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ENVIRONMENTAL BIOTECHNOLOGY

Contaminations of organic fertilizers with antibiotic residues, resistance genes, and mobile genetic elements mirroring antibiotic use in livestock?

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Abstract Pig manures are frequently used as fertilizer or co-substrate in biogas plants (BGPs) and typically contain antibiotic residues (ARs), as well as bacteria carrying resistance genes (RGs) and mobile genetic elements (MGEs). A survey of manures from eight pig fattening and six pig breeding farms and digestates from eight BGPs in Lower Saxony, Germany was conducted to evaluate the link between antibiotic usage and ARs to RGs and MGEs present in organic fertilizers. In total, 11 different antibiotics belonging to six substance classes were applied in the farms investigated. Residue analysis revealed concentrations of tetracycline up to 300 mg kg^{-1} dry weight (DW) in manures and of doxycycline up to 10.1 mg kg^{-1} DW in digestates indicating incomplete removal during anaerobic digestion. RGs (*sul1*, *sul2*, *tet*(A), *tet*(M), *tet*(X), *qacEΔ1*) were detected in total community DNA of all samples by PCR-Southern blot hybridization. Broad-host range plasmids (IncP-1, IncQ, IncN, and IncW) and integron integrase genes (*intI1*, *intI2*) were found in most manure samples with IncN and IncW plasmids being more abundant in manure from pig breeding compared to pig fattening farms. *IntI1*, IncQ, and IncW plasmids were also detected in all digestates, while IncP-1, IncN, and LowGC plasmids were detected only sporadically. Our findings strongly reinforce the need for further research to identify mitigation strategies to reduce the level of contamination of organic fertilizers with ARs and transferable RGs that are applied to soil and that might influence the mobile resistome of the plant microbiome.

Keywords Antibiotic resistance genes · Mobile genetic elements · Antibiotics · Pig husbandry · Manures · Digestates

Introduction

Conventional livestock husbandry is due to increasing numbers of animals kept in a constantly decreasing number of farms (Udo et al. 2011) inevitably linked to the application of antibiotics (Boxall et al. 2004; Sarmah et al. 2006; Widyasari-Mehta et al. 2016a). An estimated total of 1619 tons of antibiotics was used in Germany in 2012 (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) Germany 2015). Among the most frequently applied antibiotic classes are the first generation antibiotics of tetracyclines and sulfonamides. Depending on the target animals, antibiotic application patterns and frequencies typically differ. The fate of the antibiotics, however, mainly depends on their physicochemical characteristics. Thus, sulfonamides and tetracyclines are partially excreted as parent compounds and/or metabolites (up to 96 and 90 %, respectively) (Hirsch et al. 1999; Sarmah et al. 2006; Lamshöft et al. 2007; Heuer et al. 2008). Recent studies showed that antibiotic residues (ARs) in pig manures reached up to 770 mg oxytetracycline kg^{-1} dry weight (DW) concentrations (Martínez-Carballo et al. 2007; Gans et al. 2010; Ratsak et al. 2013; Spielmeyer et al. 2014, 2015; Widyasari-Mehta et al. 2016a). Furthermore, it was revealed by Heuer et al. (2008) that, due to deacetylation of one major metabolite, the concentration of ^{14}C-labeled sulfadiazine even increased

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during the storage of manure from ^{14}C-labeled sulfadiazine treated pigs over time. Besides the direct application of manure as fertilizer on agricultural soil, its usage as co-substrate in biogas plants (BGPs) is of increasing importance. Digestates resulting from the biogas production are also applied as organic fertilizers. ARs were recently reported from digestates as well, e.g. sulfadimidine up to 76.2 mg kg^{-1} DW (Ratsak et al. 2013; Spielmeyer et al. 2014, 2015; Widyasari-Mehta et al. 2016a), suggesting that their application to soil may also contribute to selection of resistant bacteria in digestate treated soils.

Livestock manure does not only frequently contain ARs but it also represents a reservoir of diverse resistance genes (RGs), integrons, and mobile genetic elements (MGEs) such as transferable plasmids (Binh et al. 2008; Heuer et al. 2012; Zhu et al. 2013; Marti et al. 2013; Kyselková et al. 2015). Also, several RGs and integrons were detected in samples from biogas digesters (Diehl and LaPara 2010; Wolters et al. 2016). When manures or digestates are applied as fertilizers to field soil, these RGs and MGEs might be transferred to the indigenous bacterial soil communities (Jechalke et al. 2014). For instance, the repeated application of pig manure spiked with different concentrations of sulfadiazine was shown under microcosm conditions to enhance the abundance of sulfonamide RGs (*sul* genes) up to 2 months after their entry into soils (Heuer et al. 2011). Several transferable plasmids conferring resistances toward antibiotics that are typically associated with livestock manure have a broad host range (BHR) (Götz et al. 1996; Binh et al. 2008; Heuer et al. 2012) and were shown to transfer to bacteria indigenous to the rhizosphere of plants (Musovic et al. 2006) or to *Pseudomonas putida* when introduced with manure into field soil (Götz and Smalla 1997). Recently, BHR plasmids belonging to the IncP-1ε plasmid subgroup were captured (based on sulfadiazine or tetracycline resistances conferred) from BGP digestate bacteria into *Escherichia coli* and *P. putida* recipients (Wolters et al. 2015). Thus, following the application of manure or digestate as fertilizer, these plasmids may potentially also be taken up by bacteria associated with crops and may subsequently be transferred to consumers. As the intestine is considered to be a hot spot for horizontal gene transfer (HGT) (Kurokowa et al. 2007; Baquero 2012), in a worst case scenario, manure fertilization might contribute via this route to the increasing hazard posed by multiple antibiotic resistant bacteria in particular pathogens of clinical relevance (Forsberg et al. 2012). The combined introduction of RGs, MGEs, and ARs into agricultural soil via manure spread might in this context be of particular relevance, as antibiotics present in soil at subinhibitory concentrations might foster HGT processes and the proliferation of antibiotic-resistant bacteria (Gullberg et al. 2011, 2014; Jechalke et al. 2014).

Therefore, the aims of the current study were to link data on the application of antibiotics in 19 pig fattening and breeding farms without/with farm-owned BGPs in Lower Saxony, Germany to ARs found via liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis and to the presence of RGs, integrons, and MGEs in manures and digestates of BGPs. In the present study, we have used PCR-Southern blot hybridization for highly sensitive and specific detection of RGs and MGEs in total community (TC-) DNA. Additionally, we evaluated whether RGs, integrons, and MGEs present in manures were also detectable in digestates of BGPs fed with manures as co-substrate.

Materials and methods

Sampling, farm operating data, and antibiotic applications

In order to investigate pig manures and digestates from farms located in Lower Saxony, Germany, in autumn 2012, samples from eight pig fattening and six breeding farms, as well as digestates from eight BGPs (five pig breeding farms with farm-owned BGPs and three corporate BGPs without the corresponding antibiotic application data) fed with manure as co-substrate, were collected. The details of samplings were described by Widyasari-Mehta et al. (2016a). Manures were sampled from cellars, silos, or lagoons using a probe sampler, a bypass sampler or in backflush mode from the vacuum tanker. Digestate samples were taken from closed or open silos via the outlet valves. At each farm, four 8-L samples were taken. Sample aliquots of 300 mL were transferred into polyethylene bottles and kept in cooling boxes during transport to the laboratory. In addition, farm operating data and information on antibiotic applications from spring 2012 until autumn 2012 (Table 1) were gathered. A complete data set of antibiotic applications and antibiotic residues for samples from spring 2012 to 2013 is detailed in Widyasari-Mehta et al. (2016a).

Residue analysis

The manure and digestate samples were analyzed for 16 antibiotics and three metabolites, namely, oxytetracycline, tetracycline, chlortetracycline, doxycycline, sulfadiazine, acetyl-sulfadiazine, sulfadimidine, acetyl-sulfadimidine, sulfamerazine, sulfadoxine, sulfadimethoxine, sulfamethoxypyridazine, trimethoprim, enrofloxacin, ciprofloxacin, danofloxacin, marbofloxacin, tiamulin, and tylosin. The analytical procedure is detailed by Widyasari-Mehta et al. (2016a, b). All samples were characterized for pH and DW content. Thereafter, aliquots of 25-g fresh samples were treated with ethylenediaminetetraacetic acid solution, citrate buffer, and hydrochloric acid to adjust pH 3.0. After lyophilization, ARs in the solids were

Table 1 Applications of antibiotics at eight pig fattening farms (F1-F8), six pig breeding farms (B1-B6), five pig breeding farms with farm-owned BGPs (BGP1-BGP5) and three corporate BGPs (BGP6-BGP8) from spring 2012 to autumn 2012 and the respective antibiotic residues in manure and digestate samples taken in autumn 2012

Farm	Antibiotic	n_A/n_P	In manure [mg kg^{-1}][d]	Farm	Antibiotic	n_A/n_P	In manure [mg kg^{-1}][d]
F1	TC	8/140–208	300	B1	DOXY	1/100	50.0
1000 FP	TYL	9/20–355	n.d.	200 S	OXY	4/8–10	n.d.
F2	DOXY	1/120	20.3		SDZ	1/4	n.d.
320 FP	TYL	1/200	n.d.		TMP	1/4	n.d.
F3	TC	3/100–200	52.9		TYL	1/125	n.d.
300 FP				B2	DOXY	n.a.	19.0 MC; 9.4 MS
F4	TC	2/242–249	161	220 S,	TC	n.a.	4.6 MC; n.d. MS
400 FP				2000 FP			
F5	TC	3/150–299	263	B3	CTC	1/35	n.d. S; 15.8 FP
1100 FP				130 S,	OTC	7/1–60	211 S; 14.9 FP
F6	TC	1/220	287	800 FP	ENF	1/30	n.d.
350 FP	SDZ	1/220	0.7	B4	CTC	1/5 kg	26.4
	$ASDZ^M$	n.a.	11.5	100 S,	DOXY	3/5 kg	27.7
	TMP	1/220	n.d.	650 FP	SDZ	1/5 kg	n.d.
F7	TC	1/385	5.5		TMP	1/5 kg	n.d.
1545 FP	CTC	2/580–820	26.6		DOXY	2/500–700	101
	SDZ	1/580	n.d.		ENF	3/4–175	n.d.
	TMP	1/580	n.d.	B5	TC	1/400	1.5
	ENF	1/150	n.d.	130 S,	DOXY	1/400	n.d.
F8	TYL	1/300	n.d.	800 FP	ENF	4/4–50	1.3
380 FP					TIA	1/50	1.4
					SDZ	1/400	n.d.
					TMP	1/400	n.d.
					TYL	1/1	n.d.
				B6	DOXY	2/85–180	52.6
				220 S	ENF	2/30–65	n.d.
					SDM	7/3–10	23.0
					SDZ	2/85–180	n.d.
					TMP	9/3–180	n.d.
Farm	Antibiotic	n_A/n_P	In digestate [mg kg^{-1}][d]	Farm	Antibiotic	n_A/n_P	In digestate [mg kg^{-1}][d]
BGP1	TC	n.a.	1.6	BGP3	DOXY	3/200–300	2.1[c]
100 S,	DOXY	n.a.	1.3	550 S,			
1000 FP				550 FP			
BGP2	CTC	1/17	n.d.	BGP4	DOXY	21/150–400	10.1
475 S	DOXY	5/1300–2000	7.4	265 S, 2100 FP	ENF	6/100–275	0.2
	ENF	7/12–56	n.d.	BGP5	DOXY	n.a.	3.2
	SDX	7/6–28	n.d.	600 S			
	TMP	7/6–28	n.d.	BGP6[a]	TC	–	6.4
	TYL	1/500	n.d.		DOXY	–	2.2
				BGP7[b]	TC	–	0.4
				BGP8[a]	TC	–	n.d.

FP fattening pig, *S* sow (producing approximately 27 piglets a^{-1}), n_A/n_P number of application/number of pigs, *TC* tetracycline, *CTC* chlortetracycline, *OTC* oxytetracycline, *DOXY* doxycycline, *ENF* enrofloxacin, *SDZ* sulfadiazine, $ASDZ^M$ acetyl-sulfadiazine (metabolite), *SDM* sulfadimidine, *SDX* sulfadoxine, *TMP* trimethoprim, *TIA* tiamulin, *TYL* tylosin, – unknown application, *n.a.* not applied, *n.d.* not detected, *MC* manure from cellar, *MS* manure from silo

[a] Farm independent BGP

[b] Cattle farm

[c] Corresponded to the detections of doxycycline residues at 166 mg kg^{-1} in manure and 0.4 mg kg^{-1} in digester material

[d] In dry weight

extracted using methanol/ethyl acetate mixture. For clean-up, liquid-liquid partition with *n*-hexane/water followed by solid phase extraction was conducted. Target compound analysis was finally performed using LC-MS/MS with electrospray ionization in multiple reaction monitoring mode. Single-point standard addition technique was applied for quantification purpose. Method quantitation limits (MQLs) of all target compounds were 0.2 mg kg^{-1} DW in manure and digestate samples.

Bioanalytics

Extraction of TC-DNA

TC-DNA was extracted from manure samples and BGP digestates using the spin kit for soil (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's recommendation. Freshly homogenized samples (14 mL) were centrifuged in 15-mL Falcon tubes (Sarstedt, Nümbrecht,

Germany) at 3100×*g* for 10 min; the supernatants were discarded and pellets were homogenized with a sterile spatula. Homogenized sample material (0.1 g) was transferred into lysis tubes and used for extraction of TC-DNA. Prior to PCRs, TC-DNA was diluted 1:5 with 1× TE buffer (pH 8.0).

Detection of sequences specific for antibiotic resistance genes, quaternary ammonium compound resistance genes, integron integrase genes, and plasmids

PCR assays used for the detection of RGs, integrons, and plasmid-specific sequences of IncN, IncP-1, IncQ, IncW, and LowGC plasmids were previously described and are listed in Table 2. To increase the sensitivity and specificity of the detection method, PCR products were Southern blotted and hybridized with the corresponding digoxigenin-labeled probes. The generation of the probes and the Southern blot hybridization were done as described in detail by Götz et al. (1996) and Binh et al. (2008). PCR amplicons obtained from reference plasmids (Table 2) were labeled with digoxigenin using the DIG-labeling Kit (Roche Diagnostic, Mannheim, Germany).

Results

Antibiotic application patterns and antibiotic residues in manures and digestates

Antibiotics from tetracycline, sulfonamide, diaminopyrimidine, pleuromutiline, and macrolide classes were used on 19 pig husbandry farms with conventional manure management (Table 1). Seven antibiotics from these classes were applied at the eight pig fattening farms F1 to F8. The highest usage rates were found for tetracycline at farm F1 keeping 1000 fattening pigs, where 140–208 pigs were treated eight times. Here, tetracycline was consistently found in manure at 300 mg tetracycline kg^{-1} DW. Tetracyclines were also used to treat up to 385 pigs at five other pig fattening farms (except for F2 and F8). At these farms, tetracycline was detected in manures, ranging from 5.5 to 287 mg kg^{-1} DW. Doxycycline and chlortetracycline were found only in single manure samples from farms F2 and F7, respectively. These findings correlated with the data on the rate of antibiotic usage. A single application of the sulfonamide sulfadiazine in combination with trimethoprim was only recorded for farms F6 and F7. At farm F6 where 220 pigs were treated with sulfadiazine/trimethoprim, 0.7 mg sulfadiazine residues were detected per kilogram DW of manure. In addition, acetyl-sulfadiazine was simultaneously detected at 11.5 mg kg^{-1} DW. Even though tylosin was frequently applied to 20–355 pigs at farm F1, it could not be detected in the corresponding manure samples.

Broader application patterns were observed at six pig breeding farms (B1 to B6), where up to 11 antibiotics were administered. On those farms, sulfonamides in combination with trimethoprim were most frequently applied, nine times to 180 pigs, while tetracycline was applied only once to 400 pigs at B5. Alternatively, doxycycline was delivered to four pig breeding farms, while chlortetracycline and oxytetracycline were less frequently administered. In manure from pig breeding farms, doxycycline was most frequently detected at concentrations from 9.4 to 101 mg kg^{-1} DW of manure. The detection of doxycycline up to 19.0 mg kg^{-1} DW in both cellar and silo manures of farm B2, however, did not reflect doxycycline usage. The highest concentration of oxytetracycline with 211 mg kg^{-1} DW manure was detected at farm B3. Moreover, the application of sulfadimidine to a larger animal group, up to 180 pigs, resulted in sulfadimidine residues of 23.0 mg kg^{-1} DW manure of B6. Here, the corresponding acetyl-sulfadimidine was not detected above MQL. Enrofloxacin was applied at four pig breeding farms but was detected only in manure from farm B5 which reported the highest application frequency (4 times/4–50 pigs) at 1.3 mg kg^{-1} DW. In contrast, 1.4 mg tiamulin kg^{-1} DW was found in the manure from farm B5 after the single application to 50 pigs.

On pig breeding farms with farm-owned BGPs (BGP1 to BGP5), frequent applications of doxycycline (21 times) were recorded to treat up to 400 pigs at BGP4. On those farms, residues of doxycycline up to 10.1 mg kg^{-1} DW digestate were detected. More doxycycline was applied to larger animal groups at BGP2, where up to 2000 pigs were treated and 7.4 mg doxycycline kg^{-1} DW of digestate could be detected. Particularly for enrofloxacin, up to seven applications were administered at BGP2 and BGP4; however, there was only one positive digestate sample (BGP4 at 0.2 mg kg^{-1} DW digestate). In addition, digestate samples from BGP6 to BGP8, fed with input materials from different farms, were also analyzed. At BGP6, tetracycline and doxycycline were detected in the digestate at 6.4 and 2.2 mg kg^{-1} DW, respectively. At BGP7, 0.4 mg tetracycline kg^{-1} DW digestate was found. The digestate from BGP8 was free of residues of all 19 antibiotics analyzed.

Antibiotic RGs and disinfectant RGs in manures derived from different pig producing systems and digestates of BGPs

A very similar occurrence of RGs was detected via PCR/Southern blot hybridization for manures originating from different pig production systems and for digestates of BGPs fed with manures as co-substrate (Tables 3 and 4). Except for the sulfonamide resistance gene *sul3*, all antibiotic RGs investigated in this study were detected in all samples analyzed. Thus, genes conferring all three known mechanisms conferring resistance toward tetracycline (efflux, ribosomal protection, enzymatic modification) were detected in manures and in BGP digestates. In most cases, signal intensities

Table 2 Primer systems used in this study for detection of transferable plasmids, integrase genes specific for class 1 and class 2 integrons, disinfectant RGs and antibiotic RGs, and reference plasmids used for generation of gene-specific probes

Target gene	Primers	Sequence [5′–3′]	Amplicon size [bp]	Annealing temp. (°C)	Reference (primer)	Reference plasmids
IncN (*rep*)	IncN-rep-1	agttcaccacctactcgctccg	165	55	Götz et al. (1996)	
	IncN-rep-2	caagttcttctgttgggattccg				
IncP-1 (*trfA*)	trfA 733 f (α, β, ε)	ttcacsttctacgagmtktgccaggac	281	60	Bahl et al. (2009); Wolters et al. (2015)	IncP-1α: RP4
		gwcagcttgcggtacttctccc				IncP-1β: R751
	trfA 1013 r	ttcacttttacgagctttgcagcgac				IncP-1γ: pQKH54
	trfA g-F (γ)	gtcagctcgcggtacttctccca				IncP-1γ-like: pKS208
	trfA g-R	ttcaccttctacgaactgtgtaat				IncP-1δ: pEST4011
	trfAg-208f (γ-l.)	gtcaaggcccgatacttctccca				IncP-1ε: pKJK5
		ttcacgttctacgagctttgcacagac				IncP-1ζ: pMCBF1
	trfAg-208r	gacagctcgcggtacttttccca				
	trfA d-F (δ)	ttcactttctacgaaatctgcaaagac				
	trfA d-r	gatagcttccgatacttttccca				
	trfA z-f (ζ)					
	trfA z-r					
IncQ (*oriV*)	IncQ-oriV-1	ctcccgtactaactgtcacg	436	57	Götz et al. (1996)	RSF1010
	IncQ-oriV-2	atcgaccgagacaggccctgc				
IncW (*oriV*)	IncW-oriV-1	gacccggaaaaccaaaaata	1140	57	Götz et al. (1996)	R388
	IncW-oriV-2	gtgagggtgagggtgctatc				
LowGC (*rep*)	V216repF	aattgaccgatttagttgtgacctgc	912	56	Heuer et al. (2009)	pHHV216
	V216repR	tgatttgytttggagatac				
intI1	intI1F	cctcccgcacgatgatc	280	55	Kraft et al. (1986)	pKJK5
	intI1R	tccacgcatcgtcaggc				
intI2	intI2F	ttattgctgggattaggc	233	52	Goldstein et al. (2001)	pGT527
	intI2R	acggctaccctctgttatc				
qacE	qacE (F1)	gccctacacaaattgggaga	359	67	Jechalke et al. (2013)	R751
	qacERmod	ttagtgggcacttgctttggaaag				
qacEΔ1	qacEΔ1 F	atcgcaatagttggcgaagt	226	50	Sandvang et al. (1997)	pKJK5
	qacEΔ1 B	caagcttttgcccatgaagc				
tet(A)	TetA-L	ggcggtcttcttcatcatgc	502	64	Lanz et al. (2003)	pKJK5
	TetA-R	cggcaggcagagcaagtaga				
tet(M)		gtggacaaaggtacaacgag	406	55	Ng et al. (2001)	pAT101
		cggtaaagttcgtcacacac				
tet(X)	tetX-1	ttagccttaccaatgggtgt	242	56	Bartha et al. (2011)	pHHV1-107
	tetX-2	caaatctgctgtttcactcg				
sul1	Sul 1-F	cggcgtgggctacctgaacg	433	68	Kerrn et al. (2002)	R388
	Sul 1-B	gccgatcgcgtgaagttccg				
sul2	Sul 2-F	gcgctcaaggcagatggcatt	293	68	Kerrn et al. (2002)	RSF1010
	Sul 2-B	gcgtttgataccggcacccgt				
sul3	sul3-F	cagataaggcaattgagcatgctctgc	569	55	Wu et al. (2010)	pUVP4401
	sul3-R	agaatgatttccgtgacactgcaatcatt				

were similar for manures from pig breeding and fattening farms. However, more intense signals for *tet*(M) were found in manures from pig fattening farms than in manures from breeding farms. While a high abundance of the sulfonamide resistance genes *sul1* and *sul2* was indicated by strong hybridization signal intensities for all samples, *sul3* was exclusively detected (with considerably less intense signals) in samples from BGP3.

The complete disinfectant RG *qacE* was not detected in any of the manure samples derived from pig fattening or in digestates, but it was found in one manure sample from the pig breeding B2 (Table 3). In contrast, the functional fragment of this gene, *qacEΔ1*, was present in all manure samples and digestates, and strong hybridization signals of Southern blotted amplicons indicated a high abundance of *qacEΔ1*.

Detection of sequences specific for transferable plasmids and integron integrases in manures derived from different pig production systems and BGP digestates

Manure

A higher diversity of transferable plasmids was revealed in TC-DNA of manures from pig breeding facilities than from pig fattening farms (Table 3). All manure samples contained both IncP-1 and IncQ plasmids, and hybridization signal intensities were comparable except for considerably weaker hybridization signals obtained from manure samples derived from pig fattening farms F5 and F7. Furthermore, plasmids of the incompatibility group IncW were detected in TC-DNA from eight of nine

Table 3 Detection of antibiotic RGs (*sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M), *tet*(X)), disinfectant RGs (*qacE*, *qacEΔ1*), transferable plasmids of different incompatibility groups and of class 1 and class 2 integron integrase genes (*intI1 and intI2*) in manure samples (n = 1) from pig fattening (F1–F8) and pig breeding farms (B1–B6) via PCR and Southern blot hybridization

Farm	*sul1*	*sul2*	*sul3*	*tet*(A)	*tet*(M)	*tet*(X)	*qacE*	*qacEΔ1*	IncN	IncP-1	IncQ	IncW	LowGC	*intI1*	*intI2*
F1	+++	+++	−	+++	+++	++	−	+++	−	++	+++	−	−	+++	+++
F2	+++	+++	−	+++	+++	++	−	+++	−	++	+++	+++	−	+++	+++
F3	+++	+++	−	+++	+++	++	−	+++	−	++	+++	++	−	+++	+++
F4	+++	+++	−	+++	+++	++	−	+++	−	++	+++	−	−	+++	+++
F5	+++	+++	−	++	+	++	−	+++	−	+	+	−	−	+++	+++
F6	+++	+++	−	+++	++	++	−	+++	−	++	+++	−	−	+++	+++
F7	+++	+++	−	++	+	++	−	+++	−	++	+	−	−	+++	+++
F8	+++	+++	−	+++	++	++	−	+++	−	++	+++	+++	−	+++	+++
B1	+++	+++	−	+++	++	++	−	+++	+	++	+++	+++	+	+++	+++
B2															
Cellar	+++	+++	−	+++	++	++	(+)	+++	−	++	+++	+++	−	+++	+++
Silo	+++	+++	−	+++	+	++	−	+++	+	++	+++	+++	−	+++	+++
B3															
Piglets	+++	+++	−	+++	++	++	−	+++	−	++	+++	+++	−	+++	+++
Fatten.	+++	+++	−	+++	++	++	−	+++	+	++	+++	++	−	+++	+++
B4															
Silo a	+++	+++	−	+++	++	++	−	++	+	++	+++	−	−	+++	+++
Silo b	+++	+++	−	+++	++	++	−	+++	+	++	+++	++	−	+++	+++
B5	+++	+++	−	+++	++	++	−	+++	++	++	+++	++	−	+++	+++
B6	+++	+++	−	+++	+++	++	−	+++	+	++	+++	+++	−	+++	+++

− not detected, *(+)* weak positive signal after long exposure time, + positive hybridization signal, ++ strong hybridization signal, +++ very strong hybridization signal. As signal intensities depend on exposure time of X-ray films and on probes used, listed intensities are only suitable for comparison of different samples but not for comparison of different PCRs

manures from pig breeding farms and three of eight manures from pig fattening farms. IncN plasmids were detected as well but exclusively in manures derived from pig breeding farms (Tables 3 and 4), whereas LowGC plasmids were detected only in one manure sample (B1) originating from a pig breeding facility. Class 1 and 2 integron integrase genes were detected in TC-DNA from all manure samples investigated with very strong hybridization signals indicating a high abundance.

Table 4 Detection of antibiotic RGs (*sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M), *tet*(X)), disinfectant RGs (*qacE*, *qacEΔ1*), transferable plasmids of different incompatibility groups and of class 1 (*intI1*) and class 2 integron integrase genes (*intI2*) in digestates (n = 4) of biogas plants (BGPs) via PCR and Southern blot hybridization

Farm	Source	*sul1*	*sul2*	*sul3*	*tet*(A)	*tet*(M)	*tet*(X)	*qacE*	*qacEΔ1*	IncN	IncP-1	IncQ	IncW	LowGC	*intI1*	*intI2*
BGP1	Digestate	+++	+++	−	+++	++	+++	−	+++	−	+	+++	+++	−	+++	+++
BGP2	Digestate	+++	+++	−	+++	++	+++	−	+++	−	+	+++	+++	++	+++	+++
BGP3	Manure	+++	+++	+	+++	++	+++	−	+++	+	++	+++	+++	++	+++	+++
	Fermenter	+++	+++	(+)	+++	++	+++	−	+++	−	+	+++	+++	++	+++	+++
	Digestate	+++	+++	(+)	+++	++	+++	−	+++	(+)	+	+++	+++	−	+++	+++
BGP4	Digestate	+++	+++	(+)	+++	++	+++	−	+++	−	+	+++	+++	−	+++	+++
BGP5	Digestate	+++	+++	−	+++	++	+++	−	+++	−	(+)	+++	+++	(+)	+++	+++
BGP6	Digestate	+++	+++	−	+++	++	+++	−	+++	−	(+)	+++	+++	+	+++	+++
BGP7	Digestate	+++	+++	−	+++	++	+++	−	+++	−	−	+++	+++	−	+++	+++
BGP8	Digestate	+++	+++	−	++	++	+++	−	++	−	(+)	+++	+++	−	+++	+++

− not detected, *(+)* weak positive signal after long exposure time, + positive hybridization signal, ++ strong hybridization signal, +++ very strong hybridization signal. As signal intensities depend on exposure time of X-ray films and on probes used, listed intensities are only suitable for comparison of different samples but not for comparison of different PCRs

BGP digestates

PCR-Southern blot hybridization indicated that IncW and IncQ plasmids were abundant in all digestate samples taken from BGPs. The *trfA*-based detection system for IncP-1 plasmids revealed that plasmids belonging to the IncP-1 group were detectable in TC-DNA from digestates of all BGPs except BGP7. A more diverse range of plasmids was observed in biogas digestate samples compared to farm manures due to the frequent detection of LowGC plasmids BGP digestates (Table 4). Plasmids belonging to the incompatibility group IncN were only detected in one BGP digestate with very weak signal intensity. Furthermore, integrase genes of class 1 and 2 integrons were also detected in TC-DNA from BGPs samples. The hybridization signal intensity indicated a similar abundance in the TC-DNA from different BGPs.

Discussion

The current study aimed to link data on antibiotic usage from a survey conducted at 19 pig farms without/with farm-owned BGPs in Lower Saxony, Germany to the occurrence of ARs, RGs, and plasmid- and integron-specific sequences in liquid manures and digestates of BGPs feeding manure as co-substrate. The survey provides unique data on the extent of antibiotic usage in conventional pig farms and reveals that a more diverse range of antibiotics is applied in pig breeding farms and in farms with BGPs.

The AR concentrations measured were higher in manures compared to digestates. The results often showed a clear dependency of the AR detected on antibiotic application data. The frequent detection of different tetracycline residues at rather high concentrations may be explained by the higher frequency of tetracycline application at the different farms and the concomitantly high excretion rates of those production animals (up to 90 %) (Table 1). However, the detected residues did not always reflect the application rates. For example, low concentrations of tetracyclines were detected in manure samples from farm B2 where no tetracyclines were used in the time period surveyed, and thus might indicate background contaminations with tetracyclines from previous applications. Although sulfonamide antibiotics were applied at 7 out of 19 farms, they were less often detected in the respective manure samples. In addition to the rather lower recovery rates of 46 ± 10 % in manures indicating a rapid formation of non-extractable residues (Kreuzig and Höltge 2005), sampling time and the application frequency might have contributed to the detection of sulfonamide residues in manures. Furthermore, sulfadiazine may be transformed into the phase II metabolite acetyl-sulfadiazine in the digestive systems of animals (Lamshöft et al. 2007) and then may be excreted with the remaining parent compound. High recovery rates of 90 ± 10 % for acetyl-metabolites in manures may also be the reason why acetyl-sulfadiazine was found at higher concentration, which is 11.5 mg kg^{-1} DW of manure. Although trimethoprim was applied as a synergist of sulfonamides, it was not detected in any sample, which might be explained by rapid biodegradation as already reported by Haller et al. (2002), Mohring et al. (2009), and Spielmeyer et al. (2015). Similar to the sulfonamides, enrofloxacin residues were detected only once. Single animal treatments and high dilution factors may be one reason contributing to the rare detection of enrofloxacin. Another factor might be the strong sorption of enrofloxacin to the biosolids of manure (Tolls 2001; Uslu et al. 2008). Tiamulin, however, was reported to remain unchanged during a 180-day degradation experiment (Schlüsener et al. 2006). This might explain the finding that tiamulin was detected in manure of B5, even though tiamulin was applied only once to a group of 50 pigs. Moreover, due to rapid degradation and strong sorption to biosolids (Kolz et al. 2005), tylosin residues could not be detected in any samples even though it was frequently applied.

In comparison to manure, the concentrations of ARs detected in digestates from the mesophilic BGPs under study were clearly lower, ranging from 0.2 to 10.1 mg kg^{-1} DW (Table 1). The current results supported the survey studies conducted in the BGPs as well as laboratory testing which showed that ARs are only partially removed during anaerobic digestion (Mohring et al. 2009; Ratsak et al. 2013; Spielmeyer et al. 2014, 2015). Corresponding to the frequent applications of enrofloxacin in the farm with the BGP4, enrofloxacin was also detected in one digestate sample from BGP4. Tetracycline residues were also detected at low mg kg^{-1} DW concentrations in digestates. The highest concentrations of doxycycline were observed in BGP4, where 21 applications of doxycycline to bigger groups of pigs were recorded. At BGP7 fed with cattle manures, only a trace of tetracycline antibiotic was detected. This is consistent to the regular practice at the dairy farm where limited amounts of antibiotics are applied (Spielmeyer et al. 2015). Various elimination rates of tetracyclines in the BGP feeding manures as co-substrate were reported ranging from 14 to 89 % (Arikan et al. 2006; Arikan 2008; Spielmeyer et al. 2015). Besides biodegradation, strong sorption to biosolids and dilution factors from other co-substrates of the BGP, such as maize silage up to 65 %, might have contributed to the lower concentrations of ARs detected in digestates.

The detection of sequences specific for antibiotic RGs, *qac* genes, integron integrase genes, and for a range of transferable plasmids confirmed previous data by Binh et al. (2008) for farm-scale manures taken from farms in different regions in Germany, supporting their conclusion that manure from conventional farms is a reservoir of transferable antibiotic RGs. While Binh et al. (2008) determined the occurrence of *sul1*, *sul2*, and *TEM-1* genes, in the present study, we focused on

the detection of genes conferring resistances toward tetracycline antibiotics. We selected three *tet* genes (*tet*(A), *tet*(M), and *tet*(X)) that were previously reported to occur in manure (Lanz et al. 2003; Thames et al. 2012; Chee-Sanford et al. 2001; Kyselkova et al. 2013; Zhang et al. 2013) and that represent the three major resistance mechanisms toward tetracyclines (efflux, ribosomal protection protein, and enzymatic degradation, respectively). Although the Southern blot hybridization provided only semi-quantitative results, hybridization signal intensities indicated that all *tet* genes with a few exceptions were highly abundant in manure bacteria. The high abundances of *tet* genes and the detection of tetracycline residues in the same manures suggest that selective pressure increased the abundance of *tet* genes carrying bacteria. Likely, vertical and horizontal gene transfer processes might have played an important role in the dissemination of tetracycline RGs. In addition, these genes might have been subject to co-selection, as for instance, *tet*(A) and *tet*(X) were previously reported as linked with various RGs on transferable plasmids (Heuer et al. 2009; Wolters et al. 2015), while *tet*(M) was shown to co-occur with a diversity of other RGs in *Staphylococcus aureus* ST398 isolates from livestock related sources (Argudín et al. 2011). The sulfonamide RGs *sul1* and *sul2* seemed to be even more abundant than the *tet* genes, although sulfonamide residues were only detected in manure from F6 and B6. Explanations for this strikingly high abundance of *sul* genes and the absence of detected sulfonamide residues could be either their co-selection or their analytical problems due to the tight binding of sulfonamides to organic matter (Heuer et al. 2008). Also, in all digestates investigated in the present study, *sul1*, *sul2*, and *tet* genes were highly abundant; although in none of the samples, sulfonamide residues were detected, while in contrast, tetracycline residues were found in digestates from all BGPs except BGP8, but at approximately tenfold lower concentrations compared to manures. This high abundance of *sul1*, *sul2*, and *tet*(M) in the digestate samples was recently confirmed by quantitative real-time PCR with relative abundances in the range of Log −2.16 to Log −3.03, Log −1.80 to Log −2.75 and Log −1.94 to Log −2.52, respectively (Wolters et al. 2016). In the present study, we showed that *qacEΔ1* genes (conferring resistance toward quaternary ammonium compounds that are frequently used as disinfectants in animal husbandries) were detected in high abundance in all manure samples. Recently, Heuer et al. (2012) and Jechalke et al. (2014) reported on IncP-1ε plasmids that were exogenously isolated from manure and manure treated soils. The molecular analysis of a collection of 50 IncP-1ε plasmids revealed the presence of *tet*(A) and class 1 integrons with the presence of *qacEΔ1* and *sul1* genes at the 3′ end of the gene cassette region. Thus, a co-selection not only of *sul* genes but also of *qacEΔ1* can be assumed. In addition, the exogenous isolation of IncP-1ε plasmids from the digestates investigated in the presented study was recently reported by Wolters et al. (2015). All IncP-1ε plasmids captured carried *tet*(A) and class 1 integrons with *sul1* and *qacEΔ1* at the 3′end of the gene cassette region, providing further support for the assumption of co-selection. The high proportion of IncP-1ε among the collection of exogenously captured plasmids that were obtained based on the sulfonamide or tetracycline resistance conferred was rather surprising as they were also obtained from BGPs with no IncP-1 plasmids detected in the present study (BGP7; Table 4). This finding also shows that plasmids with low abundance can be significant in the dissemination of RGs. The ability of IncP-1ε plasmids to efficiently transfer in soil and their BHR (Heuer et al. 2012; Musovic et al. 2014) makes them likely important vectors contributing to the dissemination of RGs in the agroecosystem. In digestates, the abundance of *intI1*, *sul1*, and *qacEΔ1* was high, while IncP-1 plasmids seemed much less abundant (Table 4). These findings were recently confirmed by qPCR (Wolters et al. 2016). Interestingly, the relative abundances of IncP-1ε plasmid-specific *trfA* gene was low (Log −4.62 to Log −6.67 that is close to or below the detection limit) compared to the relative abundances of *intI1* (Log −3.12 to Log −3.50), *sul1* (Log −2.16 to Log −3.03), or *qacEΔ1* (Log −2.26 to Log −2.88). Integrons can, however, also be localized chromosomally or on plasmids belonging to other incompatibility groups such as IncW plasmids (Revilla et al. 2008) that were detected in the digestates from all BGPs but not in all manures. In contrast to the study by Binh et al. (2008), IncN plasmids seemed to be less abundant in the manures from pig fattening farms and the digestates. Another plasmid type that seemed to be highly abundant in manure and in digestates were IncQ plasmids. Like the BHR plasmids, IncP-1, IncN, and IncW, plasmids belonging to the IncQ group have a BHR but they can only be transferred by mobilizing plasmids. IncQ plasmids typically carry *sul2* genes. Smalla et al. (2000) showed not only the presence of mobilizing plasmids in pig manure but also the isolated IncQ plasmids conferring resistances toward tetracycline, streptomycin, sulfonamide, gentamycin, and streptothricin antibiotics. In contrast to recent reports on the frequent occurrence of LowGC plasmids (Binh et al. 2008; Heuer et al. 2009; Kopmann et al. 2012), these plasmids seemed to be less abundant in the present study and were only detected in a few manure and digestate samples, e.g., from BGP2.

The broad-host range plasmid groups detected in our study (IncP-1, IncN, IncW, and IncQ) in manures or digestates were previously also reported from clinical isolates. Indeed, many of these groups were originally discovered in clinical isolates (Datta et al. 1971; Loftie-Eaton and Rawlings 2012). Furthermore, they were frequently detected among Gram-negative bacteria isolated from wounds, urine, or sputum of hospital patients in a recent study by Zhang et al. (2014).

Comparing the antibiotic application patterns, detected ARs in the samples and corresponding detected RGs and

MGEs, it was not only found that pig breeding facilities had the broadest overall antibiotic application patterns (Table 1) and that the spectrum of ARs detected in corresponding manures was larger than in pig fattening farms (Table 1) but also that the diversity of MGEs detected was higher (Table 3). As antibiotic treatment of livestock is inevitably impacting the composition of bacterial communities resident in the animals intestine and hence also in respective excrements (Heuer et al. 2011; Reichel et al. 2013), antibiotic application patterns may also influence the occurrence of RGs and MGEs in manure not necessarily accompanied by subsequent detection of ARs.

Although the detection of RG- and MGE-specific sequences in TC-DNA by means of Southern blot hybridization provides only semi-quantitative results, it was observed that the signals detected for *tet*(M) were often stronger in manures derived from pig fattening compared to pig breeding (Table 3). At the same time, the highest concentrations of tetracyclines were detected in pig fattening manures. Among the more than 50 known tetracycline RGs, of which only a small subset was monitored within the present study, *tet*(M) appears to have the broadest host range (http://faculty.washington.edu/marilynr/tetweb2.pdf&/tetweb3.pdf). In contrast to the studies of Kopmann et al. (2012), Heuer et al. (2012), and Jechalke et al. (2013), who reported on direct correlations of antibiotic treatment and antibiotic RGs (*sul1* and *sul2*) and MGEs (IncP-1 and LowGC plasmids) in manures and manure treated soil, no differences related to concentrations of the antibiotics detected were found for RGs monitored in the present study, except for *tet*(M) as already mentioned.

The present study clearly showed that antibiotics such as tetracyclines that are frequently applied in conventional pig husbandry systems were detectable in liquid pig manures and digestates of BGPs, where they provide a selective advantage for bacterial populations carrying RGs and likely foster horizontal gene transfer. The fate and effects of veterinary antibiotics entering soils might strongly depend on the physicochemical characteristics of the antibiotics. Therefore, when being spread onto fields, manures and digestates might pose a potential risk of RG transfer via plasmids to bacteria associated with soil and plant environments.

The detection of antibiotics, RGs, and MGEs in digestates showed that the mesophilic fermentation process in BGPs is not an effective mitigation strategy. In order to reduce contamination of farm fertilizers with ARs and antibiotic resistant bacteria, the frequency of antibiotic usage needs to be reduced by optimizing the conditions of conventional pig husbandry systems. Furthermore, additional factors co-selecting antibiotic resistance such as disinfectants or metal compounds used as feed additives need to be better understood. It will be important to gain better understanding of the uptake of ARs by plants and to determine whether root exudates in the presence of ARs facilitates the horizontal spread of multiple antibiotic resistances. Raw leafy greens are not only transiently but also endophytically colonized by a subset of the soil microbiome, and thus, their mobile resistome might provide a link to the human gut microbiome.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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