**ORIGINAL ARTICLE**

Journal of
Applied Microbiology

# Spread of antimicrobial resistance genes via pig manure from organic and conventional farms in the presence or absence of antibiotic use

Rupert Bassitta[1] | Annika Nottensteiner[1] | Johann Bauer[1] |
Reinhard K. Straubinger[2] | Christina S. Hölzel[3]

[1]School of Life Sciences, Technical University of Munich, Freising, Germany

[2]Institute for Infectious Diseases and Zoonoses, LMU Munich, Munich, Germany

[3]Institute for Animal Breeding and Husbandry, Kiel University, Kiel, Germany

**Correspondence**
Christina S. Hölzel, Institute for Animal Breeding and Husbandry, Kiel University, 24098 Kiel, Germany.
Email: choelzel@tierzucht.uni-kiel.de

## Abstract

**Aims:** Antibiotic-resistant bacteria affect human and animal health. Hence, their environmental spread represents a potential hazard for mankind. Livestock farming is suspected to be a key factor for spreading antibiotic resistance; consumers expect organic farming to imply less environmental health risk. This study aimed to assess the role of manure from organic and conventional farms for spreading antimicrobial resistance (AMR) genes.

**Methods and Results:** AMR-genes—namely *tet*(A), *tet*(B), *tet*(M), *sul2* and *qacE/qacEΔ1* (potentially associated with multiresistance) were quantified by qPCR. Antimicrobial use during the study period was qualitatively assessed from official records in a binary mode (yes/no). Median concentrations were between 6.44 log copy-equivalents/g for *tet*(A) and 7.85 for *tet*(M) in organic liquid manure, and between 7.48 for *tet*(A) and 8.3 for *sul2* in organic farmyard manure. In conventional manure, median concentrations were 6.67 log copy-equivalents/g for *sul2*, 6.89 for *tet*(A), 6.77 for *tet*(B) and 8.36 for *tet*(M). Integron-associated *qac*-genes reached median concentrations of 7.06 log copy-equivalents/g in organic liquid manure, 7.13 in conventional manure and 8.18 in organic farmyard manure. The use of tetracyclines or sulfonamides increased concentrations of *tet*(A) and *tet*(M), or of *sul2*, respectively. Comparing farms that did not apply tetracyclines during the study, the relative abundance of *tet*(A) and *tet*(M) was still higher for conventional piggeries than for organic ones.

**Conclusions:** Relative abundances of AMR genes were higher in conventional farms, compared to organic ones. Antibiotic use was linked to the relative abundance of AMR-genes. However, due to the bacterial load, absolute concentrations of AMR-genes were comparable between fertilizers of organic and conventional farms.

**Significance and Impact of Study:** To our knowledge, this is the first absolute quantification of AMR-genes in manure from organic farms. Our study underlines the importance of long-term reduction in the use of antimicrobial agents in order to minimize antibiotic resistance.

# INTRODUCTION

The use of antimicrobial drugs in livestock plays an important role in the evolution and spread of antibiotic resistance (Mathew et al., 2007). According to Innes et al. (2020) considerable societal costs—excess costs of "resistant human infections" over "susceptible infections"—might be attributable to antimicrobial use in livestock. The selection of bacterial resistance does not stop when the antibiotic treatment of animals ends. Far more, selection extends into the environment when farm-produced fertilizers containing antibiotic residues and antibiotic resistance genes are spread onto agricultural farmland. Horizontal gene transfer—facilitating the exchange and acquisition of antibiotic resistance genes—is an important factor in the evolution of soil microbial communities (Alonso et al., 2001). Previous studies have shown that fertilizers like pig manure contribute to the selection of resistant bacterial populations in soil (Heuer & Smalla, 2007). Such effects are not limited to agriculturally used areas but extend into groundwater (Chee-Sanford et al., 2001). Caused by the application of wastewater and fertilizers there is a constant (re-)entry of (potential) pathogenic microorganisms of human and animal origin into the environment (Baquero et al., 2008; Heuer et al., 2011). By coining the term "environmental antibiotic resistome", which consists of all antibiotic resistance genes found either free, in bacteria that live in the environment, or in commensals of other environmental organisms, Wright (2010) underlined that scientific focus on antibiotic resistance should not be limited to pathogenic bacteria. Non-pathogenic environmental microorganisms may represent a massive reservoir for antibiotic resistance genes (Baquero et al., 2008).

Previous studies have shown that consumers tend to perceive conventionally produced food products as being environmentally more harmful compared to organic ones (Hughner et al., 2007). To substantiate this assumption, we hypothesized that farm-produced fertilizers from conventional and organic pig farm management systems differ in the environmental spread of antibiotic resistance determinants. We underlaid the fact that the abundance of different antibiotic resistance genes is associated with antimicrobial use, so we aimed to compare also samples from differently managed farms which did both not use antibiotics during the study term. Repeated samples from 58 different Bavarian piggeries were included, representing different types of farm-produced fertilizers (manure, liquid manure and farmyard manure). To our knowledge, this is the first quantitative metagenomic study on resistance genes in manure from organic farms.

# MATERIALS AND METHODS

## Study farms and sampling

The study included 35 conventional and 23 organic pig husbandries from the same geographic region (Bavaria, Germany). Farms categorized as "organic" fulfilled at least the legal regulations of (EU) 834/2007, now repealed by (EU) 2018/848. Farms were recruited for voluntary participation by aid of local stakeholders and had to be fattening farms (preferred) or piglet producers. Fifty-four farms were fattening farms, four farms were piglet producers. Fifteen Farms had less than 300 fattening places, 25 had between 300 and 1000, and 24 farms had more than 1000 fattening places. Small farms were more often run in an organic management system (1 conventional vs. 14 organic), large farms were more often conventionally managed (22 conventional vs. 2 organic).

Over an 18-month-period, at least two representative samples ($n = 158$) of farm-produced fertilizers from each farm were collected in autumn and spring. Samples differed between farm types, since conventionally managed farms do not use straw beddings and thus collect manure consisting of mixed faeces and urine. In contrast, farms managed with an organic production model collect the liquids separately as liquid manure and, in addition, produce a farmyard manure, which mainly consists of straw bedding mixed with faeces (but also parts of the urine soaked up in the straw). From these two different samples per organic farm, DNA was extracted separately; extracts were not pooled.

## Antimicrobial usage

Data pertaining to the antibiotic usage in these farms was assessed by others for the whole investigation period. For this purpose, official records as mandatory for any antibiotic treatment of livestock in Germany were collected and pseudonomyzed. For this study, usage data per class of agents (tetracyclines, sulfonamides) were provided on a yes/no-level for each farm and each collecting term of manure samples.

## DNA-extraction and qPCR

Sample preparation was performed as described elsewhere (Dorn-In et al., 2015). DNA extraction followed the instructions of PowerSoil DNA Isolation Kit (MO BIO Laboratories). Using a metagenomic qPCR approach, total DNA extracts were screened for resistance genes *tet*(A), *tet*(B), *tet*(M), *sul*2, as well as for *qac*EΔ1, which indicates the presence of potential multiresistance elements, namely integrons (Carattoli, 2001). As a standard for qPCR assays 10 ml of liquid pig manure were irradiated with 450 kGy gamma rays at Synergy Health Synergy Allershausen GmbH prior to spiking with control strains carrying the target antimicrobial resistance (AMR) genes (Hölzel et al., 2010). All qPCR experiments were conducted using a LightCycler 480 Instrument II (Roche Diagnostics Deutschland GmbH). Each well was loaded with 6 μl of Nuclease-free water (Roche), 1 μl of the 20-μM forward and reverse primer (Table 1), 10 μl LightCycler® 480 SYBR® Green I Master (Roche) and 2 μl template. All DNA extracts and the serial dilution of the standard were quantified in duplicate. Nuclease-free water served as a negative control. Cycling protocols were: one cycle of preincubation (95°C, 600 s), 45 cycles of amplification (95°C, 10 s; annealing with primer-specific temperature and hold-time, see Table 1; elongation at 72°C with primer-specific hold-time, Table 1), and one final cycle of melting curve acquisition (95°C, 5 s; 65°C, 60 s; 95°C, followed by cooling at 40°C for 10 s).

## Assessment of recovery rates

Recovery rates for *tet*(B) were assessed for all sample types in a pretrial, in which the DNA was extracted from irradiated material spiked with a *tet*(B)-positive control strain. This control strain was also used as the standard for assessing 16S-rRNA-gene-copy equivalents. We did not correct the data for the copy number of the reference strain (seven copies). For determining recovery rates, extraction was performed in duplicates and qPCR was performed in duplicate for each extraction duplicate. Each qPCR was run twice on the same day. Recovery rates were then calculated as means of eight values per sample and concentration (log 4–8). That resulted in a $2 \times 5 \times 2 \times 2$ design, with 2 extractions per sample and concentration, 5 spiking concentrations, 2 qPCR replicates within the same run and 2 qPCR runs. Mean efficiencies and standard deviations were calculated from all runs (158 per gene).

## Cut-off and limit of quantification

We used SYBR Green I for quantification, which is prone to false-positive signals due to unsepcific binding of the intercalating dye, e.g. to primer-dimers. For that reason, and in order to monitor contamination, we included extraction controls (PCR reagents added to extracts of PCR-grade water) in every run. The signal of these controls, multiplied by a safety factor of 2, was set as cut-off. Concentrations below this cut-off were not included in further analysis. The same was true if melting-curve analysis failed to prove the identity between the PCR product and the target (standard). The limit of quantification (LOQ) was set as the lowest concentration of the standard which allowed amplification with sufficient efficiency (i.e. which did not lower the slope of the standard curve when included). Cut-offs and LOQs were run-specific.

**TABLE 1** Primers used for qPCR assays

| Primer name | Target | Primer sequence (5′–3′) | Amplicon size (bp) | Reference | Annealing temp. (°C) | Hold time (s)[a] |
|---|---|---|---|---|---|---|
| Com1 | 16S-rRNA genes | CAGCAGCCGCGGTAATAC | 270 | Dorn-In et al. (2015) | 59 | 10/14 |
| R789 | | ATCCTGTTTGMTMCCCVCRC | | | | |
| *tet*(A) fw | *tet*(A) | GTGAAACCCAACATACCCC | 888 | Maynard et al. (2003) | 54 | 10/30 |
| *tet*(A) rv | | GAAGGCAAGCAGGATGTAG | | | | |
| *tet*(B) fw | *tet*(B) | TACGTGAATTTATTGCTTCGG | 206 | Aminov et al. (2002) | 60 | 5/8 |
| *tet*(B) rv | | ATACAGCATCCAAAGCGCAC | | | | |
| *tet*(M) fw | *tet*(M) | ACAGAAAGCTTATTATATAAC | 171 | Aminov et al. (2001) | 52 | 10/8 |
| *tet*(M) rv | | TGGCGTGTCTATGATGTTCAC | | | | |
| *sul*2 fw | *sul*2 | CGGCATCGTCAACATAACC | 722 | Maynard et al. (2003) | 59 | 10/30 |
| *sul*2 rv | | GTGTGCGGATGAAGTCAG | | | | |
| *qac*Eall fw | *qac*E/*qac*EΔ1 | CGCATTTTATTTTCTTTCTCTGGTT | 69 | Jechalke et al. (2014) | 60 | 5/6 |
| *qac*Eall rv | | CCCGACCAGACTGCATAAGC | | | | |

[a]Annealing/elongation.

## Statistics

All statistical analysis was performed using 'R' (http://www.r-project.org/). Normality was assessed and non-parametric tests were used. Absolute concentrations of AMR genes were adjusted by recovery rates, log-transformed and compared between all sample types in a Kruskal–Wallis One-Way-analysis of variance, followed by Holm-Sidak post hoc test. A Mann–Whitney-$U$-test was used to compare relative abundances in manure and liquid manure: (i) between farms which did or did not use the respective antibiotics, and (ii) between differently managed farms, which did not use the respective antibiotic.

## RESULTS

### qPCR efficiency, cut-offs and limit of quantification

Mean qPCR efficiencies were between $1.82 \pm 0.05$ for $tet$(A) and $2.01 \pm 0.06$ for $qac$E/$qac$EΔ1. Recovery rates of $tet$(B) were 72.2% in irradiated manure, 77.4% in irradiated liquid manure and 124.5% in irradiated farmyard manure. Due to this pronounced difference, all absolute values were corrected for recovery rates.

Run-specific PCR-cut-offs were between 0.0 (minimum, all genes except 16S rRNA-gene) and 5.95 log copies/g (maximum; 16S rRNA-gene), with median values between 0.23 [$tet$(A)] and 2.99 (16S rRNA-gene). Run-specific LOQs were between 1.27 (minimum; $sul$2) and 5.84 [maximum, $tet$(A)] log copies/g, with median values between 2.48 ($sul$2) and 4.1 ($tet$(B)).

### Absolute concentration in the manure

Samples of organic farms contained significantly higher absolute concentrations for $tet$(B), $sul$2 and $qac$EΔ1, together with a significantly higher bacterial load compared to manure samples from conventionally managed farms. Median values for log 16S rRNA-gene-concentrations (*Escherichia coli* equivalents; *Ec* equ) were 10.58 log *Ec* equ/g in organic farmyard manure, 10.07 log *Ec* equ/g in organic liquid manure and 9.57 log *Ec* equ/g in conventional manure. Considerable contents of tetracycline and sulfonamide resistance genes were found in pig farm-produced fertilizers, together with considerable contents of $qac$EΔ1, an indicator for integrons. The highest concentration was measured for $tet$(M), being 8.45 log copies/g manure. Figure 1 shows absolute resistance gene contents in fertilizer samples obtained from conventional and organic management systems farms.
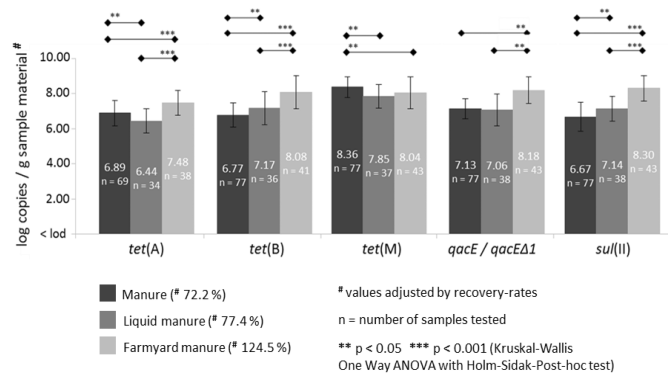


**FIGURE 1** Absolute concentrations of antimicrobial resistance genes in manure (conventional farms) and liquid/farmyard manure (organic farms). Written numbers represent median values. Error bars represent standard deviation of log-transformed data.

**TABLE 2** Median relative abundance of antibiotic resistance genes in manure (conventional farms), liquid manure and farmyard manure (organic farms), given as (copy equivalents/16S-rRNA gene copies) $\times 10^{-3}$

| Resistance gene | Conventional manure | Organic liquid manure | Organic farmyard manure |
|---|---|---|---|
| $tet$(A) | 1.42 | 0.24 | 0.46 |
| $tet$(B) | 1.30 | 0.99 | 2.58 |
| $tet$(M) | 71.60 | 4.44 | 1.13 |
| $sul$2 | 0.96 | 1.00 | 2.72 |
| $qac$E/$qac$EΔ1 | 3.60 | 1.03 | 1.13 |

## Relative abundance of AMR genes

While the absolute load with resistance genes is more appropriate to assess direct environmental impacts, the relative abundance [resistance gene copies, tet(M) or copy equivalents, other AMR genes, in relation to 16S-rRNA gene copy equivalents of *E. coli*, *Ec* equ] is a useful tool for assessing dynamics within bacterial populations. The maximum relative abundance of $tet$(A), $tet$(M) and $qac$EΔ1 was detected in conventional manure. The highest median value ($7.16 \times 10^{-2}$ copies per *Ec* equ) was measured for $tet$(M). By contrast, we detected the highest relative abundance of $tet$(B) and $sul$2 in farmyard manure from organic piggeries (median $2.58 \times 10^{-3}$ copy equivalents per *Ec* equ). The results of relative abundance are shown in Table 2.

## Association between relative abundance of AMR genes and antibiotic use

Usage of sulfonamides was associated with significantly higher relative abundance of $sul$2 in manure/liquid manure

samples compared to samples collected from farms which did not apply sulfonamides (Figure 2a). The same applied for the relative abundance of *tet*(A) and *tet*(M): comparing farms that either did or did not use tetracyclines on a regular basis, median concentrations of these resistance genes were significantly higher in samples of manure/liquid manure of applying piggeries (Figure 2b,c). With regard to *tet*(B), no statistically significant difference could be observed between farms that did or did not apply tetracyclines (data not shown).

With regard to tetracycline-free farms, base levels of *tet*(A) and *tet*(M) were significantly higher in conventional pig husbandries compared to organic ones (Figure 2b,c). Similar but nonsignificant results applied for *tet*(B) (data not shown), whereas *sul*2 levels were equal in both forms of management systems.

## DISCUSSION

To our knowledge, this is the first study on absolute concentrations of AMR genes in organic farms. This might not be obvious to the readership, due to the confusing wording: "organic fertilizers" are fertilizers produced by processes relying on fermentation of organic material (e.g. livestock waste), irrespective of whether this material is derived from organic or conventional farms. So, there are several quantitative metagenomic studies on organic fertilizers, but not on fertilizers derived from organic farms. In addition, there is one very valuable study dealing with intestinal contents of organic and conventional pigs, but not with fermented manure which is ready for application onto fields and pastures (Gerzova et al., 2015).

Absolute concentrations of all included genes except *tet*(M) were significantly higher in farmyard manure obtained at organic farms compared to conventional manure. The relative abundance of resistance genes was significantly higher in conventional manure for *tet*(A) and *tet*(M) compared to samples of liquid manure on organic farms ending up in significantly higher absolute concentrations of *tet*(M) and *tet*(A) despite the lower bacterial load. 16S-rRNA gene copies were measured compared to a standard of *E. coli*, and we did not correct for the copy number (*E. coli*: seven copies). Thus, the bacterial load in bacterial communities markedly rich of *Pseudomonas aeruginosa* or *Enterococcus faecalis* (each with four copies) would have been underestimated, while the bacterial load in communities markedly rich of *Bacillus cereus* (12 copies) or *Clostridium beijerinckii* (14 copies) would have been overestimated. As a result, relative abundances would have been overestimated in *Pseudomonas*- or *Enterococcus*-rich communities and underestimated in *Bacillus*-rich samples.

*tet*(M) represents the most commonly found tetracycline resistance gene and encodes a ribosomal protection protein (Roberts, 2011). Cheng et al. (2013) found similar relative abundance ($2.32 \times 10^2$ copy equivalents/16S-rRNA gene copies) in manure samples from Chinese pig farms. Kyselkova et al. (2015) found that the abundance of *tet*(M) in soil samples that were previously treated with cattle manure obtained in immediate proximity to the stable yards were even higher compared to *tet*(M) concentrations in the manure itself. Heuer et al. (2011) stated that the application of farm-produced fertilizers to agricultural land contributes significantly to an increase in antibiotic resistance and boosts the selection processes of resistant bacteria populations in soil. Thus, spread of resistance gene-containing materials such as farm-produced fertilizers may have selective effects on soil bacterial communities, resulting in higher levels of antibiotic resistance in the environment. However, Danish authors concluded that such effects caused by addition of pig manure slurry can be of temporarily limited duration (Sengeløv et al., 2003).

Our results indicate that pig farms may be of crucial importance for the emergence of antibiotic resistance within environmental bacterial populations, which is in accordance with previous studies (e.g., Sengeløv et al., 2003; Zhu et al., 2013). However, no specific data for manure from organic farms had been available up to now. With regard to indicator species and livestock samples, Österberg et al. (2016) published that phenotypic AMR was less common in organic pigs, as Schwaiger et al. (2008, 2010) had reported for poultry already. Consequently, one would suggest that farm-produced fertilizers originating from organic management systems generally have lower relative abundance of antibiotic resistance genes, due to fewer treatments with antimicrobial substances, as indeed seen for *tet*(A) and *tet*(M). Yet, this hypothesis is refuted by the fact that for *tet*(B) and *sul*2 highest relative abundances were detected in farmyard manure from organic piggeries. This is in certain accordance with the fact that Gerzova et al. (2015) did not find significantly lower relative abundances for AMR genes in intestinal samples of organic pigs compared to conventional pigs. Interestingly, relative abundances of *tet*(A), *tet*(B) and *sul*2 in Bavaria (this study) were generally at the upper level when compared to data from Italy, France, Sweden and Denmark (Gerzova et al., 2015).

Absolute concentrations were highest in organic farms for all investigated genes except *tet*(M). This was associated with the fact that organic farmyard samples had the highest bacterial load, and was not related to antibiotic selection. However, the samples were investigated when they were applied to land, so the amount of resistance genes is an indicator of potential environmental risk, irrespective
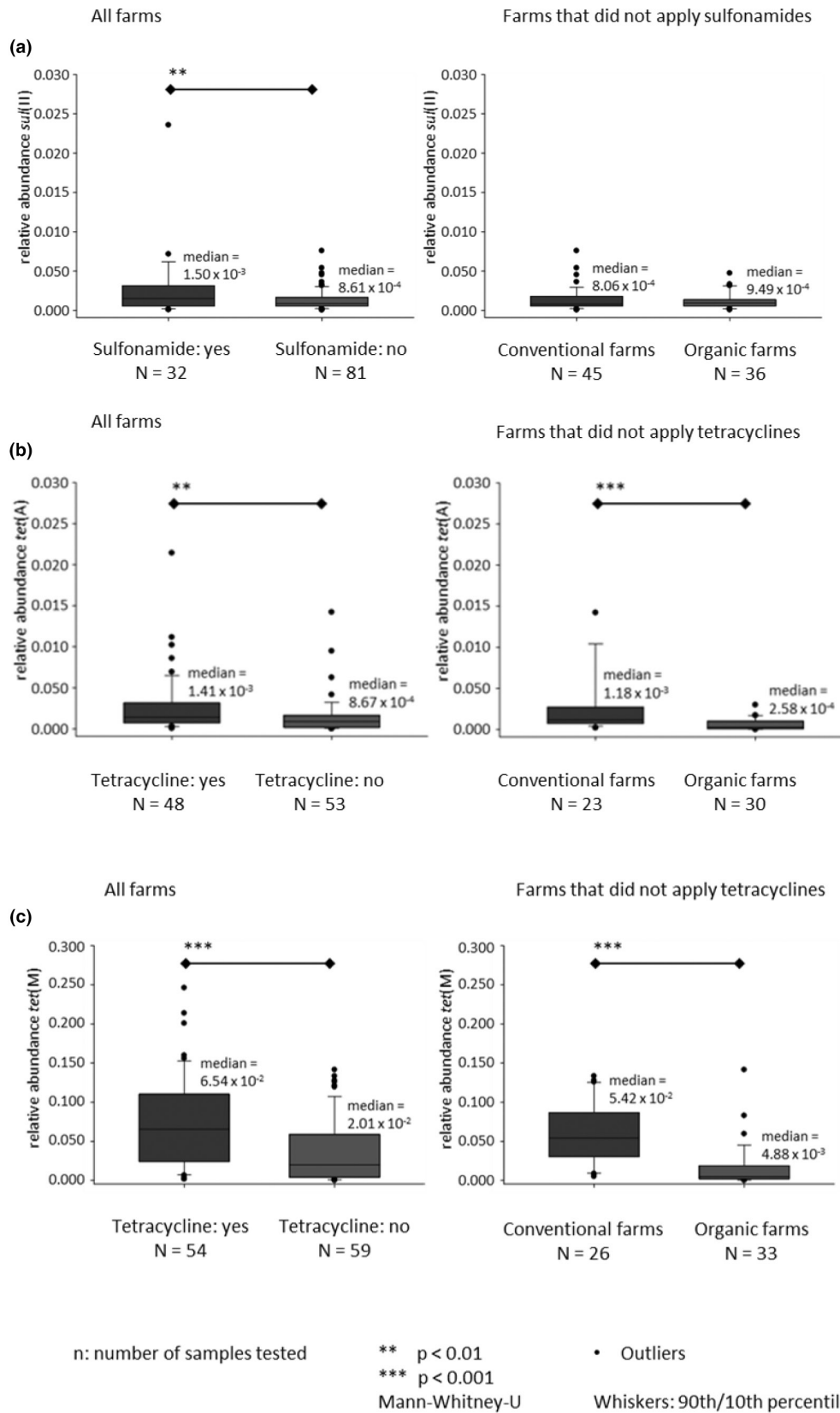
**FIGURE 2** Relative abundance (copy equivalents/16S-rRNA gene copies) of *sul*2 (a), *tet*(A) (b) and *tet*(M) (c) in farm-produced fertilizer samples; left: use of tetracyclines yes/no, right: conventional and organic tetracycline-free farms. White line = median. n: number of samples tested. **p < 0.01, ***p < 0.001 (Mann-Whitney-U-test). Dot: outliers. Whiskers: 90th/10th percentile.

of its cause. In order to consolidate our findings, we exemplarily compared the recovery rate of *tet*(B) in all types of samples. Contraintuitively, recovery rates in irradiated farmyard manure were above 100%. This applied to all investigated log concentrations (4–8) and might have been caused by the fact that 10 g of farmyard manure were extracted, compared to 1 g of manure/liquid manure. Thus, concentrations per gram are equal to total amounts in the manure/liquid manure extraction, while total amounts were 10-fold the value per g in the extraction of farmyard manure. This offers the opportunity that unequal distribution might increase (not only decrease) the calculated concentration per gram. Recovery rates were assessed in a limited number of replicates ($2 \times 5 \times 2 \times 2$), which might have impaired the validity of our assessment. However, significant differences were present regardless of whether we corrected for this recovery rate or not. Hence, resistance genes are present to a considerable extent in organic samples as well, and this difference is not levelled off by different amounts brought to land (related to different nutrient density of the fertilizers), as obvious from the following calculation. The allowed amount of conventional manure/liquid manure to reach the maximum N per area is approx. 1.5- to 1.7-fold the amount of farmyard manure, while the differences in median absolute resistance gene contents is 43-fold for *sul*2.

To date and to our knowledge, there is no quantitative data available which describes the absolute amount of resistance genes found in manure from organic farms. Above all, there is a lack of comparative data from organic and conventional farms in the same region. Though, Armalytė et al. (2019) investigated soils from organic and conventional farms and found no clear preference of resistance genes depending on conventional or organic farming.

The application of antibiotics clearly contributed to the relative abundance of resistance genes found in the samples: concentrations of *sul*2, *tet*(A) and *tet*(M) were significantly higher in farms that applied the respective antibiotic during the study period, even though we could not consider the frequency of treatments, the amount of antibiotics or the number of animals treated. These data suggest that the use of antibiotics has selective impact on the quantitative abundance of corresponding antibiotic resistance genes. Results of a previously conducted study by Peak et al. (2007) support this hypothesis: the use of antibiotics clearly influenced corresponding *tet*-resistance gene levels in lagoons at cattle feedlots in the United States of America with significantly higher values in high-end usage pens. Bearing in mind that excrementitious matter is spread to agricultural farmland in a downstream step, this exerts direct effects on the environment. This is especially the case if resistance genes are located on plasmids or integrons, which are likely to spread AMR within

bacterial populations (Carattoli, 2001; Wang et al., 2020). The fact that considerable amounts of integron-associated *qac*EΔ1 were detected in all of the tested samples—no matter whether obtained from conventional or organic piggeries—suggests that bacterial gene capture plays an important role in pig husbandry.

Due to structural differences, comparisons between farms of organic or conventional production types were hard to unify. Farm size, for example, was pronouncedly different between both production types. However, due to the fact that the choice of specific antimicrobial substances is mainly ruled by individual preferences, the use of particular substances (like tetracyclines and sulfonamides) was not likely to be associated with farm size, and comparable numbers of organic and conventional farms did not use these substance classes (see results).

We also had to compare different types of samples between the different production types (conventional/organic), due to a general unavailability of sample types, related to differences in stalling. Organic farms use straw beddings; most conventional farms do not. In farms that use straw beddings, the straw mixed with faeces is collected as "farmyard manure", while the liquids are passively separated by seeping and form "liquid manure" (German word: Jauche). In conventional systems with fully slatted floor or mats, faeces and liquids are collected together as manure (German word: Gülle) of more mushy consistence. While farmyard manure (containing straw) is a very different matrix, conventional manure and liquid manure are quite similarly composed of faeces and urine; they differ mainly by a certain difference in dry matter. Indeed, matrix features were very comparable between both sample types, and a very similar recovery rate of *tet*(B) was found in both types of samples (72.2% vs. 77.4%). Accordingly, for direct comparison of relative abundances, we stuck to manure and liquid manure, disregarding the straw-mixed farmyard manure. We then specifically compared organic and conventional farms that did not use the respective antibiotics during the study period, in order to assess differences between production types which might be unrelated to recent antibiotic use. Relative abundances of *tet*(M) and *tet*(A) were significantly higher in samples from conventional farms, despite the temporary abandonment of the respective antibiotic. According to general usage data, more conventionally than organically managed farms used other antibiotics during the study period or used the respective antibiotic before study onset (personal information, data not shown). This might hint towards a role of coselection, which is supported by a prior study on pig manure (Hölzel et al., 2010). However, significantly higher concentrations in conventional management

systems might have also been connected to the term of abandonment. More conventional than organic farms had abandoned certain or all antibiotics during only one sampling period (data not shown), or might have purchased piglets from raisings which applied antibiotics. Thus, higher concentrations or AMR genes in the samples of conventional farms might be caused by the fact that AMRs is only slowly reversed in the absence of fitness costs (Merker et al., 2020). When we exemplarily checked the liquid chromatography–mass spectrometry/mass spectrometry-data of tetracycline-analysis provided by courtesy of colleagues—we chose tetracyclines since these substances are comparatively stable in organic fertilizers (Harms & Bauer, 2012), we did not see indications for a major role of antibiotic residues transferred from prior fattening periods or with piglets at stalling, except few cases. Of 108 fertilizers with no reported use of tetracyclines on farm, only 4.6% of samples ($n = 5$) were positive for tetracyclines, with a median of 179 µg/kg wet weight and a range of 66–1891 µg/ kg (K. Harms, personal communication). Farms that did not use tetracyclines, but were analytically positive, reported use of tetracyclines in prior periods ($n = 3$) and/or purchase of pigs with unknown treatment state ($n = 5$). The maximum value of 1891 µg/kg manure was reached in a farm where pigs of the prior period had experienced stock treatment with 13.86 kg of tetracyclines (tetracycline-HCl, in this case). That treatment was carried out 4.5 months before sampling of the manure pit, which had been emptied once between both dates. To conclude, the application of farm-produced fertilizers to agricultural farmland results in a considerable spread of tetracycline resistance genes [up to 8.45 log copies *tet*(M)/g manure] and sulfonamide resistance genes as well as integron-associated *qac*EΔ1—no matter, whether the way of livestock farming was conventional or organic. In fertilizers from organic farms, a lower relative abundance was partly accompanied by a higher bacterial load, resulting in equal or even higher absolute concentrations of resistance genes. The absolute spread of AMR genes into the environment via manure is not exclusively linked to antibiotic selection. However, a clear link between use of antibiotics and the relative abundance of corresponding resistance genes could be shown. Thus, the results underline that a long-term reduction in general use of antimicrobial agents might play an important role in the mission of minimizing antibiotic resistance in the livestock-impacted environment.

## CONFLICT OF INTEREST
No conflict of interest declared.

## ORCID
*Christina S. Hölzel* https://orcid.org/0000-0001-8540-5928

## REFERENCES
Alonso, A., Sánchez, P. & Martínez, J.L. (2001) Environmental selection of antibiotic resistance genes. *Environmental Microbiology*, 3(1), 1–9. Available from: https://doi.org/10.1046/j.1462-2920.2001.00161.x

Aminov, R.I., Chee-Sanford, J.C., Garrigues, N., Teferedegne, B., Krapac, I.J., White, B.A. et al. (2002) Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Applied and Environmental Microbiology*, 68(4), 1786–1793. Available from: https://doi.org/10.1128/aem.68.4.1786-1793.2002

Aminov, R.I., Garrigues-Jeanjean, N. & Mackie, R.I. (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Applied and Environmental Microbiology*, 67(1), 22–32. Available from: https://doi.org/10.1128/AEM.67.1.22-32.2001

Armalytė, J., Skerniškytė, J., Bakienė, E., Krasauskas, R., Šiugždinienė, R., Kareivienė, V. et al. (2019) Microbial diversity and antimicrobial resistance profile in microbiota from soils of conventional and organic farming systems. *Frontiers in Microbiology*, 10, 892. Available from: https://doi.org/10.3389/fmicb.2019.00892

Baquero, F., Martínez, J.L. & Cantón, R. (2008) Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*, 19(3), 260–265. Available from: https://doi.org/10.1016/j.copbio.2008.05.006

Carattoli, A. (2001) Importance of integrons in the diffusion of resistance. *Veterinary Research*, 32(3–4), 243–259. Available from: https://doi.org/10.1051/vetres:2001122

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N. & Mackie, R.I. (2001) Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology*, 67(4), 1494–1502. Available from: https://doi.org/10.1128/AEM.67.4.1494-1502.2001

Cheng, W., Chen, H., Su, C. & Yan, S. (2013) Abundance and persistence of antibiotic resistance genes in livestock farms: a comprehensive investigation in eastern China. *Environment International*, 61, 1–7. Available from: https://doi.org/10.1016/j.envint.2013.08.023

Dorn-In, S., Bassitta, R., Schwaiger, K., Bauer, J. & Hölzel, C.S. (2015) Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *Journal of Microbiological Methods*, 113, 50–56. Available from: https://doi.org/10.1016/j.mimet.2015.04.001

Gerzova, L., Babak, V., Sedlar, K., Faldynova, M., Videnska, P., Cejkova, D. et al. (2015) Characterization of antibiotic resistance gene abundance and microbiota composition in feces of organic and conventional pigs from four EU countries. *PLoS One*, 10(7), e0132892. Available from: https://doi.org/10.1371/journal.pone.0132892

Harms, K. & Bauer, J. (2012) Detection and occurrence of antibiotics and their metabolites in pig manure in Bavaria. In: Montforts, M. & Keen, P. (Eds.) *Antimicrobial resistance in the environment*. Hoboken: John Wiley & Sons, pp. 293–309.

Heuer, H., Schmitt, H. & Smalla, K. (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. *Current Opinion in Microbiology*, 14(3), 236–243. Available from: https://doi.org/10.1016/j.mib.2011.04.009

Heuer, H. & Smalla, K. (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environmental Microbiology*, 9, 657–666.

Hölzel, C.S., Harms, K.S., Küchenhoff, H., Kunz, A., Müller, C., Meyer, K. et al. (2010) Phenotypic and genotypic bacterial antimicrobial resistance in liquid pig manure is variously associated with contents of tetracyclines and sulfonamides. *Journal of Applied Microbiology*, 108(5), 1642–1656. Available from: https://doi.org/10.1111/j.1365-2672.2009.04570.x

Hughner, R.S., McDonagh, P., Prothero, A., Shultz, C.J., II & Stanton, J. (2007) Who are organic food consumers? A compilation and review of why people purchase organic food. *Journal of Consumer Behaviour*, 6, 94–110. Available from: https://doi.org/10.1002/cb.210

Innes, G.K., Randad, P.R., Korinek, A., Davis, M.F., Price, L.B., So, A.D. et al. (2020) External societal costs of antimicrobial resistance in humans attributable to antimicrobial use in livestock. *Annual Review of Public Health*, 41, 141–157. Available from: https://doi.org/10.1146/annurev-publhealth-040218-043954

Jechalke, S., Schreiter, S., Wolters, B., Dealtry, S., Heuer, H. & Smalla, K. (2014) Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis. *Frontiers in Microbiology*, 4, 420. Available from: https://doi.org/10.3389/fmicb.2013.00420

Kyselkova, M., Jirout, J., Vrchotova, N., Schmitt, H. & Elhottova, D. (2015) Spread of tetracycline resistance genes at a conventional dairy farm. *Frontiers in Microbiology*, 6, 536. Available from: https://doi.org/10.3389/fmicb.2015.00536

Mathew, A.G., Cissell, R. & Liamthong, S. (2007) Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne Pathogens and Disease*, 4(2), 115–133. Available from: https://doi.org/10.1089/fpd.2006.0066

Maynard, C., Fairbrother, J.M., Bekal, S., Sanschagrin, F., Levesque, R.C., Brousseau, R. et al. (2003) Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrobial Agents and Chemotherapy*, 47(10), 3214–3221. Available from: https://doi.org/10.1128/aac.47.10.3214-3221.2003

Merker, M., Tueffers, L., Vallier, M., Groth, E.E., Sonnenkalb, L., Unterweger, D. et al. (2020) Evolutionary approaches to combat antibiotic resistance: opportunities and challenges for precision medicine. *Frontiers in Immunology*, 27(11), 1938. Available from: https://doi.org/10.3389/fimmu.2020.01938

Österberg, J., Wingstrand, A., Nygaard Jensen, A., Kerouanton, A., Cibin, V., Barco, L. et al. (2016) Antibiotic resistance in *Escherichia coli* from Pigs in organic and conventional farming in four European countries. *PLoS One*, 11(6), e0157049. Available from: https://doi.org/10.1371/journal.pone.0157049

Peak, N., Knapp, C.W., Yang, R.K., Hanfelt, M.M., Smith, M.S., Aga, D.S. et al. (2007) Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environmental Microbiology*, 9(1), 143–151. Available from: https://doi.org/10.1111/j.1462-2920.2006.01123.x

Roberts, M.C. (2011) Environmental macrolide-lincosamide-streptogramin and tetracycline resistant bacteria. *Frontiers in Microbiology*, 2, 40. (eCollection 2011). Available from: https://doi.org/10.3389/fmicb.2011.00040

Schwaiger, K., Schmied, E.M. & Bauer, J. (2008) Comparative analysis of antibiotic resistance characteristics of Gram-negative bacteria isolated from laying hens and eggs in conventional and organic keeping systems in Bavaria, Germany. *Zoonoses Public Health.*, 55(7), 331–341. Available from: https://doi.org/10.1111/j.1863-2378.2008.01151.x

Schwaiger, K., Schmied, E.M. & Bauer, J. (2010) Comparative analysis on antibiotic resistance characteristics of *Listeria* spp. and *Enterococcus* spp. isolated from laying hens and eggs in conventional and organic keeping systems in Bavaria, Germany. *Zoonoses and Public Health*, 57(3), 171–180. Available from: https://doi.org/10.1111/j.1863-2378.2008.01229.x

Sengeløv, G., Agersø, Y., Halling-Sørensen, B., Baloda, S.B., Andersen, J.S. & Jensen, L.B. (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environment International*, 28(7), 587–595. Available from: https://doi.org/10.1016/s0160-4120(02)00084-3

Wang, P., Qiao, Z., Li, X., Wu, D. & Xie, B. (2020) Fate of integrons, antibiotic resistance genes and associated microbial community in food waste and its large-scale biotreatment systems. *Environment International*, 44, 106013. Available from: https://doi.org/10.1016/j.envint.2020.106013

Wright, G.D. (2010) Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology*, 13(5), 589–594 Available from: https://doi.org/10.1016/j.mib.2010.08.005

Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D. et al. (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences*, 110(9), 3435–3440. Available from: https://doi.org/10.1073/pnas.1222743110