumequine, doxycycline and oxytetracycline the concentration was higher in the two upper soil layers (0–10 cm and 10–30 cm) compared to the deepest soil layer (30–60 cm). Furthermore, these 3 components were generally already detected in the soil layers at T1. Remarkably, flumequine was detected in all the soil samples but only in half of the slurry samples. The concentration of flumequine remained quite stable over time in all the soil samples. More specifically, it was found in the soil in the 2 upper layers

Table 3
Number of detections (%) and percentage of detection above the theoretical LOD (%) of ABRs in slurry and soil samples.

Antibiotic residue	Number of detections in soil samples (%), $N = 288$	Percentage of detection above the LOD relative to the number of detections in soil samples (%)	Number of detections in slurry samples (%), $N = 8$	Percentage of detection above the LOD relative to the number of detections in slurry samples (%)
Flumequine	100	85	50	100
Doxycycline	84	93	100	100
Oxytetracycline	58	68	50	100
Sulfadiazine	44	62	75	100
Lincomycin	36	33	88	86
Ciprofloxacin	22	21	25	100
Tiamulin	21	25	0	–
Enrofloxacin	18	4	25	100
Tylosin	8	59	13	100
Sulfamerazine	5	0	0	–
Marbofloxacin	4	0	38	100
Ampicillin	3	38	0	–
Benzylpenicillin	2	0	0	–
Danofloxacin	2	0	0	–
Cefapirin	2	100	0	–
Trimethoprim	1	33	38	67
Cefalonium	0.3	100	0	–
Tulathromycin	0	–	25	0
Chlortetracycline	0	–	13	0
Tylvalosin	0	–	13	100

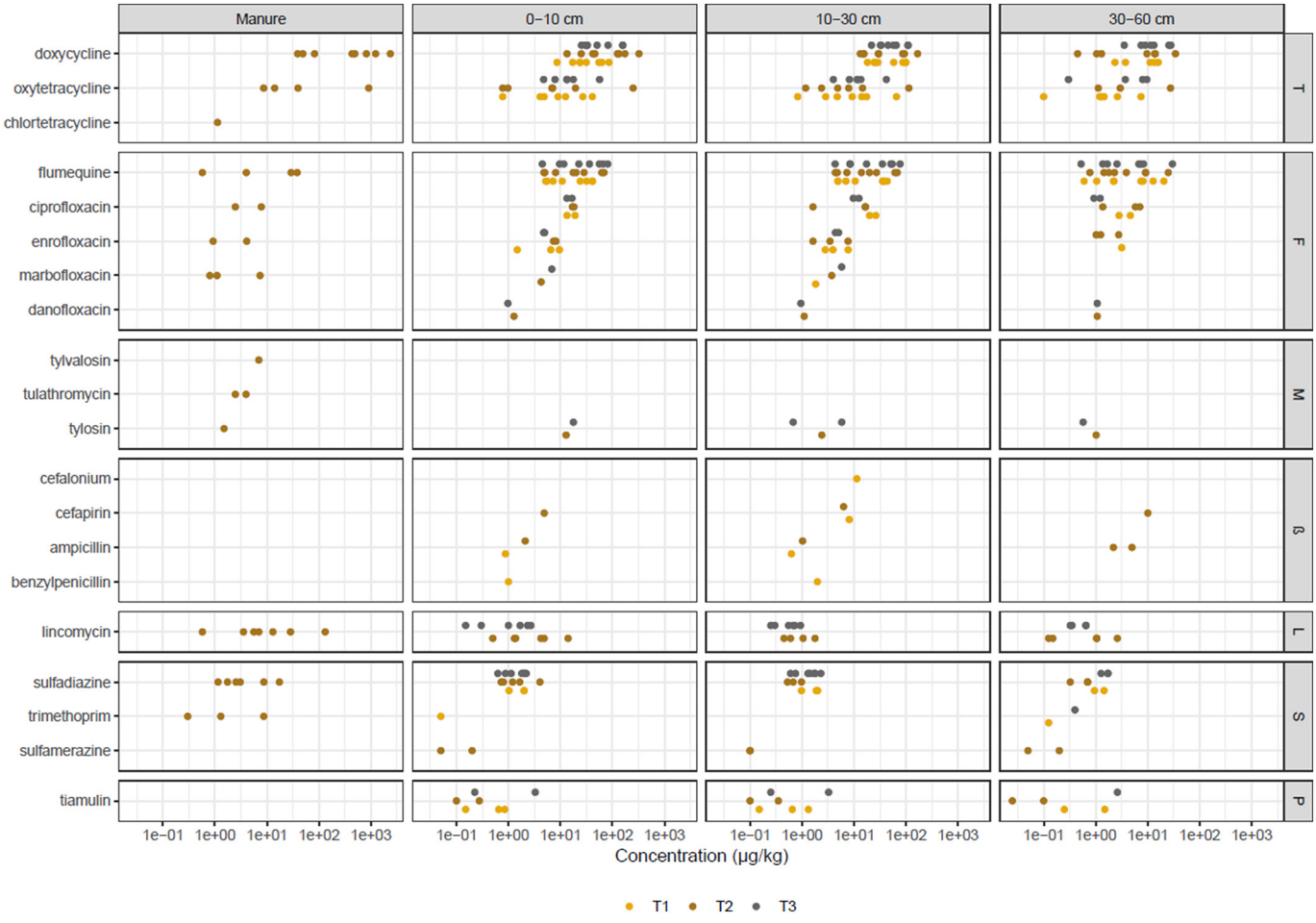

Fig. 1. Concentration (μg/kg) of ABRs in slurry samples and soil samples of 8 farms at 3 depths (0–10 cm, 10–30 cm, 30–60 cm) and 3 times (T1 = before fertilization (yellow), T2 = just after fertilization (brown) and T3 = 2 to 3 weeks after fertilization (gray)). For the soil samples, the dots represent the mean value of the observations of 4 plots at one location after removal of samples with no detection of the respective antibiotic. For the slurry, the dot represents the concentration in one sample. The position of the dots is adjusted vertically by dodging overlaps.

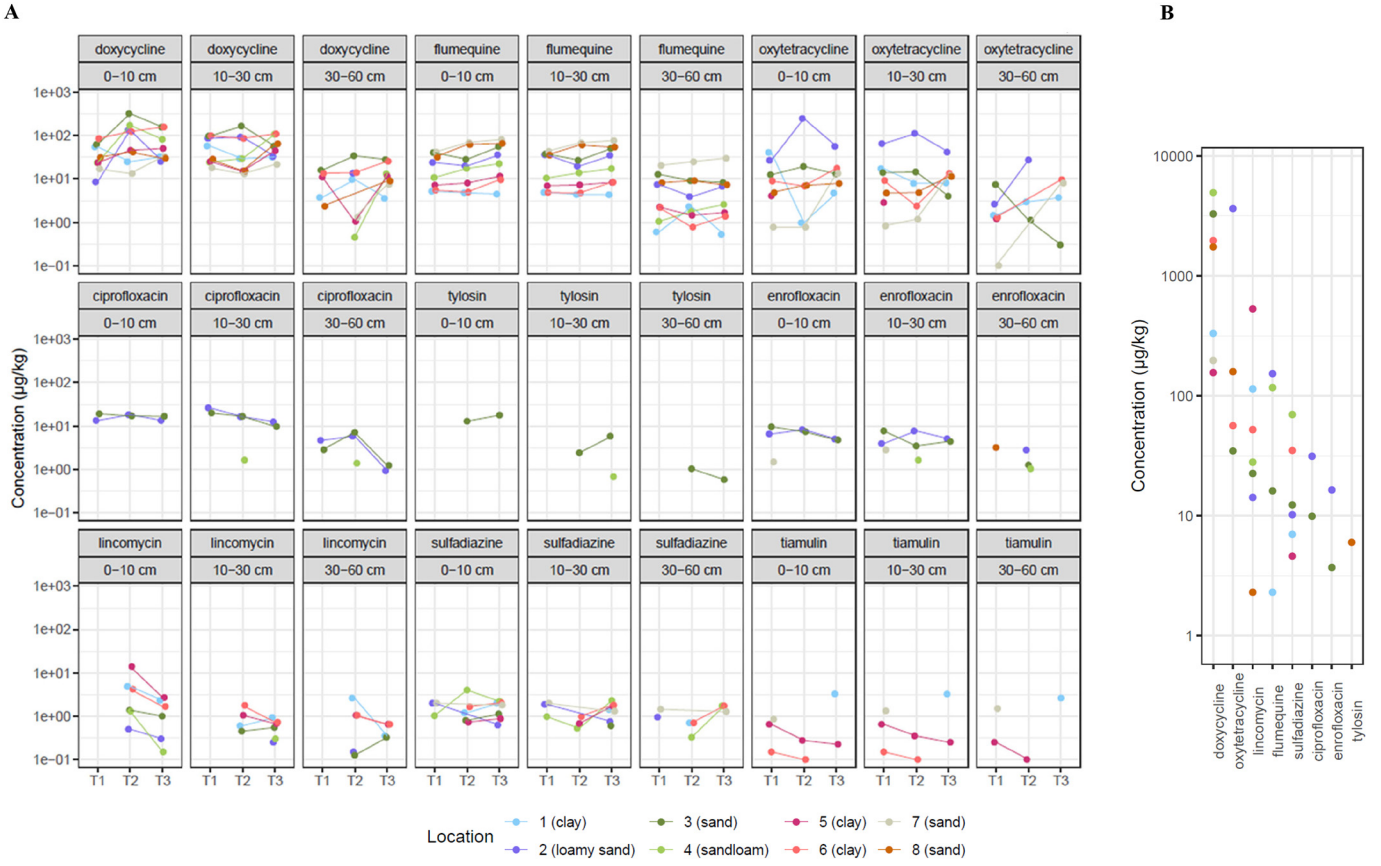

Fig. 2. A. Variability of the presence of the main ABRs in soil represented by the mean concentration (µg/kg in the log-scale) of 9 ABRs in soil samples of 4 plots at 3 depths (0–10 cm, 10–30 cm, 30–60 cm) and 3 times (T1 = before fertilization, T2 = just after fertilization, T3 = 2–3 weeks after fertilization) and at 8 locations with different soil types (clay, sand, loamy sand, sandy loam). **B**. Concentration (µg/kg) of ABRs in corresponding slurry samples.

in concentrations (mean values of 4 plots) between 5 µg/kg and 100 µg/kg and in the deepest layer below 1 µg/kg and up to 50 µg/kg. Similar concentrations were detected in slurry. Doxycycline and oxytetracycline were recovered from all soil types in concentrations (mean values of 4 plots) between 0.1 µg/kg and 500 µg/kg. For doxycycline a slight increase was observed in the top soil layer at T2 when the component was present in the slurry.

Enrofloxacin and its metabolite ciprofloxacin were present in both veal farms, with sandy soil at both farms. The components were already present in the soil at T1 in all soil layers, except enrofloxacin, which was not present on the farms at T1 in the lower soil layer. Both components were present in the slurry and soil in relatively low concentrations between 1 µg/kg and 50 µg/kg. No strong increase in concentration was observed in the soil after fertilization.

Lincomycin was introduced into the soil at T2 and was still present at T3 on 6 different farms. The highest concentrations in the soil were observed in the top layer of 3 locations (soil type clay) where the concentration was also the highest in the corresponding slurry. Lincomycin was present in the soil in relatively low concentrations (mean values of 4 plots ranging between 0.1 µg/kg and 15 µg/kg) and in the corresponding slurry samples in a concentration ranging from 10 µg/kg to 1000 µg/kg.

Sulfadiazine was detected in the soil at 7 locations and was present in all 3 soil depths in very low concentrations (below 10 µg/kg). Additionally, sulfadiazine was present in most corresponding slurry samples (between 5 µg/kg and 100 µg/kg).

Furthermore tylosin was found at a veal farm (sandy soil) in every soil layer starting at T2, even though this component was not detected in the corresponding slurry.

Finally, tiamulin was only detected in the soil at 4 locations in very low concentrations (below 10 µg/kg). Similar detections were observed in all soil layers. Most of the detections were made in clay soil.

3.2. Quantification of antibiotic resistance genes in soil and slurry samples

Fig. 3 visualizes the variation of the 16S rRNA gene copy number in the different soil layers at different time points. A lower number of 16S rRNA gene copies was observed in the 30–60 cm layer compared to the 0–10 cm and 10–30 cm layer. The 16S rRNA gene copy number/g ranged from 7.5 log to 9.9 log in soil and from 9.9 to 10.6 log in slurry. Fig. 4 represents the 9 investigated ARGs and their estimated marginal mean concentrations (normalized to the 16S rRNA gene copy number) across the 8 locations with confidence intervals and significant differences in soil at different times and depths. Fig. S2 plots the concentration of each antibiotic resistance gene for each observation in the soil against time. Similar concentration patterns were observed for the different resistance genes at different times and depths between the modelled concentrations and the observed concentrations, indicating a good model for our observed data. This allows us to discuss the modelled concentrations in what follows.

At T1 no significant differences in gene copy numbers for the different resistance genes were observed between the upper layer (0–10 cm) and middle layer (10–30 cm). The gene copy number of the resistance genes was significantly lower at T1 in the deepest layer (30–60 cm), except for *erm*(F), *tet*(B), *tet*(Q) and *tet*(L) where no significant difference was observed between the layers. At T2, the gene copy number of each antibiotic resistance gene was significantly higher in the upper soil layer (0–10 cm) as compared to the two lower layers, except for *erm*(B).

In general, *sul*2, *erm*(B), *erm*(F) and *tet*(M) were detected in animal slurry in the highest concentrations (approximately −2 log and more). This resulted in higher concentrations of the respective resistance genes in all the soil layers at T2 and T3. For *sul*2, *erm*(B), *erm*(F), *tet*(M),*tet*(W), tet(L), *tet*(Q) and *tet*(B) a significant increase in gene copy number was observed in the upper layer between T1 and T3 and between T1 and T2. In the

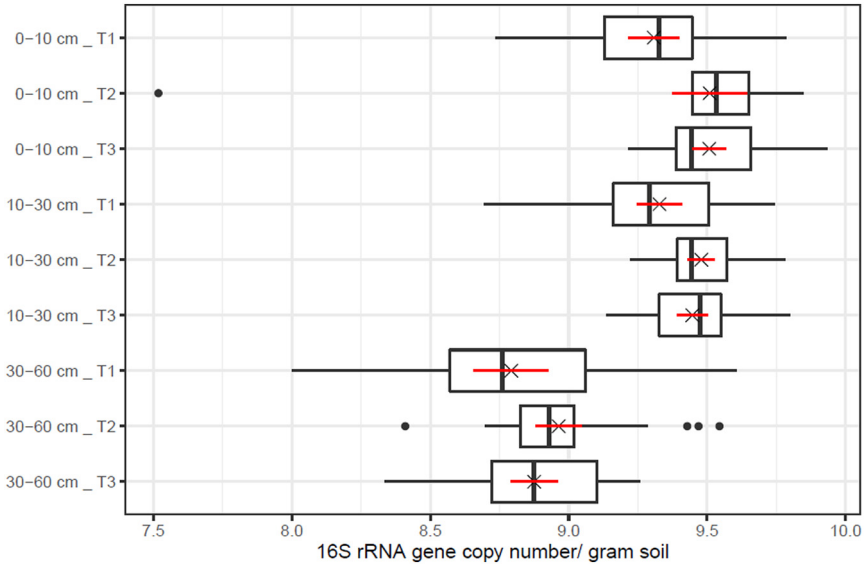

Fig. 3. The variation in 16S rRNA gene copy number expressed per gram soil between the 8 locations (in log-scale) for the different depths (0–10 cm, 10–30 cm, 30–60 cm) and time points (T1 = before fertilization, T2 = just after fertilization, T3 = 2 to 3 weeks after fertilization). The box plots demonstrate the minimum, lower quartile, median, upper quartile and maximum, respectively. The dots represent the outliers. The crosses represent the mean value and the red lines represent the 95% confidence interval on the mean value.

middle layer (10–30 cm), a significant increase in gene copy number was observed between T1 and T2 for *sul*2, *erm*(B), *erm*(F), *tet*(M) and *tet*(W), *tet*(L) and *tet*(Q). Furthermore, a significant increase in gene copy number in the middle layer was observed between T1 and T3 for all the ARGs, except for *tet*(O). Even in the deepest layer (30–60 cm), the gene copy number increased significantly between T1 and T3 for all ARGs, except for *tet*(Q) and *tet*(B). Moreover, in the deepest layer an increased gene copy number of all resistance genes was observed between T1 and T2, except for *tet*(L) and *tet*(B).

Fig. S3 shows the abundance of samples where no ARGs were detected, subdivided by soil type for each gene separately. Similar abundance patterns of the ARGs were observed for each soil type. Generally, *tet*(B), *tet*(Q) and *tet*(L) were less abundant in the soil. In addition, fewer ARGs were detected in the deepest soil layer (30–60 cm) compared to the other soil layers. Furthermore, *erm*(F) was less abundant in the soil before fertilization (T1) while the gene was frequently present in the soil samples after fertilization (T2 and T3).

3.3. Association between antibiotic resistance genes and antibiotic residues in soil

The tetracyclines, specifically doxycycline, were most frequently detected in higher concentrations; presumably the total antibiotic load is mainly determined by the tetracyclines (Fig. S1).

The association between ARGs and the total antibiotic load in soil is shown in Fig. 5. A significant and increasing trend was found between all the ARGs and the total antibiotic load in the soil. The highest slopes were found for *erm*(B), *erm*(F), *sul*2, *tet*(M), *tet*(L) and *tet*(O).

4. Discussion

In brief, the observed environmental increases in ABRs and ARGs are clearly the consequence of fertilization with animal slurry. However the presence of ABRs in soil is also depending on the properties of the soil as well as the physicochemical properties of the residue itself. The impact of this presence on resistance selection in soil is probably quite limited.

Regarding ARGs, their presence is likely due to the direct input via fertilization rather than resistance selection in soil itself. The abundance of ARGs is affected by the prevailing microbiota in soil and slurry.

Moreover a positive association between ABRs and ARGs in soil was found, which may also be explained by the simultaneous input via fertilization with animal slurry.

4.1. Antibiotic residues are frequently present in the soil

Flumequine, doxycycline, oxytetracycline, lincomycin and sulfadiazine were most frequently detected in the soils and slurry, in accordance with other studies (Huygens et al., 2021; Rasschaert et al., 2020; Van den Meersche et al., 2020; Lahr et al., 2018; Mehrtens et al., 2021; Conde-Cid et al., 2020; Salvia et al., 2015). In a Dutch study, flumequine and oxytetracycline turned out to be persistent in sandy soils fertilized with pig or calf slurry with estimated half-lives of respectively 226 and 8 days (Lahr et al., 2018). Flumequine was found in all soil samples, despite a 76% decrease in veterinary use between 2015 and 2019, and only in half of the manure samples (BelVetSac Belgian Veterinary Surveillance of Antibacterial Consumption - National consumption report, 2018). The presence of flumequine in the soils can be attributed to the historical exposure of the synthetic fluoroquinolone due to fertilization, as the component has a long half-life in soil (Berendsen et al., 2021).

The tetracyclines were found to be present in all soil types (Berendsen et al., 2021; Conde-Cid et al., 2020). These compounds can strongly adsorb to the soil particles as they can form chelatic complexes with humic acids or clay particles. In addition to the multivalent cation content (e.g. Ca^{2+}), the adsorption capacity of tetracyclines can also increase by the organic matter content, clay content and the acidity of the soil (Conde-Cid et al., 2020). Although our results do not show a clear relationship between the tetracyclines and Ca^{2+} and/or pH content in soil (Table S6). Furthermore, oxytetracycline can be naturally produced in soil by *Streptomyces rimosus*, which can explain the presence of this residue in low concentrations at locations in fertilized soils where manure contained no oxytetracycline (Pickens and Tang, 2010). Data on naturally-occurring concentrations of oxytetracycline in soil are lacking.

Sulfadiazine and lincomycin were found to be less present in smaller quantities than the other antimicrobials, in accordance with previous research (Berendsen et al., 2021). The molecular structure of the sulfonamides possesses less functional groups (only aniline and amide groups) which cause a lower affinity for the soil in comparison to tetracyclines, which have more functional groups causing multiple adsorption. In general, the adsorption of the sulfonamides is dependent on the organic carbon content of the soil: in general, the higher the organic carbon content, the higher the adsorption (Conde-Cid et al., 2020). However, no large variation in organic carbon content was observed in the upper layer of the soil at the 8 locations (from 1.14 to 1.91%/dry soil) (Table S6). Sulfonamides are

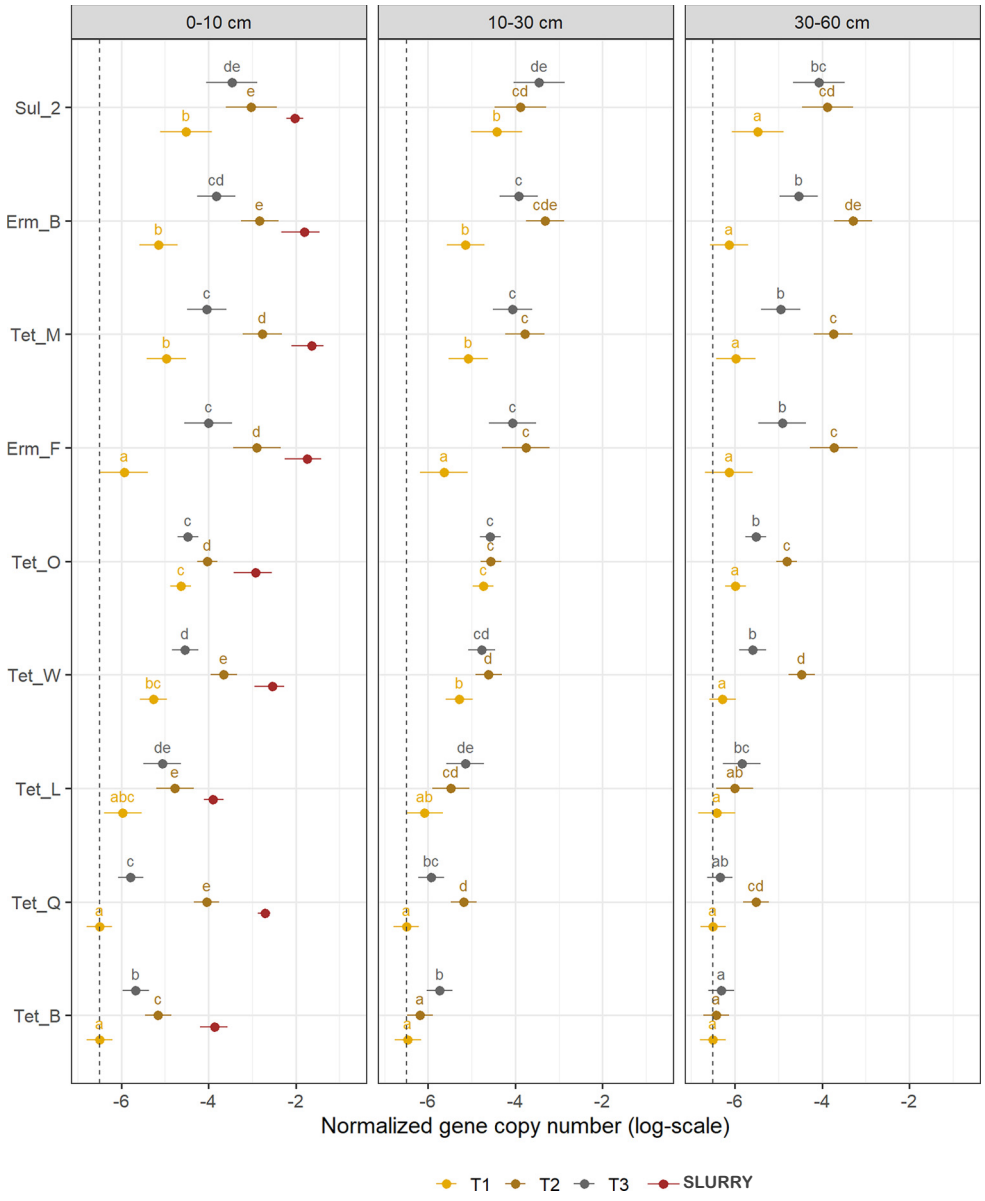

Fig. 4. Concentration (estimated marginal means and 95% confidence intervals) of ARGs in soil samples at 3 depths (0–10 cm, 10–30 cm, 30–60 cm) and 3 times (T1 = before fertilization (yellow), T2 = just after fertilization (brown) and T3 = 2 to 3 weeks after fertilization (gray)), and the concentration of ARGs in slurry samples (red). The concentration is expressed in gene copy number normalized to the 16S rRNA gene copy number in each sample. The letters indicate statistically significant differences between the estimated marginal mean concentrations in soil of the different depths and time points for each antibiotic resistance gene. *Tet*(B) was not detected in the soil layers 0–10 cm and 30–60 cm at T1 and *tet*(Q) was not detected in all the soil layers at T1. Dots on the vertical dashed line should be interpreted as not detected in the soil.

considered to be highly mobile in the soil and therefore may be found in groundwater (Kivits et al., 2018). Nevertheless, no differences in sulfadiazine concentrations among the 3 soil layers (to a depth of 60 cm) were observed in our samples. Lincomycin has a half-life time in soil between 2 and 26 days, depending on the soil type. Lincomycin is shown to be more persistent in clay than in sand (Berendsen et al., 2021; Mehrtens et al., 2021). This is consistent with our study, but as the highest concentrations were also found in the corresponding slurry, we cannot draw conclusions from these observations. Despite its low half-life time, lincomycin has been detected in all other relevant environmental compartments such as manure, surface water, groundwater and crops (Mehrtens et al., 2021).

In our study, enrofloxacin, ciprofloxacin, tiamulin and tylosin were also often detected in the soil. In a previous study enrofloxacin and its metabolite ciprofloxacin were detected in all calf slurry samples (Huygens et al., 2021). Similarly, in this study, those compounds were almost solely detected in the soil and slurry from veal farms. This demonstrates both the use of the fluoroquinolones in the calf sector, which is the largest consumer of fluoroquinolones after the poultry sector, and the persistence of this antibiotic class in slurry and soil (the half-life of enrofloxacin in sandy soil is 137 days) (BelVetSac Belgian Veterinary Surveillance of Antibacterial Consumption - National consumption report, 2018; Salvia et al., 2015; Rosendahl et al., 2012; Cuprys et al., 2018). Furthermore traces of danofloxacin were only detected in soil, which may be explained by its persistence in soil environments (half-life 87–143 days) (Reviews of Environmental Contamination and Toxicology, 2006). Fluoroquinolones are classified by the World Health Organization as one of the most critically important antimicrobials for human medicine and therefore care is recommended for their use in the animal sector (WHO Critically Important Antimicrobials for Human Medicine: 6th revision Available, online).

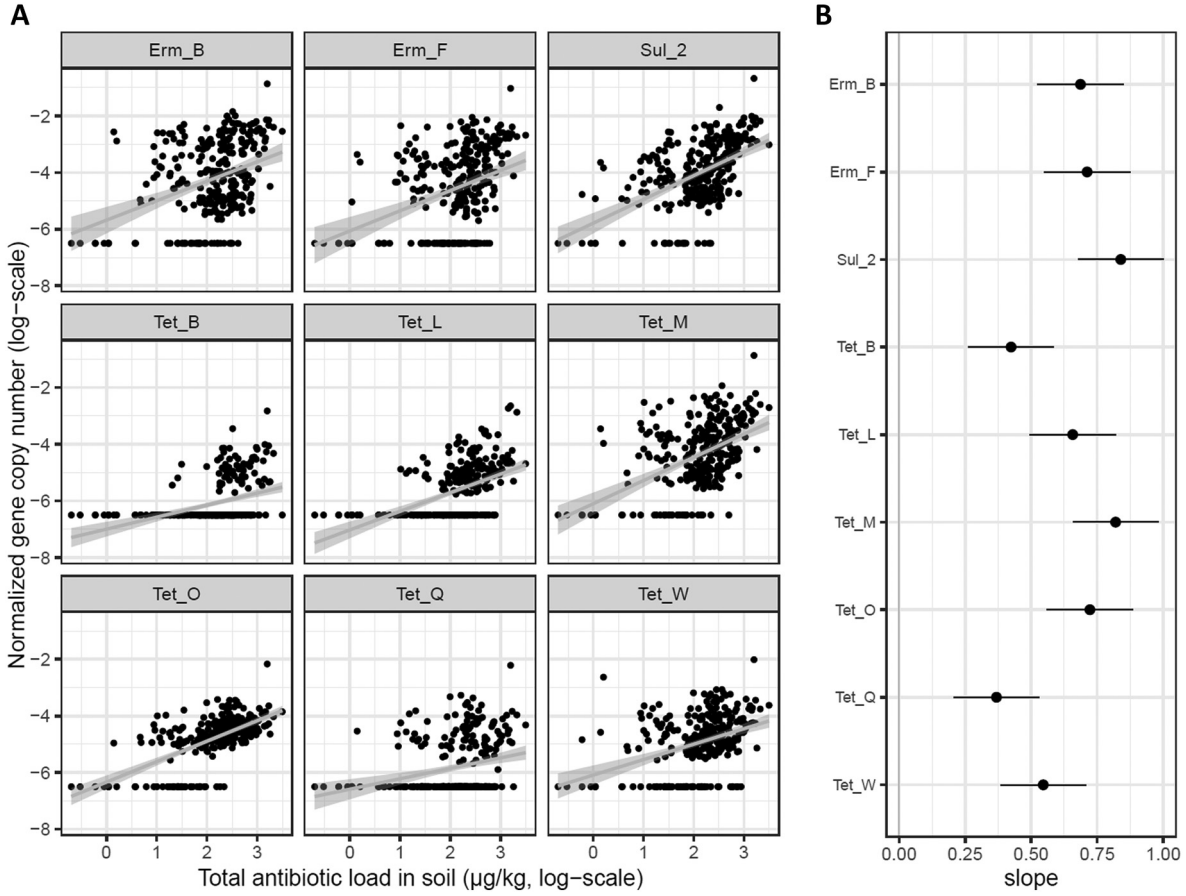

Fig. 5. A. Linear regression (and the 95% confidence interval) as an indicator for the positive trend between the normalized gene copy number (log10) of ARGs (*erm*(B), *erm* (F), *sul2*, *tet*(B), *tet*(Q), *tet*(L), *tet*(M), *tet*(O) and *tet*(W)) and the total antibiotic load in the soil (log10) of all the locations. **B**. The slope and 95% confidence interval of the linear regression model of each antibiotic resistance gene.

Tiamulin and tylosin were also often detected in this study in the soil in very low concentrations. Another study stated that tiamulin degrades rapidly in soil after fertilization until a base level is reached in which it can persist for a long time (more than 115 days) in very low concentrations (Schlüsener and Bester, 2006). The persistence of tylosin in the soil strongly depends on the soil type: half-life has been previously reported as 2 days in sand and 73 days in clay (Berendsen et al., 2021). Both components were not detected in the corresponding slurry samples. As the LOD for tylosin and tiamulin was lower in the soil than in the slurry, it cannot be excluded that the components are present in the slurry in concentrations below LOD. In addition, tylosin can be produced naturally by *Streptomyces fradiae*, which may be another explanation for the presence of low levels in soil (Table 2) (Bekker et al., 2014).

In previous studies, β-lactam antibiotics were not detected in manure and soil (Huygens et al., 2021; Rasschaert et al., 2020; Van den Meersche et al., 2020). In this study, penicillins (amoxicillin and benzylpenicillin) and cephalosporins (cefalonium and cefapirin) were sporadically detected in soil without detection in slurry. The low detection rates of these ABRs may be explained by the rapid hydrolysis of the β-lactam ring (Thiele-Bruhn, 2003). Furthermore, it can be due to different Limits of Detection (LOD) in the two different matrices, but only the limit of detection of ampicillin was lower (10 times) in soil compared to manure (Table S3). Only for benzylpenicillin the presence in soil may also be explained by the possible production by *Penicillium chrysogenum* (Table 2) (Martín, 2020). Lastly, ampicillin and cefapirin were present in the soil at T1 and T2, but at T2 the components were detected in all soil depths, indicating that the components were already present in the soil at fertilization time.

Finally, sulfamerazine was detected sporadically in some samples, but not in slurry (Fig. 1). A possible explanation is the differences in LOD. The LOD in slurry and soil were 1.8 µg/kg and 1.7 µg/kg, respectively, but the LOD was determined in sandy loam. Sulfamerazine was only detected in sandy soil samples, so it is likely that lower detections were possible there.

Furthermore it should be mentioned that no distinction was made between traces and detections above the LOD, so it is possible that a slight overestimation has been made on the quantification of ABRs at the lower concentrations; nevertheless this corresponds to a worst case scenario.

Concerning the antibiotic use data supplied by the farmers (Table 1), it was difficult to relate them to the ABRs detected in soil and slurry as the data was incomplete. There is too little data such as the frequency of antibiotic use in the past year, the time of last use, and the duration of treatment to meaningfully discuss the antibiotic use.

4.2. Antibiotic resistance selection in the soil

Research indicates that antibiotic resistance selection in soil can occur even at very low concentrations. In previous studies, the predicted no-effect concentration for antibiotic resistance selection in soil ($PNEC_{soil}$) was calculated by multiplying the K_d of the antibiotic residue with the previously estimated $PNEC_{water}$ (predicted no-effect concentration for antibiotic resistance in water) (Huygens et al., 2021; Thomaidi et al., 2016). The K_d of the antibiotic residue is the soil-water partition coefficient (L/kg). This value is highly dependent on the antibiotic residue, environmental conditions and soil properties, so the K_d may have a broad value range (Huygens et al., 2021; Thomaidi et al., 2016). In case no $PNEC_{soil}$ was described in literature, own $PNEC_{soil}$ calculations were made based on the formula described by Thomaidi et al. (2016). A worst case scenario is discussed here, as the lower limit of the $PNEC_{soil}$ range was used to estimate

antibiotic resistance selection in the soil. Furthermore, this approach fails to address certain factors, as it only takes the K_d value into account, while selection pressure is known to be dependent on the soil microflora and other soil properties (Menz et al., 2018).

Previously, a $PNEC_{soil}$ for oxytetracycline was estimated between 200 μg/kg and 500 μg/kg soil (Huygens et al., 2021). Consequently, antibiotic resistance selection in Flemish soils may only occur sporadically as only 1% of the soil samples in our study had a concentration of oxytetracycline higher than 200 μg/kg. The $PNEC_{soil}$ for sulfamethoxazole was estimated between 22 μg/kg and 224 μg/kg (Huygens et al., 2021). The sulfonamides were in all soil samples below 10 μg/kg; according to those values no resistance selection in the environment is assumed. Previous findings (Huygens et al., 2021) show that resistance selection can occur but will be rather rare as only a few calf slurry samples with high concentrations of sulfadiazine (up to 84,000 μg/kg) were detected (Huygens et al., 2021). The $PNEC_{soil}$ for enrofloxacin was estimated between 0.03 μg/kg and 359 μg/kg (Huygens et al., 2021). For its metabolite ciprofloxacin, the $PNEC_{soil}$ can be similarly estimated between 27 μg/kg and 310 μg/kg (Cycoń et al., 2019; Bengtsson-Palme and Larsson, 2016). All soil samples containing enrofloxacin (18%) were above the lower limit of the estimated $PNEC_{soil}$. Only one soil sample contained ciprofloxacin above the lower limit of the $PNEC_{soil}$. No K_d values were found in literature for flumequine, thus no $PNEC_{soil}$ was calculated.

The $PNEC_{soil}$ can be calculated for lincomycin, which is expected to be between 4 μg/kg and 420 μg/kg (Mehrtens et al., 2021; Bengtsson-Palme and Larsson, 2016). Lincomycin was detected in 4% of the soil samples (mainly in the upper layer at T2 at 3 locations with clay soils) in concentrations that can potentially cause resistance selection. The $PNEC_{soil}$ for tylosin is estimated between 22 μg/kg and 689,920 μg/kg (Cycoń et al., 2019; Bengtsson-Palme and Larsson, 2016). The concentrations for tylosin in the soil were below the lower limit of the $PNEC_{soil}$.

These are only estimations. Antibiotic resistance selection would not occur in any sample if the upper limit of the $PNEC_{soil}$ range was taken into account. With this in mind, the selective pressure of ABRs in soil dwelling bacteria is probably not the main source of antibiotic resistance in soil. This conclusion is consistent with the insights of previous studies (Heuer et al., 2011a; Van den Meersche et al., 2020; Cycoń et al., 2019).

Low abundances for *tet*(B), *tet*(L) and *tet*(Q) were observed in soil at T1 and T3. Those low abundances as well as the low abundance of *tet*(B) and *tet*(L) in slurry were also established in a previous study from our research group (Van den Meersche et al., 2020). In contrast, high abundances of *tet*(Q) were found in manure and soil in other studies (Patterson et al., 2007; Zhou et al., 2017). These differences in occurrence can be attributed to the differences in bacterial microbiota found in soil and manure samples. Microbiota are affected by farming practices as well as environmental factors such as soil properties and climate (Patterson et al., 2007; Zhou et al., 2017; Feng et al., 2018; Wang et al., 2015; Marti et al., 2014). *Tet*(B) and *tet*(L) are encoding efflux pumps, which originate mainly from Gram-negative bacteria (Roberts, 2005). The low occurrence of these genes in the slurry and corresponding soil can be related to the higher proportion of Gram-positive bacteria in the animal gut and hence in manure (Patterson et al., 2007). *Tet*(M), *erm*(B), *erm*(F) and *sul*2 were the most abundant ARGs in both soil and slurry, in accordance with similar findings (Van den Meersche et al., 2020; Zhou et al., 2017). The *tet*(M) gene encodes for a ribosomal protection protein (RPP). Most RPP genes have a low G + C % content and originate from Gram-positive bacteria. Low G + C% Gram-positive bacteria dominate in 70 to 80% of the gut bacteria (Patterson et al., 2007). Larger abundances of the *tet*(M) gene in fertilized soils may therefore be expected. *Tet*(M) can be found in both Gram-positive as Gram-negative bacteria and can be transferred easily by Tn916, a common mobile genetic element (MGE) (Chee-Sanford et al., 2009). Genes can be co-selected with co-resistance as an underlying mechanism, where genes are located close to each other on the same MGE like transposons, integrons and plasmids. Roberts et al. (1999) described genetic linkage between *tet* and *erm* genes. More specifically, the *erm*(F) gene can be linked to *tet*(Q) and *erm*(B) can be linked to *tet*(M) (Roberts et al., 1999). In another study, significant associations between tetracycline resistance genes and sulfonamide resistance genes were found in *E. coli* originating from cattle feces (Gow et al., 2008). Previous molecular studies have shown significant associations between ARGs (Gow et al., 2008; Boerlin et al., 2005).

It should be noted that the increase in abundance of ARGs in soils after fertilization with manure cannot always necessarily be attributed to the presence of ABRs or ARGs in slurry. For example, Nolvak et al. observed increased levels of *tet*(A) in soils fertilized with a mineral fertilizer (Nõlvak et al., 2016). Moreover, abundant numbers of ARGs have been observed in manure from animals never treated with antibiotics (Heuer and Smalla, 2007; Van den Meersche et al., 2020).

Concerning the abundance of bacteria in soil, the lower gene copy number of the 16S rRNA gene in the 30–60 cm layer compared to the layers above is consistent with the lower microbial density in deeper soil layers (Hao et al., 2021). Furthermore the lower normalized gene copy number of antibiotic resistance genes (*erm*(B), *sul*2, *tet*(M), *tet*(O) and *tet*(W)) in the deepest layer (30–60 cm) at T1 compared to the other layers may be due to the lower microbial density in the deepest layer, where gene transfer between bacteria is less likely. The higher normalized gene copy number of each antibiotic resistance gene in the upper layer (0–10 cm) at T2 can be explained by fertilization of the field, as all samples at T2 were taken before the incorporation of manure into the field. Incorporation practices of slurry into the soil could influence the presence of both ABRs and ARGs in the different soil layers at T3 as those practices can transport ARGs and ABRs to deeper layers. The incorporation of slurry into soil during plowing vary between 15 cm to 30 cm, so it can affect the 2 upper layers.

The impact of the antibiotic use in livestock animals and the use of animal manure as fertilizer on soil, crops and public health is not fully understood (Ashbolt et al., 2013). Studies with a link between antibiotic concentrations and ARGs in the environment are scarce and usually only concern one class of antibiotics (Heuer et al., 2011b; Heuer and Smalla, 2007; Jechalke et al., 2013). Within the current study, a positive trend between different ARGs and the total antibiotic load was found in 288 soil samples. In most cases, the soil samples contained several ABRs from different antibiotic classes. Due to the large number of variables and the limited data size in comparison to the number of variables, we could not disentangle the relationship of the individual variables. We therefore pooled the ABRs to get a picture of the total antibiotic load. This approach resulted in a positive relation between the total antibiotic load and the ARGs. In our opinion, this association is not likely to be the result of a soil process as ABRs and ARGs were measured soon after manure application; they are therefore more likely to be introduced together with the animal manure. As such, the total antibiotic load may act as an indicator for the antibiotic use in the farm, and, hence, the positive association between the total load and ARGs suggests that a high level of antibiotic use results in a higher resistance selection in the farm.

5. Conclusion

Doxycycline, oxytetracycline, flumequine, lincomycin and sulfadiazine were most frequently detected in the soil due to fertilization with animal manure. Although in all soil types, fertilization seemed to have a limited impact on the concentration of flumequine in the soil. The genes *tet*(M), *sul*2, *erm*(B) and *erm*(F) were most abundant in fertilized soil. Based on the $PNEC_{soil}$, residues of tetracyclines (oxytetracycline), fluoroquinolones (enrofloxacin and ciprofloxacin) and lincomycin might cause resistance selection in the soil, but there is little evidence for the direct influence of ABRs in the soil on the development of antimicrobial resistance. Furthermore, the association between the total antibiotic load and ARGs is presumably caused by the simultaneous input of ABRs and resistance genes into the soil by fertilization with raw manure. The ABRs found in soil samples may be considered as an indicator for the antibiotic use in animals as well as higher numbers of antibiotic resistance genes in the soil.

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CRediT authorship contribution statement

Judith Huygens: Writing—Original Draft, Methodology, Validation, Formal analysis, Investigation, Visualization, Resources. **Geertrui Rasschaert**: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Marc Heyndrickx**: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Jeroen Dewulf**: Methodology, Writing - Review & Editing, Supervision. **Els Van Coillie**: Methodology, Writing - Review & Editing. **Paul Quataert**: Methodology, Formal analysis, Writing - Review & Editing, Visualization. **Els Daeseleire**: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Ilse Becue**: Conceptualization, Methodology, Validation, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153518.

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