



Longitudinal screening of antibiotic residues, antibiotic resistance genes and zoonotic bacteria in soils fertilized with pig manure

Tina Van den Meersche^{1,2} · Geertrui Rasschaert¹ · Thijs Vanden Nest¹ · Freddy Haesebrouck² · Lieve Herman¹ · Els Van Coillie¹ · Stephanie Van Weyenberg¹ · Els Daeseleire¹ · Marc Heyndrickx^{1,2}

Received: 27 May 2019 / Accepted: 29 April 2020 / Published online: 14 May 2020
© Springer-Verlag GmbH Germany, part of Springer Nature 2020

Abstract

Fertilization with animal manure is one of the main routes responsible for the introduction of antibiotic residues, antibiotic resistance genes, and zoonotic bacteria into the environment. The aim of this study was to assess the effect of the use of pig (swine) manure as a fertilizer on the presence and fate of six antibiotic residues, nine antibiotic resistance genes, and bacteria (zoonotic bacteria *Salmonella* spp. and *Campylobacter* spp. and *E. coli* as indicator for Gram-negative bacterial species of the microbiota of livestock) on five fields. To the best of our knowledge, the present study is the first to assess a multitude of antibiotic residues and resistance to several classes of antibiotics in pig manure and in fertilized soil over time in a region with an intensive pig industry (Flanders, Belgium). The fields were sampled at five consecutive time points, starting before fertilization up to harvest. Low concentrations of antibiotic residues could be observed in the soils until harvest. The antibiotic resistance genes studied were already present at background levels in the soil environment prior to fertilization, but after fertilization with pig manure, an increase in relative abundance was observed for most of them, followed by a decline back to background levels by harvest-time on all of the fields studied. No apparent differences regarding the presence of antibiotic resistance genes in soils were observed between those fertilized with manure that either contained antibiotic residues or not. With regard to dissemination of resistance, the results presented in this study confirm that fertilization with animal manure directly adds resistance genes to the soil. In addition, it shows that this direct mechanism may be more important than possible selective pressure in soil-dwelling bacteria exerted by antibiotic residues present in the manure. These results also indicate that zoonotic bacteria detected in the manure could be detected in the soil environment directly after fertilization, but not after 1 month. In conclusion, although some antibiotic residues may be present in both manure and soil at concentrations to exert selective pressure, it seems that antibiotic resistance is mostly introduced directly to soil through fertilization with animal manure.

Keywords Antibiotic resistance · Antibiotic residues · Agriculture · Manure · Zoonotic pathogens · Environment

Responsible editor: Diane Purchase

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11356-020-09119-y>) contains supplementary material, which is available to authorized users.

✉ Marc Heyndrickx
marc.heyndrickx@ilvo.vlaanderen.be

¹ Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology and Food Science Unit, Brusselsesteenweg 370, 9090 Melle, Belgium

² Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Introduction

Belgian pig production is an important national economic activity, with 6.08 million pigs produced in Flanders (the northern region) of Belgium in 2016. In that year, the pigs produced about 39.5 kt of nitrogen (N); in total, 126.7 kt N from animal manure were produced. Animal manure was mostly used to fertilize grasslands and fields planted with maize, as well as a smaller number of vegetable fields (Vlaamse Landmaatschappij 2017). Concern about the high levels of antibiotic use in intensive pig production has led to increased awareness about the occurrence and dissemination of antibiotic residues into the environment. Depending on the antibiotic used, 30–90% of the administered antibiotics are excreted unchanged in the urine or the feces (Kumar et al. 2005; Sarmah

et al. 2006). It has been hypothesized that these residues may select for antibiotic-resistant bacteria in the soil when manure is used to fertilize the field (Chee-Sanford et al. 2009). Antibiotic use in pigs may also lead to selective pressure in the pigs' gastrointestinal tract. In the gut, resistance genes may be transferred between different bacteria which are then excreted via the feces (Chee-Sanford et al. 2012). When that manure is used for fertilization, antibiotic-resistant bacteria and antibiotic resistance genes are disseminated into the environment which may lead to a possible exchange of resistance with bacteria present in soil as demonstrated for *Salmonella* (Pornsukarom and Thakur 2017). Another aspect of increasing importance is the possible dissemination of zoonotic bacteria upon fertilization with manure (Nicholson et al. 2005). It has been reported previously that zoonotic bacteria such as *Salmonella* spp. and *Campylobacter* spp. can survive in pig manure (Mannion et al. 2007; Bui et al. 2011). Zoonotic bacteria can also survive in the environment for several weeks to months, thereby posing a potential risk for re-infection of animals or humans (Baloda et al. 2001; Holley et al. 2006). Additionally, pathogenic bacteria can be (multi)resistant to some of the antibiotics used, possibly threatening successful antibiotic treatment (Jechalke et al. 2014). *Salmonella* Typhimurium often shows a high resistance to ampicillin and a weaker resistance to sulfonamides, streptomycin, and tetracyclines. Multiresistant *Salmonella* Typhimurium has been reported to be associated with pigs (Gebreyes and Altier 2002; Sisak et al. 2006; Vanholme et al. 2015). This resistance may be transmitted to commensal bacteria or to other pathogenic bacteria through horizontal gene transfer (Vanholme et al. 2015).

To the best of our knowledge, no data on the parallel presence and fate of different antibiotic residues and antibiotic resistance genes in soil fertilized with animal manure have been reported. In general, studies on the presence of antibiotic residues and antibiotic resistance genes in the environment are scarce. Studies reporting antibiotic resistance in combination with antibiotic residues mostly report resistance linked to only one class of antibiotic (Heuer and Smalla 2007; Heuer et al. 2011b; Jechalke et al. 2013).

The aim of this research was to assess the effect of the use of pig manure as a fertilizer by carrying out a longitudinal screening on the presence and fate of six antibiotic residues, nine antibiotic resistance genes, and bacteria (*Salmonella* spp., *Campylobacter* spp., and *E. coli* as indicator) on five arable fields in Flanders, a region with an intensive pig industry.

Materials and methods

Sample collection

Soil samples were collected on five arable fields (1–5) connected to five respective pig farms (1–5) in East and West

Flanders (Belgium) in 2016 and 2017 at five consecutive time points between March and November. The samplings were carried out before manure application, on the day of manure application, after 1 month, after 2 months, and after harvest (5 to 7 months after fertilization). On the date of manure application (May 9, 2016, March 30, 2017, April 5, 2017, April 10, 2017, and April 13, 2017 for farms 1, 2, 3, 4, and 5, respectively), a sample from the pig manure was taken directly from the manure tank on the respective farm as those animals had produced the manure used to fertilize the field linked to that farm. For practical reasons, timing of the soil samples taken before manure application was as follows: for field 1, 2 months prior; for fields 2, 3, and 5, about 2 weeks prior; and for field 4, on the same day as the manure application. Fields 1, 3, 4, and 5 were planted with maize; field 2 was planted with potatoes. Field 2 was harvested at the beginning of October while the other fields were harvested on dates ranging from mid-October to mid-November. Soil type for all maize-planted fields was sandy loam; field 2 was clay.

Soil was sampled using a gouge auger on four plots of 10 × 10 m located at least 30 m from the headland and at least 10 m from the border of the field. The same plots were sampled for each consecutive sampling. Within each plot, a mixed soil sample of 15 individual core samples was produced for each depth, i.e., 0–10 cm, 10–30 cm, and 30–60 cm. Upon arrival in the lab, subsamples were taken and stored at – 20 °C for further analysis of the antibiotic residues and at 4 °C for DNA extraction, which was carried out within 72 h after sampling. The remainder of the samples was stored at 4 °C for bacteriological analysis within 24 h after sampling.

Field 1 was fertilized with manure from animals with no history of antibiotic use as none of the animals contributing manure for fertilization had been treated either prophylactically or metaphylactically during the 5 years preceding the first sampling. For the animals that did receive curative antibiotics, their manure was deposited into a separate manure pit. The other fields were all fertilized with manure originating from animals treated with antibiotics in the 3 months preceding manure application as shown in Table 1.

Physico-chemical analysis of the manure and soil samples

The physico-chemical analyses of the soil samples were carried out by ILVO (Merelbeke, Belgium). Iron, potassium, magnesium, calcium, manganese, sodium, and phosphorus levels were determined using the methods described in BAM part 1/11 (VITO 2015). Total nitrogen was assessed according to ISO 13878, organic carbon according to ISO 10694, and pH according to ISO 10390 (Anonymous 2015a, 2015b, 2016). The analyses of the manure samples were performed by INAGRO, the extension research center for research and advice in agriculture and horticulture (INAGRO,

Table 1 Overview of the antibiotic residue concentrations ($\mu\text{g}/\text{kg}$) recovered from the pig manure samples used to fertilize the fields under study. For each farm, the antibiotic treatment of animals in the 3 months preceding manure application is noted

| Compound | Farm 1 | Farm 2 | Farm 3 | Farm 4 | Farm 5 |
|-----------------|--------|---------------------|----------|--------------------|-------------------------|
| | None | AMX, DOX, SUL, TMP* | AMX, DOX | AMX, DOX, SUL, TMP | AMX, DOX, SUL, TMP, TYL |
| Ceftiofur | - | - | - | - | - |
| Colistin | - | - | - | - | - |
| Doxycycline | - | 1 604 | 8 712 | 12 093 | 9 268 |
| Oxytetracycline | - | - | - | - | - |
| Sulfadiazine | - | 83 | < LOQ | 49 | 25 |
| Trimethoprim | - | < LOQ | - | < LOD | < LOD |
| Tylosin | - | - | - | - | NQ |

NQ, compound detected but not quantifiable due to high measurement uncertainty; < LOD, below limit of detection; < LOQ, below limit of quantification; “-”, compound not detected; *AMX, amoxicillin; DOX, doxycycline; SUL, sulfadiazine; TMP, trimethoprim; TYL, tylosin

Rumbeke-Beitem, Belgium). Dry matter, total nitrogen, calcium, phosphorus, potassium, magnesium, sodium, and ammoniacal nitrogen were determined using the methods described in BAM parts 3 and 4 (VITO 2015). The organic carbon and organic matter were determined as described in NEN 7432 (NEN 1998). Both laboratories are accredited for the abovementioned analyses.

Quantification of antibiotic residues in manure and soil samples

Reagents and materials

Acetonitrile (MeCN, LC-MS grade) and formic acid 99% (FA, ULC-MS grade) were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Ammonium formate and trichloroacetic acid (TCA, analytical grade) were purchased from Sigma-Aldrich (Diegem, Belgium). Water (H_2O) was high-performance liquid chromatography (HPLC) grade (generated by a Milli-Q Gradient purification system, Millipore, Brussels, Belgium).

The reference standards of ceftiofur, sulfadiazine, tylosin tartrate, roxithromycin (internal standard, I.S.), trimethoprim, doxycycline hyclate, oxytetracycline hydrochloride, and methacycline hydrochloride (I.S.) were purchased from Sigma-Aldrich (Diegem, Belgium). Sulfadimethoxine- $^{13}\text{C}_6$ (I.S.) and trimethoprim- d_9 (I.S.) were purchased from WITEGA Laboratorien Berlin-Adlershof GmbH (Berlin, Germany), and ceftiofur- d_3 hydrochloride (I.S.) was purchased from Toronto Research Chemicals (Toronto, Canada).

Polyvinylidene fluoride (PVDF) filters were purchased from Merck-Millipore (Carrigtwohill, Ireland), polypropylene (PP) tubes and PP inserts were procured by Novolab (Geraardsbergen, Belgium) and Grace Alltech Associates Inc. (Lokeren, Belgium), respectively.

Liquid chromatography-mass spectrometry

The liquid chromatographic system consisted of an Acquity UPLC H-class system (Waters, Milford, MA). Separation was achieved on a reversed-phase Kinetex C_{18} column (100 mm \times 2.1 mm i.d., 1.7 μm) with a SecurityGuard Ultra guard cartridge system (Phenomenex, The Netherlands, Utrecht). The mass spectrometric equipment consisted of a Xevo TQ-S (Waters) equipped with a Z-spray system.

Sample extraction and quantification

The concentration of the different antibiotic residues (colistin, sulfadiazine, trimethoprim, doxycycline, oxytetracycline, ceftiofur, and tylosin A) in each sample was determined as described previously by Van den Meersche et al. (2016) for the manure samples. Briefly, the extraction was carried out by means of shaking and centrifugation using MeCN and trichloroacetic acid, followed by ultrasonication and filtration. Minor modifications were made for the soil samples: MeCN/ H_2O (90/10) was used as the extraction solvent, and a one-point standard addition was used instead of a matrix-matched calibration curve as the soil samples analyzed during one run were too dissimilar to be able to use one standard curve for all the samples. Therefore, the results obtained using this method are semi-quantitative. The samples were also not diluted prior to filtration. Additionally, the compound colistin was not included in this method as recovery from spiked soil samples could not be achieved.

Quantification of antibiotic resistance genes in manure and soil samples

DNA extraction

The DNA extraction was carried out within 72 h after arrival in the lab. DNA was extracted from 0.25 g of each manure or sieved soil sample using the PowerSoil DNA isolation kit

(MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. DNA quantity and quality were determined with the NanoDrop® ND-1000 (Thermo Scientific) and the Quantus™ fluorometer (Promega, Madison, WI, USA).

Quantitative real-time polymerase chain reaction (qPCR)

The abundance of nine antibiotic resistance genes belonging to three antibiotic classes (tetracyclines, sulfonamides, and macrolides) and the 16S rRNA gene was quantified in each sample using qPCR. The 16S rRNA gene was used to normalize antibiotic resistance gene levels to total bacteria within each sample for comparative analysis. For the tetracycline resistance genes, different mechanisms of resistance were included: two efflux pump genes (*tet(B)* and *tet(L)*) and four genes encoding for ribosomal protection proteins (*tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*) were selected due to their abundance in animal fecal matter (Aminov and Mackie 2001; Patterson et al. 2007; Peak et al. 2007; Roberts 2012). *Erm(B)* and *erm(F)* were selected for the macrolide resistance genes as they are the most abundant and they are commonly associated with pig excrement (Patterson et al. 2007; Knapp et al. 2010). Finally, *sul2* was chosen as a representative for the sulfonamide resistance genes as it is predominant in pig manure (Heuer et al. 2009). Previously published primers and probes for these resistance genes were compared and aligned with several GenBank sequences to ascertain whether they were located in conserved regions. Consecutively, a plasmid (for the 16S rRNA gene) (Integrated DNA Technologies, Inc (IDT), Coralville, IA, USA) and two gBlock® gene fragments (one for the tetracycline resistance genes and one for the sulfonamide and macrolide resistance genes) (IDT, Coralville, IA, USA) containing the consecutive sequences of interest separated by ATAT were designed and used as standards for quantification purposes.

First, these gBlock® gene fragments and the plasmid were used to optimize the concentrations of the primers and the probes for the selected resistance genes. All primers and probes were ordered from IDT (IDT, Coralville, IA, USA). For the sulfonamide and macrolide resistance genes, a gradient polymerase chain reaction (PCR) was carried out to determine the optimal annealing temperature. Target efficiency was 90–110% with linearity (R^2) of at least 0.985. The impact of inhibitory substances in the different matrices was assessed by making three consecutive 1:10 dilutions of the test samples and analyzing the differences in threshold values. These experiments revealed that for the resistance genes, a tenfold and a 100-fold (1000-fold for *tet(M)*) dilution of each DNA extract was required for the soil and the manure samples, respectively. For the 16S rRNA gene, a 100-fold dilution of each DNA extract had to be executed to stay in the range of the standard curve. qPCR analyses were performed using a LightCycler® 480 System (Roche).

Total 16S rRNA gene abundance and the abundance of the two macrolide resistance genes, *erm(B)* and *erm(F)*, and the sulfonamide resistance gene, *sul2*, were quantified using SYBR® Green technology. Each reaction mixture consisted of 12.5 µl of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad, Temse, Belgium), the appropriate amount of each primer (Table S1), 5 µl of DNA template, and nuclease-free water to create a reaction volume of 25 µl. For the amplification of the 16S rRNA gene, an activation step of 10 min at 95 °C was followed by 30 cycles of 15 s at 95 °C and 1 min at 60 °C. For the macrolide and sulfonamide resistance genes, an activation step of 10 min at 95 °C was followed by 40 cycles of 15 s at 95 °C and 1 min at the appropriate annealing temperature (Table S1). The specificity of the reaction products was assessed by melting curve analysis. This was performed by gradually increasing the temperature with 0.1 °C/s to 95 °C, with acquisition of data every 2 s. Samples with anomalous melt curves were further analyzed using gel electrophoresis. Samples without a clear band of the expected size were not retained.

The abundances of the six tetracycline resistance genes, *tet(B)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*, were quantified using TaqMan™ Assays. The primers and ZEN™ Double-Quenched Probes are listed in Table S1. A total reaction volume of 25 µl was achieved by adding 5 µl of DNA template to a reaction mixture with the appropriate primers and probe, 12.5 µl TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), and nuclease-free water. The tetracycline resistance genes were amplified by an activation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C.

Within each run, a standard curve was constructed in duplicate using a tenfold serial dilution of the plasmid or a gBlock® gene fragment, with a range of 10–10⁵ gene copy numbers for the resistance genes and 10³–10⁷ gene copy numbers for the 16S rRNA gene used for quantification. The relative abundance of the different antibiotic resistance genes was calculated by dividing the abundance of the respective gene by the 16S rRNA gene abundance. Normalization was performed to account for differences in extraction and in total bacterial community.

Detection of *Salmonella* spp., *Campylobacter* spp. and enumeration of *E. coli* in manure and soil samples

Reagents and materials

Buffered peptone water (BPW) (CM0509), Bolton Broth (CM0983), modified Bolton Broth selective supplement (SR0208E), Ringer solution tablets (BR0052), modified Semi-solid Rappaport Vassiliadis medium base (MSRV) (CM0910), MSRV selective supplement (SR016E), Xylose-Lysine-Desoxycholate Agar (XLD) (CM0469),

Campylobacter Blood-Free Selective Agar (CCDA) (CM0739), and CCDA selective supplement (SR0155E) were all purchased from Oxoid (Basingstoke, UK). RAPID' *E. coli* 2 agar (356-4024) was purchased from BioRad (Marnes-la-Coquette, France). Defibrinated horse blood (DHB100) was purchased from International Medical products (Oudergem, Belgium).

Detection of *Salmonella* spp.

Detection of *Salmonella* spp. was done according to the ISO 6579-1:2017E protocol with minor modifications (Anonymous 2017). For the raw manure and the soil samples, 99 ml of BPW was added to 11 g of sample. After homogenization for 1 min, three consecutive 1:10 dilutions of the samples were prepared in BPW. The samples and the dilutions were then incubated at 37 ± 1 °C for 18 ± 2 h. Subsequently, 3 drops of the pre-enrichment culture were inoculated on MSR/V agar plates supplemented with MSR/V selective supplement and incubated at 41.5 ± 1 °C for 24 ± 3 h. Negative plates were incubated for an additional 24 ± 3 h. From the positive plates, a 10- μ l loop from the edge of the migration zone was inoculated on XLD and incubated at 37 ± 1 °C for 24 ± 3 h. Presumptive *Salmonella* colonies were further confirmed by means of a multiplex *Salmonella* and *Salmonella* Typhimurium PCR as described by Aabo et al. (1993) and Lin and Tsen (1999), respectively.

Detection of *Campylobacter* spp.

For the raw manure and the soil samples, 99 ml of Bolton Broth was added to 11 g of sample. After homogenization for 1 min, three consecutive 1:10 dilutions of the samples were prepared in Bolton Broth. The samples and the dilutions were then incubated under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) in a Forma Series II 3110 Water-Jacketed CO₂ incubator (Thermo Scientific, Waltham, MA) at 41.5 °C for 24 and 48 h. After 24 h of enrichment, 10 μ l of the culture was plated on CCDA plates supplemented with CCDA selective supplement (mCCDA). The plates were incubated under microaerobic conditions at 41.5 °C for 24 and 48 h. If no growth of *Campylobacter* was observed after 24 h, 10 μ l of the enrichment incubated for 48 h was plated on mCCDA plates. These plates were also incubated under microaerobic conditions at 41.5 °C for 24 and 48 h. Colonies of presumptive *Campylobacter* were confirmed by means of a multiplex *Campylobacter jejuni* and *Campylobacter coli* PCR as described by Linton et al. (1997).

Enumeration and detection of *E. coli*

For the enumeration of *E. coli*, 1:10 dilution series of the original samples with BPW were made in Ringer's solution.

In total, 100 μ l of the original samples and the dilutions were plated on RAPID' *E. coli* 2 plates and incubated at 44 °C for 24 h. For the few samples where enumeration was not possible, detection of *E. coli* after enrichment was also performed. To do so, 10 μ l of the pre-enriched samples and dilutions with BPW ("Detection of *Salmonella* spp." section) were plated on RAPID' *E. coli* 2 plates and incubated at 44 °C for 24 h.

Statistical analysis of the data

A log transformation of the data was carried out for the selected resistance genes. Linear regression models were fitted for each farm with the different antibiotic resistance genes (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*B, *erm*F, and *sul*2) as dependent variables and time point and depth (0–10 cm, 10–30 cm, and 30–60 cm) and the interactions between the two variables as independent variables. Statistical significance was considered at *P* values ≤ 0.05 . Analyses were performed with Statistical Analysis System (SAS version 9.4, SAS Institute Inc., Cary, NC, USA).

Results

Physico-chemical analysis of the manure and soil samples

Physico-chemical analyses were carried out on the soil samples taken before the application of manure on the field and on the manure samples taken the day of fertilization. The results are presented in Tables S2 and S3.

Quantification of antibiotic residues

Manure samples

No antibiotic residues were found in the manure sample on farm 1 (Table 1). For the other farms (2–5), sulfadiazine and doxycycline were recovered in the manure sample taken. For sulfadiazine, the concentrations were ranging from < LOQ (limit of quantification) to 83 μ g/kg, and for doxycycline from 1604 to 12,093 μ g/kg. Trimethoprim was recovered on farm 2 but it was below the LOQ. Tylosin was only found in the manure from farm 5 at an estimated concentration in the range of 100 μ g/kg but exact quantification could not be carried out for this compound due to high measurement uncertainty (Van den Meersche et al. 2016). Colistin, oxytetracycline, and ceftiofur were not recovered in any of the manure samples.

In order to calculate the concentration of antibiotics applied through fertilization per kg of soil, assumptions about the amount of manure applied (39,535 kg pig slurry/ha) and the amount of soil present in the upper 30 cm in 1 ha of field (4,350,000 kg soil/ha) had to be made. In addition, as the

concentrations for the residues recovered from the soil samples are semi-quantitative, these calculations are rough approximations. For doxycycline 14.6, 79.2, 109.9, and 84.2 μg doxycycline/kg soil is expected to be applied on fields 2, 3, 4, and 5, respectively. Regarding sulfadiazine, 0.75, 0.45, and 0.23 μg sulfadiazine/kg soil is expected to be applied on fields 2, 4, and 5, respectively.

Soil samples

None of the selected antibiotics were found in any of the soil samples taken on field 1. Only sulfadiazine and doxycycline were recovered from the soil samples taken on fields 2–5. The results are presented in Table 2 and were obtained by means of a standard addition and thus are semi-quantitative. On field 2 (clay), doxycycline and sulfadiazine residues were present in the first (0–10 cm) and the second (10–30 cm) layers studied for the duration of the experiment. Sulfadiazine residues were present at very low concentrations (below 1 $\mu\text{g}/\text{kg}$), and concentrations for doxycycline ranged from 39 to 78 $\mu\text{g}/\text{kg}$. Samples taken on field 3 contained sulfadiazine and doxycycline residues in the first and the second layers prior to manure application. After manure application, doxycycline was found in all of the layers while sulfadiazine was only found in the upper and middle layers. The concentrations of sulfadiazine were very low (in the range of 1 $\mu\text{g}/\text{kg}$), and the concentrations of doxycycline ranged from 17 to 118 $\mu\text{g}/\text{kg}$ throughout the duration of the study. For the soil samples collected on field 4, no antibiotic residues were detected prior to manure application. Sulfadiazine was only detected in the first and the third layers after manure application and in the first layer after harvest, at concentrations below 10 $\mu\text{g}/\text{kg}$. Doxycycline residues were detected in the upper (105 $\mu\text{g}/\text{kg}$) layer after manure application, and although they were not detected 1 month after fertilization, they were again detected 2 months after manure application (115 $\mu\text{g}/\text{kg}$) and after harvest (70 $\mu\text{g}/\text{kg}$). In the soil samples of field 5, no antibiotic residues were detected prior to manure application. After fertilization, both doxycycline (42–733 $\mu\text{g}/\text{kg}$) and sulfadiazine (0.3–8 $\mu\text{g}/\text{kg}$) were observed in all of the layers investigated. For sulfadiazine, residues were no longer detected after 1 month, while doxycycline residues were still present until harvest in the first two layers in the range of 35–321 $\mu\text{g}/\text{kg}$.

Quantification of antibiotic resistance genes

Manure samples

The results of the antibiotic resistance genes in the manure samples are shown in Table 3 as the mean relative abundances which means the ratio between the resistance gene copy number and the 16S rRNA gene copy number in a sample. No apparent differences were observed between the manure

samples studied of the different farms as also shown in Fig. 1 for farm 1 (with no antibiotic treatment of the animals) and farm 5 (with amoxicillin, doxycycline, sulfadiazine, trimethoprim and tylosin treatment of the animals). The results show that the mean relative abundances of the efflux pump genes *tet(B)* and *tet(L)* were lower than the ones encoding for ribosomal protection proteins (*tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*). Within the genes encoding for the ribosomal protection proteins, *tet(M)* was the most abundant. The macrolide and sulfonamide resistance genes, *erm(B)*, *erm(F)*, and *sul2* respectively, were present in the same range as *tet(M)*.

Soil samples

With regard to the relative abundances of the studied antibiotic resistance genes in the soil environment, an overview of the mean value per time point over all farms and soil layers is presented in Table 3. The complete dataset per farm with statistical differences can be found in the supplementary information (Table S4). The range in 16S rRNA gene copy numbers/g soil was 8.3–9.6 log. The results show that for most of the antibiotic resistance genes studied, there were no apparent differences between the soil layers studied. *Tet(B)* was the least abundant resistance gene in the soil samples analyzed prior to fertilization (-7.7 ± 0.8 log) followed by *tet(Q)* (-6.3 ± 0.9 log) and *ermF* (-5.6 ± 1.2 log.) The other resistance genes studied (*tet(L)*, *tet(M)*, *tet(O)*, *tet(W)*, *ermB*, and *sul2*) were present at about the same background levels in the range of -4.4 to -5.0 log. The background resistance gene levels observed in the soil environment prior to manure fertilization were several orders of magnitude lower than the relative abundances found in the manure, with the exception of *tet(L)* where this difference was the lowest.

Immediately after fertilization with swine manure, a significant increase of the relative abundance of the antibiotic resistance genes was observed. These fertilized soil levels were still about 1 to 3 log lower than the relative abundances found in the manure applied on the respective farms for (nearly) all of the resistance genes studied. The exception was *tet(L)* for which no statistically significant increase nor decrease was observed during the study over all the layers studied (fields 1, 2, and 3) or for which the increase after fertilization was statistically significant but small (fields 4 and 5) (Table S4). In Fig. 2, the evolution of the normalized *tet(L)* abundance is shown for 2 extremes, farm 1 representing a situation without antibiotic treatment of the animals, and farm 5 representing a high antibiotic use of the animals (amoxicillin, doxycycline, sulfadiazine, trimethoprim and tylosin); for the other farms, a similar pattern was observed. For the tetracycline resistance genes encoding for ribosomal protection proteins, the highest increase immediately after fertilization was observed for *tet(M)* and *tet(Q)*. The increase observed for *ermB* and *ermF* was comparable to the increase observed for *tet(M)* and *tet(Q)*, respectively.

Table 2 Overview of the doxycycline and sulfadiazine residue concentrations ($\mu\text{g}/\text{kg}$) recovered from the soil samples during the present study. Field 1 was not shown as it contained no residues. These

results are obtained by means of a standard addition (semi-quantitative) and are a mean (\pm standard deviation) of the four plots studied on each field

| Doxycycline | Field 2 | | | Field 3 | | | Field 4 | | | Field 5 | | |
|---------------------------|----------------|----------------|---|-----------------|--------------|------------|-----------------|---|---------------|-----------------|---------------|---------------|
| | Clay soil | | | Sandy loam soil | | | Sandy loam soil | | | Sandy loam soil | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Before manure application | 55 \pm 5 | 33 \pm 2 | - | 32 \pm 4 | 35 \pm 7 | - | - | - | - | - | - | - |
| After manure application | 59 \pm 9 | 34 \pm 5 | - | 82 \pm 24 | 35 \pm 6 | 17 \pm 9 | 105 \pm 43 | - | 252 \pm 118 | 733 \pm 933 | 42 \pm 5 | 398 \pm 380 |
| After 1 month | 50 \pm 8 | 78 \pm 9 | - | - | 118 \pm 48 | 18 \pm 3 | - | - | - | 35 \pm 27 | 321 \pm 107 | 84 \pm 93 |
| After 2 months | 44 \pm 5 | 76 \pm 26 | - | - | 80 \pm 20 | - | 115 \pm 145 | - | - | 52 \pm 21 | 313 \pm 342 | - |
| After harvest | 39 \pm 1 | 56 \pm 6 | - | 59 \pm 8 | 51 \pm 18 | - | 70 \pm 16 | - | - | 45 \pm 5 | 84 \pm 33 | - |
| Sulfadiazine | Field 2 | | | Field 3 | | | Field 4 | | | Field 5 | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Before manure application | 0.2 \pm 0.04 | 0.1 \pm 0.02 | - | 1 \pm 0.4 | 1 \pm 0.4 | - | - | - | - | - | - | - |
| After manure application | 0.6 \pm 0.2 | 0.1 \pm 0.02 | - | 2 \pm 0.8 | 1 \pm 0.4 | - | 3 \pm 0.7 | - | 9 \pm 5 | 8 \pm 5 | 0.3 \pm 0.5 | 5 \pm 3 |
| After 1 month | 0.3 \pm 0.2 | 0.4 \pm 0.09 | - | - | - | - | - | - | - | - | - | - |
| After 2 months | 0.1 \pm 0.02 | 0.3 \pm 0.08 | - | - | - | - | - | - | - | - | - | - |
| After harvest | 0.1 \pm 0.02 | 0.2 \pm 0.03 | - | 3 \pm 0.3 | 2 \pm 0.3 | - | 2 \pm 1.7 | - | - | - | - | - |

“-”, Compound not detected; 1, upper layer (0–10 cm); 2, middle layer (10–30 cm); 3, lower layer (30–60 cm)

In the studied monitoring period, after fertilization, the relative abundance of all studied antibiotic resistance genes gradually dropped to background levels over time: no significant difference was observed between the relative abundance of antibiotic resistance genes before the application of manure and after harvest in the different layers studied. The only exception was *tet(O)* which showed a statistically significant but very minor difference on field 1 (Table S4). In Fig. 3, the evolution of the normalized *tet(M)* abundance is shown again for the two extremes in antibiotic use, farm 1 and farm 5, showing an equal pattern of increase followed by a decline of *tet(M)* to background levels; for the other farms a similar pattern was observed.

Presence of *Salmonella* spp., *Campylobacter* spp., and *E. coli*

We observed that both *Salmonella* Typhimurium and *Campylobacter coli* can be present in the pig manure. This was the case on farms 1, 2, and 5, with estimations ranging from 10 to 10^3 cfu/g manure (Table 4). Farms 3 and 4 are not shown as *Salmonella* Typhimurium and *Campylobacter coli* were not recovered from any samples. *Salmonella* spp. and *Campylobacter* spp. were not detected in the soil samples prior to the application of manure, with the exception of *Campylobacter jejuni* which was found on field 1 in the upper layer (data not shown). Immediately after fertilization, *Salmonella* Typhimurium and *Campylobacter coli* could be detected in the different layers studied if they had been present in the manure although the amount present in the soil was

lower than in the manure (0.1 to 10^2 cfu/g soil) (Table 4). One month after fertilization, these zoonotic bacteria were not detected anymore (data not shown).

As enumeration of *E. coli* was not possible in soil prior to manure application due to low numbers, enrichment of the fractions had to be carried out. The results are shown in Table 5 and a table with the complete dataset can be found in the supplementary information (Table S5). On farms 2, 3, 4, and 5, the amount of *E. coli* present in the manure was on average 5.2 ± 0.5 log cfu/g. The results show an increase in the amount of *E. coli* present on the fields immediately after manure application, from median background values of mostly –1 to 0 log cfu/g towards 2 to 3 log cfu/g as well as a gradual reduction over time until harvest back to background levels for the different soil types studied. On farm 1, with the amount of *E. coli* present in the manure about 2 log lower than in the other manure samples, a smaller increase was observed after fertilization with a gradual decline to undetectable levels at harvest.

Discussion

High concentrations of doxycycline are present in pig manure and in soil

The concentrations of the antibiotic residues recovered from the different manure samples (Table 1) are comparable to our own previously published results (Van den Meersche et al. 2016; Rasschaert et al. 2020) as well as those from another

Table 3 Overview across all farms and soil layers studied of the mean of the relative abundance (log) per time point of the different antibiotic resistance genes under study in the soil samples ($n = 60$) and in the manure ($n = 5$). The abundance of each gene is divided by the abundance

of the 16S rRNA gene copies present in the same sample for normalization. The complete dataset with statistical differences can be found in the supplementary information (Table S4)

| | <i>Tet(B)</i> | | <i>Tet(L)</i> | | <i>Tet(M)</i> | | <i>Tet(O)</i> | | <i>Tet(Q)</i> | | <i>Tet(W)</i> | | <i>ErmB</i> | | <i>ErmF</i> | | <i>Sul2</i> | |
|---------------------------|---------------|-----|---------------|-----|---------------|-----|---------------|-----|---------------|-----|---------------|-----|-------------|-----|-------------|-----|-------------|-----|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Before manure application | -7.7 | 0.8 | -5.0 | 0.6 | -4.8 | 0.7 | -4.4 | 0.4 | -6.3 | 0.9 | -4.6 | 0.5 | -4.9 | 0.6 | -5.6 | 1.2 | -4.8 | 0.7 |
| After manure application | -5.4 | 1.3 | -4.7 | 0.6 | -2.8 | 0.7 | -3.7 | 0.5 | -3.7 | 0.7 | -3.4 | 0.6 | -3.0 | 0.7 | -3.0 | 0.8 | -3.3 | 0.7 |
| After 1 month | -5.4 | 1.1 | -4.7 | 0.5 | -3.7 | 0.7 | -4.1 | 0.4 | -4.8 | 0.8 | -4.0 | 0.6 | -3.8 | 0.8 | -3.6 | 0.9 | -3.2 | 0.7 |
| After 2 months | -6.8 | 1.2 | -4.8 | 0.6 | -4.2 | 0.6 | -4.2 | 0.3 | -5.5 | 0.8 | -4.3 | 0.5 | -4.2 | 0.5 | -4.3 | 0.9 | -3.8 | 0.9 |
| After harvest | -7.6 | 0.8 | -5.0 | 0.7 | -4.8 | 0.8 | -4.4 | 0.6 | -6.4 | 1.0 | -4.6 | 0.6 | -4.9 | 0.9 | -5.4 | 1.3 | -4.6 | 0.7 |
| Manure | -3.4 | 0.3 | -3.6 | 0.3 | -0.9 | 0.1 | -2.3 | 0.4 | -2.0 | 0.1 | -1.8 | 0.2 | -1.1 | 0.2 | -1.0 | 0.2 | -1.3 | 0.3 |

SD standard deviation

recent study (Berendsen et al. 2015). The absence of trimethoprim in the manure from farms 4 and 5 may be explained by rapid metabolism of this antibiotic: only about 16% is excreted unchanged (Baggot and Giguère 2013). No residues were present in the manure samples of farm 1. These results were expected as no animals whose manure was used for fertilization were treated with antibiotics during the 5 years preceding the sampling.

The results of the antibiotic residues in soil samples are semi-quantitative; therefore, it was not possible to perform a statistical analysis of these data. A previously published study reported tetracycline residue concentrations up to 300 µg/kg on farmed land and sulfonamide concentrations that were two orders of magnitude lower (Hamscher et al. 2005). These findings are comparable to the results observed in fertilized soil in our study (Table 2). No residues were found in the soil samples of farm 1 which corresponds with their absence in the manure. On the other fields fertilized with manure from animals treated with antibiotics, we observed that sulfadiazine residues were present at very low concentrations after fertilization; and that in the sandy loam soils, these residues were no

longer detected after 1 month. In contrast, in the clay soil samples (upper layer only, 0–30 cm), they could be detected at very low concentrations during the duration of the experiment. The results for the sandy loam soils were as expected: the dissipation half-life of sulfonamides ranges between 2 and 10 days, and these antibiotics have a high mobility and a low soil sorption (Boxall 2012; Jechalke et al. 2014). The observations for the clay soil might be explained by slow release of sequestered antibiotics whereby sub-inhibitory concentrations of antibiotics may be detectable for extended periods of time or as a result of reduced leaching due to the soil type (Jechalke et al. 2014).

For doxycycline, we found that residues can be present at depths of 30 cm up until harvest in both soil types. It has been reported previously that tetracyclines may persist in soil for several months or years and that they exhibit a strong sorption to the soil (Hamscher et al. 2005; Jechalke et al. 2014). Even though the residues are bound to soil particles, they may still be biologically active (Chander et al. 2005). However, their effect on reducing the microbial population has been suggested to be minimal (Kumar et al. 2005). In the lower layer

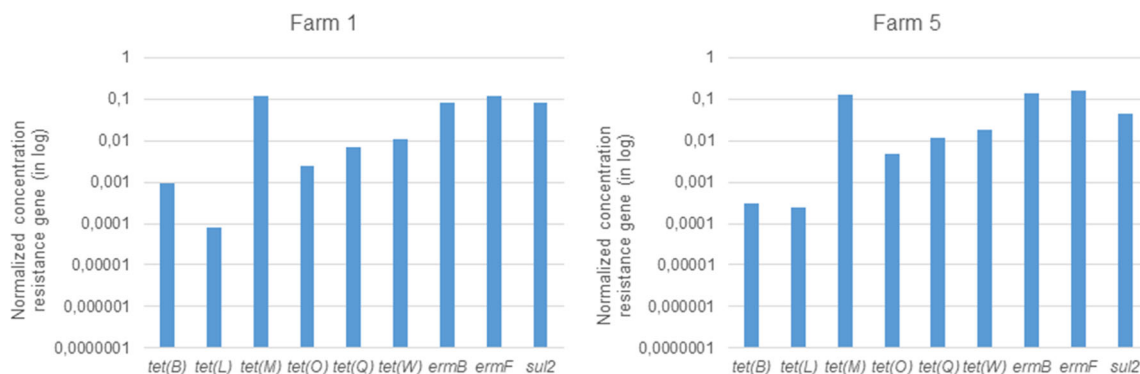


Fig. 1 Normalized resistance gene abundances (expressed as the log of the relative abundance of a resistance gene divided by the relative abundance of the 16S rRNA gene) in the manure samples of farm 1 and

farm 5. On farm 1, the pigs did not receive antibiotics; on farm 5, the animals were treated with amoxicillin, doxycycline, sulfadiazine, trimethoprim and tylosin

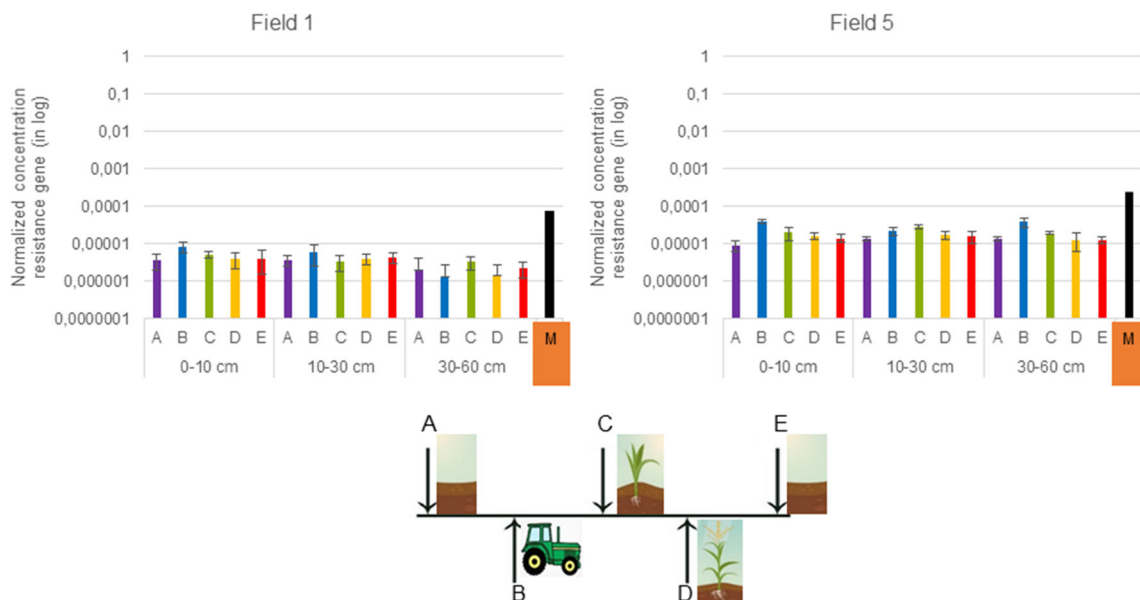


Fig. 2 Mean normalized relative abundances for *tet(L)* (expressed as the log of the relative abundance of the resistance gene divided by the relative abundance of the 16S rRNA gene) in soil at different depths and at the different time points of sampling for farm 1 (no antibiotics found in soil and in manure) and for farm 5 (doxycycline and sulfadiazine found in

manure and soil; tylosin found in manure). The different soil sampling times are as follows: A, before fertilization; B, immediately after fertilization; C, 1 month after fertilization; D, 2 months after fertilization; E, after harvest. M, manure

(30–60 cm), doxycycline residues were present in the sandy loam samples immediately after fertilization and up to 1 month later (for 2 of the 3 soils). This is in contrast to previously published results by Hamscher et al. (2005) who found that no tetracycline residues were observed in soil segments deeper than 30 cm. This may be explained either by the sampling of different soil types or the plowing of the soil in the present study, which could have transported antibiotic residues into the deeper soil layers.

For the clay soil, no increase in antibiotic residue concentration was observed after fertilization. As the background residue concentrations present on field 2 were already high, the addition of antibiotic residues with pig slurry was not

expected to increase the levels already present in the soil. Indeed, after calculations, it was expected that about 14.6 µg doxycycline/kg soil would be applied upon fertilization. This is lower than the concentration already present in the soil. The absence of any residue in the lower layer of the clay soil is probably due to restricted leaching and binding to elements such as calcium and magnesium as these elements are more abundant in clay soil (Table S2) (Kumar et al. 2005). The stronger adsorption and slow release of sequestered antibiotics may explain why antibiotic residues were already present before fertilization in this type of soil (Hamscher et al. 2002; Jechalke et al. 2014). In contrast, in the sandy loam soils, no antibiotic residues were observed before fertilization with the

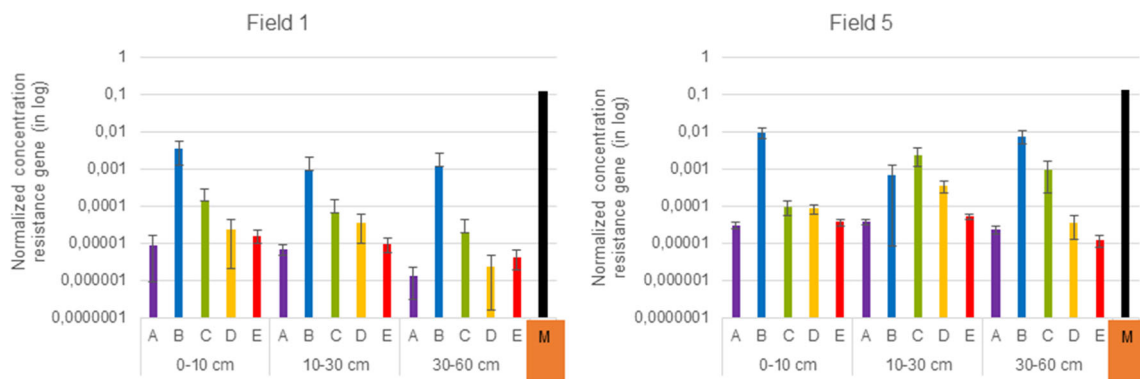


Fig. 3 Mean normalized relative abundances for *tet(M)* (expressed as the log of the relative abundance of the resistance gene divided by the relative abundance of the 16S rRNA gene) in soil at different depths and at the different time points of sampling for farm 1 (no antibiotics found in soil and in manure) and for farm 5 (doxycycline and sulfadiazine found in

manure and soil; tylosin found in manure). The different soil sampling times are as follows: A, before fertilization; B, immediately after fertilization; C, 1 month after fertilization; D, 2 months after fertilization; E, after harvest. M, manure

Table 4 Detection of *Salmonella* Typhimurium and *Campylobacter coli* (cfu/g) in pig manure and in soil samples immediately after fertilization

| | Farm 1 | | Farm 2 | | Farm 5 | |
|-----------------|---------------------------------------|-----------------------------------|---------------------------------------|-----------------------------------|---------------------------------------|-----------------------------------|
| | <i>Salmonella</i> Typhimurium (cfu/g) | <i>Campylobacter coli</i> (cfu/g) | <i>Salmonella</i> Typhimurium (cfu/g) | <i>Campylobacter coli</i> (cfu/g) | <i>Salmonella</i> Typhimurium (cfu/g) | <i>Campylobacter coli</i> (cfu/g) |
| Plot 1 0–10 cm | 0.1–1 | - | - | - | - | 0.1–1 |
| Plot 1 10–30 cm | - | - | - | - | - | - |
| Plot 1 30–60 cm | - | - | - | - | 0.1–1 | 10–10 ² |
| Plot 2 0–10 cm | 0.1–1 | - | 10–10 ² | 1–10 | 1–10 | 1–10 |
| Plot 2 10–30 cm | - | - | 0.1–1 | - | - | - |
| Plot 2 30–60 cm | - | - | - | - | 10–10 ² | - |
| Plot 3 0–10 cm | 1–10 | - | 0.1–1 | - | 0.1–1 | - |
| Plot 3 10–30 cm | 0.1–1 | - | - | 0.1–1 | - | - |
| Plot 3 30–60 cm | - | - | - | 0.1–1 | 0.1–1 | - |
| Plot 4 0–10 cm | 0.1–1 | 0.1–1 | 1–10 | - | - | 1–10 |
| Plot 4 10–30 cm | - | 0.1–1 | - | - | - | - |
| Plot 4 30–60 cm | 0.1–1 | - | - | - | - | 0.1–1 |
| Manure | 10–10 ² | 10 ² –10 ³ | 10 ² –10 ³ | 10 ² –10 ³ | 10–10 ² | 10 ² –10 ³ |

Detection limit, 0.1 cfu/g

exception of field 3. The latter could be due to the fact that prior to the first sampling, effluent had been spread onto this field (personal communication with the farmer). Effluent is an end-product of biological treatment of pig manure that may contain antibiotic residues (Van den Meersche et al. 2019). With regard to field 4 (30–60 cm) and field 5 (0–10 and 30–60 cm), higher concentrations were observed than expected based on the rough estimates. In addition, the variation in concentration between the different plots was high. This may be explained by the non-homogeneous incorporation of manure into the soil, leading to potential hot spots of antibiotic residues due to a higher manure fraction. Extrapolation of these results to other soil types is difficult because antibiotics are ionizable and may therefore be present in the soil environment with either a positive, a negative, or a neutral charge or as zwitterions, depending on the pH of the soil (Boxall 2012).

With regard to selective pressure as a result of the observed residue concentrations, the maximal sulfadiazine concentration in manure was 83 µg/kg, and the concentrations in the soil samples were below 10 µg/kg. These concentrations are considerably lower than the epidemiological cutoff values (ECOFF) for sulfamethoxazole which is between 64 and 128 mg/L (EUCAST 2020). The ECOFF of sulfamethoxazole was used, as there were no data available for sulfadiazine and both are members of the same antibiotic class (Agnoletti et al. 2018). Hence, it is expected that these sulfadiazine residue concentrations will presumably not cause selective pressure. In contrast, the concentrations of doxycycline residues present in the manure (1.6–12 mg/L) should be sufficient to be able to select for resistance as the ECOFF for doxycycline for different bacteria is between 0.5 and 8 mg/L (EUCAST 2020). In addition, predicted no-effect concentrations (PNECs) for resistance selection have been theoretically determined for an array of commonly administered antibiotics, and the predicted PNEC for doxycycline was 2 µg/kg (Bengtsson-Palme and Larsson 2016). According to these calculations, the doxycycline concentrations recovered in the soil samples (18–733 µg/kg) may also cause selective pressure. However, the bioavailability in soil is lower than in the liquid media used to determine the MIC, and it may vary depending on the soil properties (organic carbon content, ionic strength, clay content, texture, and pH) and on the concentration of the antibiotic. In addition, the PNEC is not determined experimentally. Therefore, more research is needed to extrapolate these residue values to the soil environment with regard to selective pressure (Schmitt et al. 2017). On the other hand, antibiotic residues are not distributed homogeneously in the soil, and hot spots with higher concentrations of antibiotic residues may be present (Jechalke et al. 2014; Schmitt et al. 2017).

Antibiotic resistance genes temporarily increase after fertilization with pig manure

Concerning the tetracycline resistance genes, two efflux pump genes (*tet(B)* and *tet(L)*) and four genes encoding for ribosomal protection proteins (*tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*) were selected due to their abundance in animal fecal matter (Aminov and Mackie 2001; Patterson et al. 2007; Peak et al. 2007; Roberts 2012). *Tet(B)* was found in 31 Gram-negative genera, which makes it the most widely distributed efflux pump gene among Gram-negative bacteria. In contrast, *tet(L)* has been identified in 14 Gram-negative and 19 Gram-positive genera. These efflux pump genes are associated with plasmids (Roberts 2012). The genes encoding for ribosomal protection proteins are present in both Gram-positive and Gram-negative genera and are found on conjugative transposons, with the exception of *tet(O)* which has been associated with conjugative and non-conjugative plasmids (Patterson et al. 2007; Roberts 2012). All of these resistance genes confer resistance to doxycycline, an antibiotic used on four of the selected farms (Chopra and Roberts 2001; Roberts 2012). *Erm(B)* and *erm(F)* were selected for the macrolide resistance genes as they are the most abundant and they are commonly associated with pig excrement (Patterson et al. 2007; Knapp et al. 2010). These resistance genes are also located on conjugative or non-conjugative transposons (Roberts et al. 1999). For the sulfonamide resistance, *sul2* was chosen as a representative as it is predominant in pig manure (Heuer et al. 2009). For all these selected antibiotic resistance genes, no apparent differences were observed between the manure samples under investigation (Table 3, Fig. 1). This may seem unexpected as no antibiotics were used on farm 1; however, these results are in accordance with Heuer and Smalla (2007) who reported that even manure from animals that had never received antibiotics may still contain abundant numbers of antibiotic-resistant bacteria. Additionally, it has been reported that antibiotic resistance genes can persist for many years after abolishment of antibiotic use, and resistance traits have been

identified in environmental settings in the absence of the corresponding antibiotic (Knapp et al. 2010; Heuer et al. 2011a). This can also clarify the high relative abundance of macrolide resistance genes in the different manure samples even though no macrolide antibiotics were used to treat the animals, with the exception of farm 5. The abundance of *ermB* in swine manure in the present study is comparable to previously published results (Marti et al. 2014). Another explanation is co-selection caused by the genetic linkage of *erm(B)* to *tet(M)* and *erm(F)* to *tet(Q)* on the same mobile genetic element (Roberts et al. 1999). The use of doxycycline on all farms (except farm 1) may thus be responsible for a selective pressure on both tetracycline and macrolide resistance genes, respectively.

All of the resistance genes studied were already present in the soil samples taken prior to fertilization with manure. This is not surprising as antibiotic resistance genes originally evolved from a defense mechanism in environmental ecosystems (Miao et al. 2012). However, the functions carried out by these genes in the natural ecosystems might initially not have been related to antibiotic resistance (Chee-Sanford et al. 2012). The use of antibiotics may select for these antibiotic resistance genes present in the soil environment (D'Costa et al. 2011; Chee-Sanford et al. 2012). In addition, the repeated fertilization of the fields over time may have increased the background levels of antibiotic resistance genes present in the soil. The results showed that the relative abundances present in the soil after fertilization with manure increased (except for *tet(L)*). These increased levels were in general about 1 to 3 log lower than the relative abundances found in the manure. Similar soil loading rates have been estimated by Marti et al. (2014). Jechalke et al. (2013) also observed that the differences between resistance gene levels in soils were comparable to the differences observed between the manures used for fertilization. An example from the present study may clarify this further. Rough estimates were made in order to calculate expected abundance based on the amount of manure applied per g of soil for *tet(M)* and *tet(L)* as these represent the most

Table 5 First quartile (Q1), median (Q2), and third quartile (Q3) are given for the enumeration or detection of *E. coli* in soil samples and in the manure samples (log cfu/g). The values for the different depths studied were compiled. Unless otherwise indicated, all values were obtained after enrichment

| | Before manure application | | | After manure application | | | After 1 month | | | After 2 months | | | After harvest | | | Manure |
|--------|---------------------------|-------|-------|--------------------------|-----|-------|---------------|-----|-----|----------------|-------|-------|---------------|-------|-------|--------|
| | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | |
| Farm 1 | - | - 1-0 | 0-1 | - 1-0 | 0-1 | 2-3 | - | 0 | 0-1 | - 1-0 | - 1-0 | 1-2 | - | - | - 1-0 | 3.23 |
| Farm 2 | - | - 1-0 | 0-1 | 1-2 | 2-3 | 2-3 | - 1-0 | 0 | 0-1 | - | - | - 1-0 | - 1-0 | 0-1 | 1-2 | 4.98 |
| Farm 3 | - | - 1-0 | 1-2 | 1-2 | 2-3 | 3.23* | 1-2 | 1-2 | 2-3 | - 1-0 | - 1-0 | 1-2 | - 1-0 | - 1-0 | 0-1 | 5.71 |
| Farm 4 | - | - 1-0 | - 1-0 | 1-2 | 2-3 | 3.23* | 1-2 | 2 | 2-3 | - 1-0 | 0-1 | 1-2 | - | - 1 | 0-1 | 5.51 |
| Farm 5 | - | - | - 1-0 | 1-2 | 2-3 | 2-3 | - 1-0 | 0-1 | 1-2 | - | - | - 1-0 | - | - 1-0 | - 1-0 | 4.56* |

Q₁ or first quartile is defined as the middle number between the smallest number and the median of the data set; Q₂ is the median of the data set; Q₃ or third quartile is the middle value between the median and the highest value of the data set *Enumeration without enrichment; “-”not detected

extreme values in manure. Assumptions about the amount of manure applied (39,535 kg pig slurry/ha) and the amount of soil present in the upper 30 cm in 1 ha of field (4,350,000 kg soil/ha) were made. For *tet(M)*, the expected gene copy number/g soil after fertilization was 5.3, 5.2, 5.4, 5.5, and 5.5 log for fields 1, 2, 3, 4, and 5, respectively. For the same field, the background gene copy numbers in the soil prior to fertilization were 2.9, 3.3, 2.8, 1.8, and 2.2 log, respectively; these values are 1.9 to 3.7 log lower. The use of pig slurry as a fertilizer is thus expected to increase the gene copy numbers/g soil for *tet(M)*. For *tet(L)*, the expected gene copy number/g soil after fertilization was 2.6, 2.7, 2.8, 1.9, and 2.4 log for fields 1, 2, 3, 4, and 5, respectively. The background gene copy numbers in the soil of the same fields prior to fertilization were 2.5, 2.7, 2.9, 1.5, and 1.9 log, respectively. This shows that the background levels for this antibiotic resistance gene are comparable with the expected levels after fertilization and that fertilization is thus not expected to increase the levels already present in the soil environment for *tet(L)*. Moreover, the load of *tet(L)* in manure was the lowest of all resistance genes investigated.

Direct input of resistance genes through manure is probably more important than selective pressure through antibiotic residues in manure or soil

In our study, no apparent correlation is seen between antibiotic residues present in the soil and the presence of antibiotic resistance mediated by the specifically selected antibiotic resistance genes. This is evidenced by the similar evolution of these antibiotic resistance genes in soil after fertilization irrespective of the antibiotic use on the farm or the concentrations of antibiotic residues present in the manure or soil. The decline in relative abundance of resistance genes over time can be attributed to the presence of resistance determinants in the manure-borne bacteria, which may not survive for extended periods of time in soil. It has also been postulated that the nutrients added upon fertilization may provide a temporary enrichment of some soil bacteria carrying antibiotic resistance genes (Jechalke et al. 2013; Marti et al. 2014; Leclercq et al. 2016). But as we observed a narrow range of the 16S rRNA gene copy numbers/g soil, the declining trend observed in resistance genes is not due to differences in total bacterial abundance. Another possible explanation might be the transfer of resistance determinants from manure-borne bacteria to soil bacteria. The observed return to background levels over time would indicate that potentially transconjugant bacteria might not persist in the absence of selective pressure, as the host fitness is affected by the acquisition of antibiotic resistance (Sengelov et al. 2003; Heuer et al. 2011a). According to Jechalke et al. (2013), an increase in relative abundance of *sul2* with more than three orders of magnitude was observed after manure application, followed by a decline over time.

Sengelov et al. (2003) also observed an increase of tetracycline resistant bacteria after fertilization with pig manure followed by a decline to background levels over a period of 5 months. Marti et al. (2014) noted that a 1-year waiting period after manure application would reduce the relative abundance of selected antibiotic resistance genes (*sul1*, *erm(B)*, *str(B)*) back to background levels. For individual resistance genes, all these results are in accordance with the results observed in the present study. However, to the best of our knowledge, the present study is the first to assess resistance to several classes of antibiotics in pig manure and in fertilized soil over time together with measurements of antibiotic residue concentrations. It is also the first study to report data for Flanders (Belgium), a region with an intensive pig industry.

Zoonotic pathogens can be temporarily present in manured soil

The results showed that both *Campylobacter coli* and *Salmonella* Typhimurium can be present in pig manure and that they may enter the environment through fertilization. Prior to manure fertilization, no *Salmonella* spp. nor *Campylobacter* spp. were present in the soil (with one exception for *Campylobacter jejuni*). Although the concentrations found in soil samples were lower than in the manure samples, the presence of the studied zoonotic bacteria is still cause for concern, as their presence can maintain the infection pressure on animals or humans through the contamination of the environment. One month after fertilization, no *Salmonella* spp. nor *Campylobacter* spp. were detected in the soil. This is in accordance with the results obtained by Hutchison et al. (2005), who reported that the longest survival time of *Salmonella* and *C. jejuni* in soil after fertilization with pig manure was 16 days (Hutchison et al. 2005). Bacterial survival in the soil environment depends on several factors such as temperature, moisture content, pH, soil composition, and the presence of other microorganisms in the soil (Cools et al. 2001).

E. coli can be considered as representative for Gram-negative bacterial species of the fecal commensal microbiota of livestock (Hanon et al. 2015). The results obtained for this fecal indicator organism showed an increase upon fertilization. Afterwards, a gradual return to background levels at the time of harvest was observed. It was also observed that if higher numbers of *E. coli* are present in the manure, this will lead to higher amounts in soil 1 month after fertilization which may possibly pose a higher risk of transmission to plants.

Conclusion

To the best of our knowledge, the present study is the first to assess a number of antibiotic residues and resistance to several classes of antibiotics in pig manure and in fertilized soil over

time. The doxycycline concentrations found in the pig slurry used to fertilize the field may be high enough to cause selective pressure. In addition, low concentrations of antibiotic residues might still be present in the soil at harvest. The relative abundances of the studied antibiotic resistance genes declined over time. With regard to dissemination of resistance, the results presented in this study indicate that fertilization with animal manure directly adds resistance genes to the soil. This mechanism may be more important than possible selective pressure in soil-dwelling bacteria exerted by antibiotic residues present in the manure or soil.

This is supported by the results obtained for the particular farm where no antibiotic residues were present in either the manure or the soil samples. In spite of this, the relative abundance of the different antibiotic resistance genes increased on this field after fertilization and dropped to background levels at harvest in a manner comparable with the other fields studied. Although no zoonotic bacteria were detected in the soil samples at harvest, contamination pressure on the farm may be maintained as these zoonotic bacteria may be transferred to the environment upon fertilization.

Acknowledgments We are grateful to Sofie Rauch for her help during sampling and microbial analysis and to the farmers for their participation.

Funding information This research was funded by a Ph.D. grant (IWT-SB/141290) of the Agency for Innovation by Science and Technology in Flanders (IWT).

References

- Aabo S, Rasmussen OF, Rossen L et al (1993) *Salmonella* identification by the polymerase chain reaction. *Mol Cell Probes* 7:171–178
- Agnoletti F, Brunetta R, Bano L et al (2018) Longitudinal study on antimicrobial consumption and resistance in rabbit farming. *Int J Antimicrob Agents* 51:197–205
- Aminov RI, Mackie RI (2001) Molecular Ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins Molecular Ecology of Tetracycline Resistance : development and validation of primers. *Appl Environ Microbiol* 67:22–23
- Anonymous (2015a) ISO 13878:1998 Soil Quality – Determination of Total Nitrogen Content by Dry Combustion (“elemental Analysis”)
- Anonymous (2015b) ISO 10390:2005 Soil Quality – Determination of PH
- Anonymous (2016) ISO 10694:1995 Soil Quality – Determination of Organic and Total Carbon after Dry Combustion (Elementary Analysis)
- Anonymous (2017) International Standard 6579-1. Microbiology of the Food Chain — Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella. Part 1: Detection of Salmonella Spp.
- Baggot JD, Giguère S (2013) Principles of antimicrobial drug bioavailability and disposition. In: Giguère S, Prescott JF, Dowling PM (eds) Antimicrobial Therapy in Veterinary Medicine, Fifth Edition, Fifth edn. John Wiley & Sons, Inc., p 703
- Baloda SB, Christensen L, Trajcevska S (2001) Persistence of a *Salmonella enterica* Serovar typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella* -contaminated slurry. *Appl Environ Microbiol* 67:2859–2862
- Bengtsson-Palme J, Larsson DGJ (2016) Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environ Int* 86:140–149
- Berendsen BJ, Wegh RS, Memelink J et al (2015) The analysis of animal faeces as a tool to monitor antibiotic usage. *Talanta* 132:258–268
- Boxall A (2012) Fate and transport of antibiotics in soil systems. In: Keen P, Montforts M (eds) Antimicrobial Resistance in the Environment. Wiley-Blackwell, pp 309–324
- Bui XT, Wolff A, Madsen M et al (2011) Fate and survival of *Campylobacter coli* in swine manure at various temperatures. *Front Microbiol* 2:1–8
- Chander Y, Kumar K, Goyal SM et al (2005) Antibacterial activity of soil-bound antibiotics. *J Environ Qual* 34:1952
- Chee-Sanford JC, Mackie RI, Koike S et al (2009) Fate and transport of antibiotic residues and antibiotic r resistance genes following land application of manure waste. *J Environ Qual* 38:1086
- Chee-Sanford J, Maxwell S, Tsau K et al (2012) Antibiotic resistance in swine-manure-impacted environments. In: Keen P, Montforts M (eds) Antimicrobial Resistance in the Environment. Wiley-Blackwell, pp 203–223
- Chopra I, Roberts M (2001) Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232–260
- Cools D, Merckx R, Vlassak K et al (2001) Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. *Appl Soil Ecol* 17:53–62
- D’Costa VM, King CE, Kalan L et al (2011) Antibiotic resistance is ancient. *Nature* 477:457–461
- EUCAST (2020) “European Committee on Antimicrobial Susceptibility Testing”. Data from the EUCAST MIC distribution website, last accessed 07 April 2020. <http://www.eucast.org>
- Gebreyes WA, Altier C (2002) Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* Serovar typhimurium isolates from swine. *J Clin Microbiol* 40:2813–2822
- Hamscher G, Sczesny S, Höper H et al (2002) Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem* 74:1509–1518
- Hamscher G, Pawelzick HT, Hoper H et al (2005) Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem* 24:861–868
- Hanon J-B, Jaspers S, Butaye P et al (2015) A trend analysis of antimicrobial resistance in commensal *Escherichia coli* from several livestock species in Belgium (2011–2014). *Prev Vet Med* 122:443–452
- Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* 9:657–666
- Heuer H, Kopmann C, Binh CTT et al (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G + C content. *Environ Microbiol* 11:937–949
- Heuer H, Schmitt H, Smalla K (2011a) Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microbiol* 14:236–243
- Heuer H, Solehati Q, Zimmerling U et al (2011b) Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine. *Appl Environ Microbiol* 77:2527–2530
- Holley RA, Arrus KM, Ominski KH et al (2006) *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. *J Environ Qual* 35:1170

- Hutchison ML, Walters LD, Moore T et al (2005) Fate of pathogens present in livestock wastes spread onto fescue plots. *Appl Environ Microbiol* 71:691–696
- Jechalke S, Kopmann C, Rosendahl I et al (2013) Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. *Appl Environ Microbiol* 79:1704–1711
- Jechalke S, Heuer H, Siemens J et al (2014) Fate and effects of veterinary antibiotics in soil. *Trends Microbiol* 22:536–545
- Knapp CW, Zhang W, Sturm BSM et al (2010) Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. *Environ Pollut* 158:1506–1512
- Kumar K, Gupta CS, Chander Y et al (2005) Antibiotic use in agriculture and its impact on the terrestrial environment. *Adv Agron* 87:1–54
- Leclercq SO, Wang C, Sui Z et al (2016) A multiplayer game: species of *Clostridium*, *Acinetobacter*, and *Pseudomonas* are responsible for the persistence of antibiotic resistance genes in manure-treated soils. *Environ Microbiol* 18:3494–3508
- Lin JS, Tsen HY (1999) Development and use of polymerase chain reaction for the specific detection of *Salmonella typhimurium* in stool and food samples. *J Food Prot* 62:1103–1110
- Linton D, Lawson AJ, Owen RJ et al (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* 35:2568–2572
- Mannion C, Lynch PB, Egan J et al (2007) Seasonal effects on the survival characteristics of *Salmonella typhimurium* and *Salmonella Derby* in pig slurry during storage. *J Appl Microbiol* 103:1386–1392
- Marti R, Tien Y-C, Murray R et al (2014) Safely coupling livestock and crop production systems: how rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Appl Environ Microbiol* 80:3258–3265
- Miao V, Davies D, Davies J (2012) Path to resistance. In: Keen P, Montforts M (eds) *Antimicrobial Resistance in the Environment*. Wiley-Blackwell, pp 7–14
- NEN (1998) Dierlijke Mest En Mestproducten - Bepaling van de Gehalten Aan Droge Stof En Organische Stof - Gravimetrische Methode.
- Nicholson FA, Groves SJ, Chambers BJ (2005) Pathogen survival during livestock manure storage and following land application. *Bioresour Technol* 96:135–143
- Patterson AJ, Colangeli R, Spigaglia P et al (2007) Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by microarray detection. *Environ Microbiol* 9:703–715
- Peak N, Knapp CW, Yang RK et al (2007) Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ Microbiol* 9:143–151
- Pomsukarom S, Thakur S (2017) Horizontal dissemination of antimicrobial resistance determinants in multiple *Salmonella* serotypes following isolation from the environment of commercial swine operations after manure application. *Appl Environ Microbiol* 83:e01503–17
- Rasschaert G, Elst DV, Colson L et al (2020) Antibiotic residues and antibiotic-resistant bacteria in pig slurry used to fertilize agricultural fields. *Antibiotics (Basel)* 9(1):E34. <https://doi.org/10.3390/antibiotics9010034>
- Roberts MC (2012) Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline-resistant bacteria. In: Keen P, Montforts M (eds) *Antimicrobial Resistance in the Environment*. Wiley-Blackwell, pp 93–121
- Roberts MC, Sutcliffe J, Courvalin P et al (1999) Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* 43:2823–2830
- Sarmah AK, Meyer MT, Boxall AB (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65:725–759
- Schmitt H, ter Laak T, Duis K (2017) Development and dissemination of antibiotic resistance in the environment under environmentally relevant concentrations of antibiotics and its risk assessment
- Sengelov G, Agerso Y, Halling-Sorensen B et al (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ Int* 28:587–595
- Sisak F, Havlickova H, Hradecka H et al (2006) Antibiotic resistance of *Salmonella* spp. isolates from pigs in the Czech Republic. *Vet Med (Praha)* 51:303–310
- Van den Meersche T, Van Pamel E, Van Poucke C et al (2016) Development, validation and application of an ultra high performance liquid chromatographic-tandem mass spectrometric method for the simultaneous detection and quantification of five different classes of veterinary antibiotics in swine manure. *J Chromatogr A* 1429:1–10
- Van den Meersche T, Rasschaert G, Haesebrouck F et al (2019) Presence and fate of antibiotic residues, antibiotic resistance genes and zoonotic bacteria during biological swine manure treatment. *Ecotoxicol Environ Saf* 175:29–38
- Vanholme L, Imberechts H, Braeye T et al (2015) Report on Zoonotic Agents in Belgium.
- VITO (2015) Compendium bemonsterings- en analysemethodes voor mest, bodem en veevoeder (BAM)
- Vlaamse Landmaatschappij (2017) Mestrapport 2017. Jaarlijks Rapport over Het Mestbeheer in Vlaanderen

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.