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Research Article

RESEARCH ARTICLE

Bulk soil and maize rhizosphere resistance genes, mobile genetic elements and microbial communities are differently impacted by organic and inorganic fertilization

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One sentence summary: Effects of organic and inorganic fertilization on antibiotic resistance genes, mobile genetic elements and microbial communities in maize planted soil were compared in a field plot study.

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ABSTRACT

Organic soil fertilizers, such as livestock manure and biogas digestate, frequently contain bacteria carrying resistance genes (RGs) to antimicrobial substances and mobile genetic elements (MGEs). The effects of different fertilizers (inorganic, manure, digestate) on RG and MGE abundance and microbial community composition were investigated in a field plot experiment. The relative abundances of RGs [*sul1*, *sul2*, *tet*(A), *tet*(M), *tet*(Q), *tet*(W), *qacE*Δ*1*/*qacE*] and MGEs [*intI1*, *intI2*, IncP-1, IncP-1ε and LowGC plasmids] in total community (TC)-DNA from organic fertilizers, bulk soil and maize rhizosphere were quantified by qPCR before/after fertilization and prior to maize harvest. Microbial communities were analyzed *via* Illumina sequencing of 16S rRNA gene fragments amplified from TC-DNA. Compared to inorganic fertilization, manure treatments increased relative abundances of all RGs analyzed, integrons and few genera affiliated to Bacteroidetes and Firmicutes in bulk soil, while digestate increased *sul2*, *tet*(W) and *intI2*. At harvest, treatment effects vanished in bulk soil. However, organic fertilizer effects were still detectable in the rhizosphere for RGs [manure: *intI1*, *sul1*; digestate: *tet*(W)] and *Clostridium* related sequences (digestates) with increased relative abundance. Our data indicated transient organic fertilizer effects on RGs, MGEs and microbial community composition in bulk soil with long-term history of digestate or manure application.

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INTRODUCTION

The increasing occurrence of antibiotic resistant bacterial pathogens in clinical settings raises questions and concerns about potential contributions of environmental resistance gene (RG) reservoirs and their association with mobile genetic elements (MGEs). Organic soil fertilizers, such as livestock manure and biogas plant (BGP) digestates typically harbor bacteria that carry various RGs and MGEs such as transferable plasmids and integrons (Binh *et al.* 2009; Marti *et al.* 2013; Zhu *et al.* 2013; Wolters *et al.* 2015, 2016a,b). Spread of manure increases the abundance of RGs and MGEs in soil (Sengelov *et al.* 2003; Heuer and Smalla 2007; Chee-Sanford *et al.* 2009; Byrne-Bailey *et al.* 2011; Heuer *et al.* 2011; Jechalke *et al.* 2014) and as RGs in pig manure are frequently localized on broad host range plasmids (e.g. IncP-1, IncW, IncQ) (Götz *et al.* 1996; Binh *et al.* 2008; Heuer *et al.* 2009; Heuer *et al.* 2012), these might be exchanged with bacteria associated with soil ecosystems (Hammesfahr *et al.* 2008; Heuer *et al.* 2008). For instance, the plasmids pIE723 (IncQ) and pGT527 (IncP-1α) were transferred to *Pseudomonas putida* recipients when introduced with manure into soil (Götz and Smalla 1997), and pKJK10 (IncP-1ε) spread to diverse bacteria associated with barley rhizosphere (Musovic *et al.* 2006). Integrons might further enhance resistance spread and co-selection among bacteria, as they are often localized on plasmids and accumulate diverse RGs (conferring protection towards e.g. various antibiotics or disinfectants based on quaternary ammonium compounds [QACs]) in gene cassette arrays (for review, see Gillings 2014; Mulder *et al.* 2017).

Manure application was reported to transiently change the composition of soil microbial communities (Chee-Sanford *et al.* 2009; Ding *et al.* 2014; Jechalke *et al.* 2014). Moreover, manure is assumed to foster horizontal gene transfer (HGT) in soil (Götz and Smalla 1997; Rahube and Yost 2012; Jechalke *et al.* 2013), likely due to the parallel introduction of nutrients stimulating metabolic activities of bacteria, genetic exchange and proliferation of emerging transconjugants (Van Elsas, Turner and Bailey 2003; Heuer and Smalla 2012). On the other hand, Udikovic-Kolic *et al.* (2014) and Hu *et al.* (2016) recently reported an increased abundance of ß-lactamase genes in soil after manure application triggered by indigenous soil bacteria blooms carrying RGs. Thus, the increased abundances of RGs and MGEs in soils receiving organic fertilizers might result from bacteria introduced with these fertilizer or soil bacteria proliferating in response to the nutrients. Consistently, Forsberg *et al.* (2014) reported that the soil resistome (pool of all RGs in an environment) was predominantly shaped by resident bacteria's phylogeny, whereas no impact of HGT was indicated. In contrast, recent work has shown the impressive permissiveness capacity of soil bacterial communities toward receiving and disseminating conjugative plasmids to a wide phylogenetic range of recipients (Heuer *et al.* 2012; Shintani *et al.* 2014; Klümper *et al.* 2015, 2016). Thus, the role of HGT in dissemination of RGs introduced into agricultural soils *via* organic fertilizers requires further investigation.

The increasing use of livestock manure as co-substrate in BGPs, and the concomitant growing importance of BGP digestates as organic fertilizer raises concerns about RG/MGE presence and co-occurrence, as well as their potential impacts on soil bacteria. Mesophilic anaerobic fermentation of organic substrates, as usually performed in full-scale BGPs, was shown to reduce the abundance of diverse RGs and class 1 integrons, along with shifts in bacterial community composition at different processing steps (Ghosh *et al.* 2009; Diehl and LaPara 2010; Ma *et al.* 2011; Wolters *et al.* 2016a). Recently, effects of manure, digestate and inorganic fertilizer on RGs and integrons in grassland soils were compared and revealed individual sets of genes affected differently in abundance by treatments (Nõlvak *et al.* 2016). However, knowledge on digestate application effects on RGs and MGEs abundance, as well as associated impacts on bacterial community structures in agricultural soil systems, still remains scarce (Francioli *et al.* 2016).

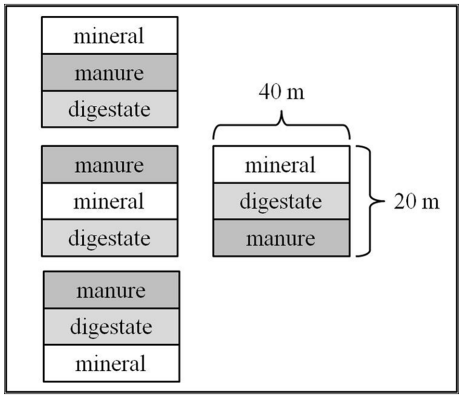

Figure 1. Field plot design. Maize was planted on all plots in May 2014 and harvested in October 2014.

In the present study, an integrated comparison between the individual effects of organic fertilization with manure or digestate *vs.* inorganic fertilizer on RGs, MGEs and bacteria was performed in an agro-ecosystem field plot study over time (bulk soil and rhizosphere of maize plants). The relative abundances (normalized 16S rRNA gene copy numbers [nr.]) of tetracycline RGs [*tet*(A), *tet*(M), *tet*(Q) and *tet*(W)], sulfonamide RGs (*sul1* and *sul2*), integron specific sequences (*intI1* and *intI2*), disinfectant RG *qacEΔ1/qacE* and IncP-1(*korB*), IncP-1ε (*trfA*) and LowGC (*traN*) plasmids were quantified by real-time PCR in total community (TC-) DNA from organic fertilizers, bulk soil samples (taken before and after fertilization and at harvest) and from rhizosphere of maize plants (at harvest). Illumina sequencing of 16S rRNA gene fragments amplified from TC-DNA of respective samples was applied to compare microbial community compositions and to identify taxa responding to specific fertilization.

MATERIALS AND METHODS

Field plot design

An agricultural field (2 ha) belonging to a pig farm located in Lower Saxony was arranged as shown in Fig. 1 to allow a direct comparison of treatment with different fertilizers (inorganic fertilizer, manure and digestate). In preceding years, this agricultural field received digestate as fertilizer. The farm (keeping 265 sows and 2100 fattening pigs) is equipped with a manure silo (as no further treatment takes place the manure is considered raw manure) and a BGP (250 kW, operated under mesophilic conditions (40°C), fed with combination of pig manure (40%) and maize silage (60%), substance residence time: 90–100 days). The fertilizers were applied *via* under-root fertilization with the following amounts per ha on respective treatment plots: 40 m^3

manure, 20 m^3 digestate and 230 kg of inorganic fertilizer (20 N, 20 P and 30 K). The grown maize cultivar was 'Dekalb'.

Sampling

Samples were taken as described below at three different time points of the experiment: prior to the application of fertilizers (t_0; 28 March 2014), shortly after fertilizer spread (t_1; 23 April 2014, six days after fertilization) and at harvest (t_2; 29 September 2014). Per plot, each 20 bulk soil samples were taken to a depth of 0–30 cm using a probe sampler (in the case of t_2 sampling between maize plants) and combined subsequently to one representative composite sample. At the time of harvest (t_2), additionally three maize plants were taken randomly from each field plots by excavation, and root systems were separated from the above ground part of the plants by cutting. In addition, prior to application (t_1), organic fertilizers were sampled (each four replicates) using the procedure described in Wolters *et al.* (2016a). A matrix characterization was performed on the soil samples taken at harvest (t_2) and organic fertilizers as described in File S1 (Supporting Information).

Extraction of TC-DNA

Immediately upon receipt, all samples were prepared for TC-DNA extraction using the spin kit for soil (MP Biomedicals, Heidelberg, Germany) according to the manufacturers' instructions and kept at −20°C until use. Bulk soil replicates were left to dry for 24 h at room temperature, and subsequently a minimum of 500 g per replicate was sieved at 2 mm and aliquots were kept at −20°C in 14 mL Falcon tubes. Of each of these homogenized soils, 0.5 g was used for TC-DNA extraction. 14 mL of freshly homogenized organic fertilizer samples (manure and digestate) were centrifuged in 15 mL Falcon tubes (Sarstedt, Nümbrecht, Germany) at 3100*g* for 10 min. The supernatants were discarded, pellets were stored as described above and TC-DNA was extracted from 0.1 g of the sample pellets (homogenized manually with sterile spatulas). Rhizosphere samples were obtained from the root systems of three maize plants sampled per field plot. Loosely adhering soil was thoroughly shaken off. Roots were cut with sterile scissors into small parts (about 10 mm), mixed thoroughly and 5 g were placed in sterile stomacher bags. Subsequently, roots were washed three times with 15 mL of sterile saline solution (0.85% NaCl) by Stomacher treatment, and supernatants were collected in sterile 50 mL Falcon tubes and centrifuged at 3780*g* and 4°C for 10 min. For TC-DNA extraction 0.5 g of obtained pellets were used. All TC-DNA extracts were further processed using the Gene Clean Spin Kit (MP Biomedicals, Heidelberg, Germany) and aliquots of cleaned DNA were diluted 1:5 with 1x TE buffer (pH 8,0) and used for PCRs and quantitative real-time PCRs.

Quantification of ribosomal 16S rRNA genes, RGs, integrons and conjugative plasmids

Abundances of 16S rRNA genes, RGs [*sul1*, *sul2*, *tet*(A), *tet*(M), *tet*(Q), *tet*(W) and *qacE*Δ*1*/*qacE*], integron-specific sequences (*intI1*, *intI2*), and IncP-1, IncP-1ε and LowGC plasmids in TC-DNA of samples derived from field plots at different sampling times were quantified using quantitative real-time PCR 5′-nuclease assays in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) as described in File S2 (Supporting Information). Based on the assumption of potential unspecific amplification appearing during the last cycles of quantitative real-time PCR assays, a detection cut-off level was set for signals detected after 35 amplification cycles (corresponding to an estimated gene copy number of 10^4–10^5/g sample for individual targets) in order to evaluate data accordingly. Obtained qPCR data were analyzed and compared by Tukey´s HSD test (p-value < 0.05). Values that were calculated based on two or more signals below the determined quantification limit were indicated as below quantification limit (b.q.) and only included into Tukey´s HSD test if a quantification of respective targets was possible in other treatments or time points to be compared. In case no signals were detected in two or more replicates, the respective set was labeled as not detected (n.d.) and excluded from statistics.

16S rRNA gene sequencing and analysis

The complete procedure describing the preparation of the 16S rRNA gene amplicon and the following sequencing, quality trimming and annotation steps are provided in File S3 (Supporting Information). Briefly, the ≈ 460bp fragment covering the hyper variable regions V3 and V4 of the small ribosomal subunit 16S rRNA gene was amplified, tagged and sequenced using 2 × 250 bp paired-end high-throughput sequencing on an Illumina® MiSeq® platform (Illumina, San Diego, CA, USA) before proceeding with the QIIME annotation workflow (Caporaso *et al.* 2010), as described in previous studies (Nunes *et al.* 2016; Jacquiod *et al.* 2017a). The statistical analysis was done using the PAST (Hammer, Harper and Ryan 2001) and Rgui softwares (R Development Core Team, 2013), as described previously (Jacquiod *et al.* 2016). The complete α- and β-diversity reports are provided in File S3 (Supporting Information). Major phylogenetic changes detected at phylum/class levels and responding genera/OTUs were extracted by the likelihood ratio test after negative binomial generalized linear modeling (nbGLM) of the raw counts with the false discovery rate (FDR) adjusted p-values (p-value <0.05, R package *edgeR*, Robinson, McCarthy and Smyth 2010). This analytical method was benchmarked and proved to be reliable for responding taxa prediction by minimizing risk of errors (Thorsen *et al.* 2016). Generalized heatmaps were generated using center-scaled counts to represent taxa showing a significant abundance shift pattern across treatments (Figs 3 and 4). Sequencing data were deposited at the Sequence Read Archive of NCBI (accession number: SRP 130 309).

To explore correlations between RGs/MGEs and microbial community structure, redundancy analysis (RDA) was done using the qPCR data as explanatory variables to discriminate 16S rRNA gene amplicon profiles. Analysis was done using the Rgui package *vegan* as previously described (Nunes *et al.* 2016). Briefly, only significantly contributing explanatory variables were displayed as vectors in the RDAs. The robustness of the models was tested with 1000 permutations, and the total percentage of variance explained is indicated by the r^2 values.

RESULTS

Effect of different fertilizers on the relative abundance of RGs, integrons and conjugative plasmids

For most tested genes, no differences in relative abundance were detected in bulk soil of field plots prior to application of fertilizers (t_0). Nevertheless, significant differences were observed for *sul1* and *sul2*, which showed higher levels in plots subsequently fertilized with digestates (Fig. 2). Likely, these observa-

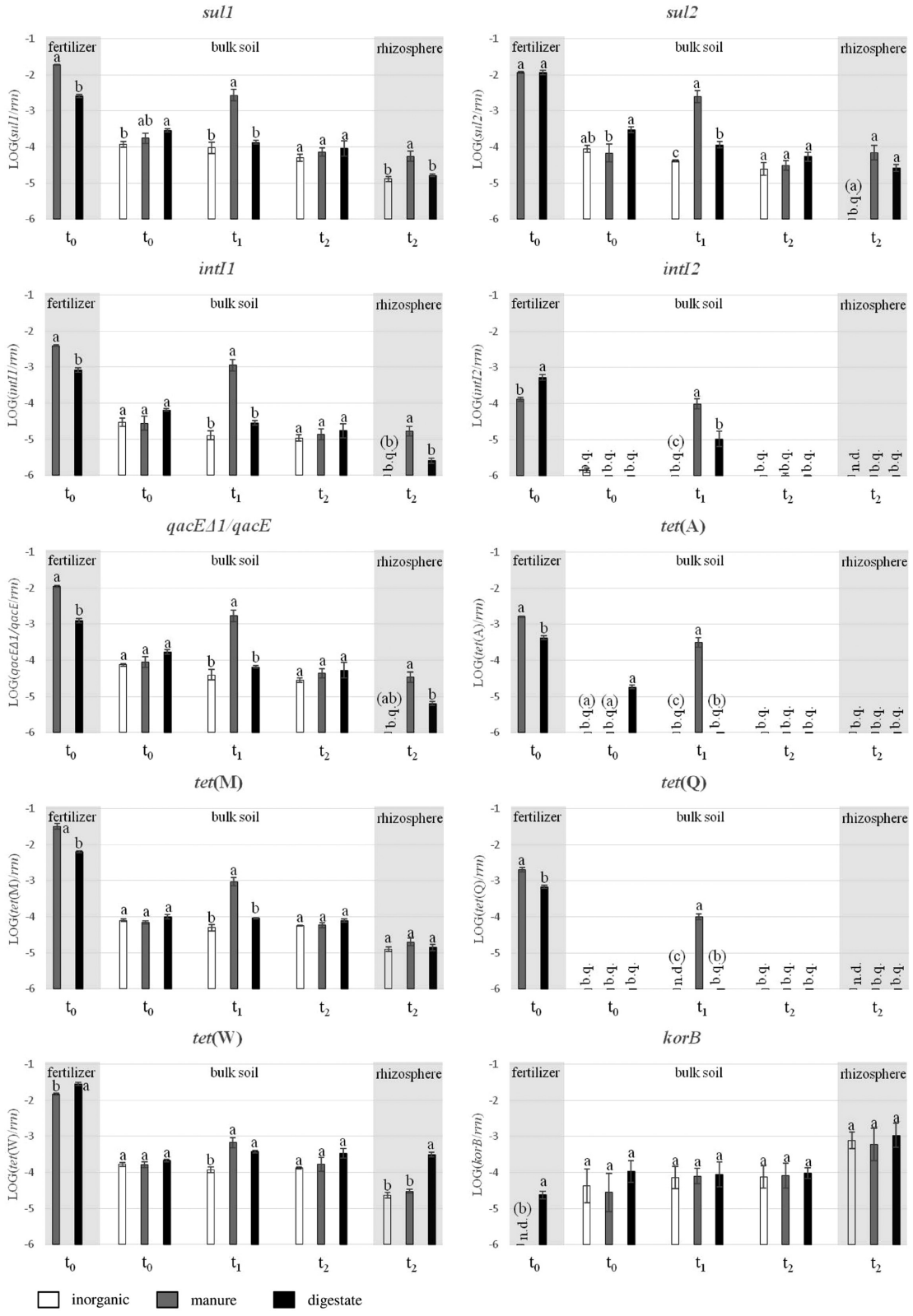

Figure 2. Relative abundances of resistance genes and mobile genetic elements in organic fertilizers and soil samples as determined by quantitative real-time PCR (n = 4). Shown are mean values and standard errors of the mean for logarithmized values correlated to 16S rRNA (*rrn*) gene copy numbers. Letters indicate statistical grouping according to Tukey's HSD testing in descending order, where 'a' is assigned to the highest mean. Different letters indicate statistically significant differences (p-value <0.05) between tested groups, while similar letters indicate no significant differences, respectively. Group assignments for values below the quantification limit are given in brackets. (b.q.: below quantification limit (signals in at least 2/4 replicates detected after cycle 35), n.d.: not detected (no signals occurring in two or more replicates, excluded from statistics)).

tions reflect intra-field variability of residing bacterial communities. Looking at differences between organic fertilizers, determined quantities of *sul1*, *qacEΔ1/qacE*, *tet*(A), *tet*(M), *tet*(Q) and *intI1* were significantly higher (p-value <0.05) in manure compared to digestate (Fig. 2). In contrast, *tet*(W), *intI2* and *korB* had higher relative abundances in digestate. The *korB* gene, indicative for IncP-1 plasmids, was below detection limit in manure TC-DNA.

Soil samples taken from field plots treated with inorganic mineral fertilizer showed no differences for the monitored genes during the experiment, except for *sul2* and *qacEΔ1/qacE*, which decreased slightly (significantly only at t_2; Table 1, Fig. 2) and *tet*(M), which was lower in relative abundance after fertilization but increased again at harvest (Table 1). In contrast, manure application significantly increased the relative abundance of all analyzed RGs and integrons and also, although not significant, IncP-1 plasmids (*korB*) at t_1. As levels of all gene targets declined again until harvest (t_2), these effects were transient (Table 1, Fig. 2).

Following digestate application, most genes decreased (significantly for *sul2* [t_1 and t_2], *intI1*, *qacEΔ1/qacE*, and *tet*(A) [all at t_2]) compared to t_0, whereas *tet*(W) and *intI2* increased slightly (Table 1, Fig. 2).

Soils from manure treated plots had a higher relative abundance of all RGs and integrons compared to inorganic fertilizer at t_1 (Fig. 2). Mean values determined for relative abundances of all analyzed genes were also higher in soils treated with digestate compared to inorganic fertilizer, but these differences were only significant for *sul2*, *tet*(A), *tet*(Q), *tet*(W) and *intI2* (Fig. 2).

At harvest (t_2; 165 days after fertilization), no differences were detectable in bulk soil for all tested genes. However, significant differences were observed in rhizosphere of maize plants grown in manure treated plots for *intI1* and *sul1* compared to other fertilizers, and *qacEΔ1/qacE* compared to digestate, whereas the rhizosphere of maize plants from digestate fertilized plots had a higher relative abundance of *tet*(W) compared to other treatments (Fig. 2).

No effects of different fertilizers were observed on the relative abundance of IncP-1 plasmids (*korB*) at all sampling times (Table 1, Fig. 2). Sequences specific for IncP-1ε (*trfA*) and LowGC (*traN*) plasmids were either below the detection limit (>35 cycles) or not quantifiable in most samples (data not shown).

Effect of different fertilizers on the microbial communities in soil based on 16S rRNA gene amplicon sequencing

Besides obvious differences found between bulk soil, rhizosphere and organic fertilizer microbes in terms of richness, evenness and overall community structure (Fig. 3; File S3, Supporting Information), a clear fertilization effect was detected (Table 2, Fig. 4). It mainly consisted in an organic fertilizer imprint onto the bulk soil microbiome (red cluster in the dendrogram, Fig. 4, Tables 2 and 3), while the rhizosphere remained very stable between treatments (Table 3). An imprint effect was most noticeable right after fertilization, with increase of Spirochaetes, Epsilonproteobacteria (Campylobacterales) and Euryarchaeota (*Methanomicrobia*) in the bulk soil of plots treated with manure and inorganic fertilizer, while both organic fertilizers enhanced Synergistetes compared to inorganic fertilizer. The manure imprint was the most striking, mostly consisting in an increase of Clostridiales and Bacteroidetes members after fertilization (Fig. 4), followed by transient receding at harvest (Tables 2 and 3, Fig. 4). The same trend was seen for the digestate application, with direct enrichment of Clostridiales and Bacteroidetes. However, unlike in manure treated plots, some identified responders were still enriched in the bulk soil at harvest and surprisingly also in the rhizosphere of maize plants grown in digestate treatments (*Clostridium XIVa* related sequences, Table 3, Fig. 4).

The second effect detected was related to time and plant growth, as shown by the green cluster of responders in the dendrogram (Fig. 4). This effect was mainly attributed to a general increase over time of specific responders until harvest. Most of them were affiliated to Bacteroidetes (*Pedobacter* sp., *Sphingobacterium* sp.), but also Gammaproteobacteria (*Lysobacter*, *Stenotrophomonas*, *Rhizobacter*, *Legionella*, *Xanthomonas*), Alphaproteobacteria (*Rhizobium*, *Devosia*, *Labrys*) and Acidobacteria (*Geothrix*, *Acidobacterium*). Some were also receding over time, including Microbacteriaceae, *Pedobacter* sp. *Ala12*, *Sphingobacterium M326*.

The RDA revealed strong correlation of the RGs *tet*(Q), *tet*(M), *sul1*, *sul2* and *intI2* to the microbial community in manure and of IncP-1 plasmids (*korB*) to the rhizosphere (Fig. 5a). In bulk soil samples, correlations of *intI2*, *sul1*, *sul2* and *tet*(M) to the soil microbial communities residing in manure treated plots after fertilization (t_1) were observed, while no connection between *korB* and neither sampling time nor treatment was identified (Fig. 5b).

DISCUSSION

Despite previous identification of manure and digestate as reservoirs of RGs and MGEs (Binh *et al.* 2008; Wolters *et al.* 2015, 2016b; Nõlvak *et al.* 2016), integrated work on the topic is still needed, as published studies on the topic remain scarce, focusing on very specific aspects (Francioli *et al.* 2016; Hupfauf *et al* 2016). To our knowledge, this is the first integrated study directly comparing effects of digestate, manure and inorganic fertilization on microbial communities by combining next generation sequencing depth of 16S rRNA gene amplicons with the sensitive quantification of RGs and MGEs in soil samples taken from a field experiment. Nevertheless, the present study is including only on a small subset of known RGs and MGEs (Zhu *et al.* 2013).

Long-term field fertilization legacy

The field experiment was performed in northwestern Germany, an area with high animal density due to intense husbandry activity. Therefore, due to long-term intensive organic fertilization history (digestate), the soil in this area was likely under a pronounced legacy effect. As an illustration of this legacy, comparable levels of extractable antibiotic residues were obtained from soil samples regardless of fertilization types (File S4, Supporting Information). Furthermore, determined values of target genes in the soil plots before fertilizer application were rather high compared to the few available reports on qPCR-analysis in pristine soils (Yang *et al.* 2010) and other reports on the so-called untreated soils not receiving organic fertilizer for several years (Kyselková *et al.* 2013; Kopmann *et al.* 2013; Chessa *et al.* 2016; Blau *et al.* 2017) in which the determined values for RGs are often below the detection limit or well below the levels detected here. Fertilization legacy effects in soil were previously characterized, with reported selection of specific microbial communities with increased richness and reduced evenness (Hartmann *et al.* 2015; Francioli *et al.* 2016). Our qPCR results revealed hardly any increase in 16S rRNA gene copy numbers regardless of fertilization (data not shown). This is contrasting previous studies

Table 1. Relative abundances of RGs and MGEs in differently fertilized bulk soil samples at different sampling times as determined by quantitative real-time PCR (n = 4).

	sul1	*sul2*	*intI1*	*intI2*	*qacEΔ1/qacE*
Inorganic					
t_0	$-3.93^{a} \pm 0.08$	$-4.05^{a} \pm 0.10$	$-4.53^{a} \pm 0.12$	b.q.	$-4.13^{a} \pm 0.04$
t_1	$-4.02^{a} \pm 0.16$	$-4.39^{ab} \pm 0.03$	$-4.89^{a} \pm 0.12$	b.q.	$-4.40^{ab} \pm 0.14$
t_2	$-4.30^{a} \pm 0.09$	$-4.61^{b} \pm 0.18$	$-4.96^{a} \pm 0.08$	b.q.	$-4.55^{b} \pm 0.06$
Manure					
t_0	$-3.76^{b} \pm 0.14$	$-4.17^{b} \pm 0.25$	$-4.56^{b} \pm 0.20$	b.q.^{b}	$-4.05^{b} \pm 0.15$
t_1	$-2.56^{a} \pm 0.16$	$-2.60^{a} \pm 0.17$	$-2.94^{a} \pm 0.16$	$-4.01^{a} \pm 0.14$	$-2.77^{a} \pm 0.17$
t_2	$-4.14^{b} \pm 0.12$	$-4.51^{b} \pm 0.13$	$-4.86^{b} \pm 0.15$	b.q.^{b}	$-4.36^{b} \pm 0.13$
Digestate					
t_0	$-3.54^{a} \pm 0.05$	$-3.52^{a} \pm 0.08$	$-4.19^{a} \pm 0.04$	b.q.^{a}	$-3.77^{a} \pm 0.06$
t_1	$-3.87^{a} \pm 0.05$	$-3.94^{b} \pm 0.09$	$-4.55^{ab} \pm 0.06$	$-4.98^{a} \pm 0.21$	$-4.19^{ab} \pm 0.04$
t_2	$-4.04^{a} \pm 0.21$	$-4.27^{b} \pm 0.13$	$-4.76^{b} \pm 0.19$	b.q.^{a}	$-4.27^{b} \pm 0.21$
	tet(A)	*tet*(M)	*tet*(Q)	*tet*(W)	*korB*
Inorganic					
t_0	b.q.	$-4.10^{a} \pm 0.03$	b.q.	$-3.78^{a} \pm 0.04$	$-4.37^{a} \pm 0.47$
t_1	b.q.	$-4.31^{b} \pm 0.08$	n.d.	$-3.93^{a} \pm 0.07$	$-4.14^{a} \pm 0.31$
t_2	b.q.	$-4.25^{ab} \pm 0.00$	b.q.	$-3.88^{a} \pm 0.03$	$-4.13^{a} \pm 0.31$
Manure					
t_0	b.q.^{b}	$-4.16^{b} \pm 0.05$	b.q.^{b}	$-3.79^{b} \pm 0.08$	$-4.55^{a} \pm 0.53$
t_1	$-3.51^{a} \pm 0.13$	$-3.02^{a} \pm 0.12$	$-4.00^{a} \pm 0.07$	$-3.18^{a} \pm 0.14$	$-4.10^{a} \pm 0.21$
t_2	b.q.^{b}	$-4.24^{b} \pm 0.07$	b.q.^{b}	$-3.77^{b} \pm 0.19$	$-4.09^{a} \pm 0.35$
Digestate					
t_0	$-4.73^{a} \pm 0.05$	$-4.00^{a} \pm 0.06$	b.q.	$-3.66^{a} \pm 0.04$	$-3.97^{a} \pm 0.30$
t_1	b.q.^{a}	$-4.04^{a} \pm 0.02$	b.q.	$-3.42^{a} \pm 0.03$	$-4.05^{a} \pm 0.34$
t_2	b.q.^{b}	$-4.10^{a} \pm 0.05$	b.q.	$-3.46^{a} \pm 0.13$	$-4.02^{a} \pm 0.16$

Listed are mean values and standard errors of the mean for logarithmized values (correlated to 16S rRNA gene copy numbers). Differing letters indicate significant differences between relative abundances at different sampling times as determined by Tukey's HSD testing (p-value <0.05), while similar letters indicate no differences between tested groups. (b.q.: below quantification limit (signals in at least two replicates detected after cycle 35), n.d.: not detected (no signals occurring in two or more replicates, excluded from statistics)).

investigating fertilizer effects on soil bacteria (Heuer and Smalla 2007; Kopmann *et al.* 2013; Nõlvak *et al.* 2016), and might be a direct consequence of the long-term utilization of organic fertilizers in the area.

Nevertheless, a significant increase in OTU richness following manure application was observed, although not sustained until harvest, indicating transient persistence of bacteria introduced with organic fertilizers into soils. Although earlier studies based on Denaturing Gradient Gelelectrophoresis, indicated soil bacterial community compositions to be resilient towards manure application, which might be explained by poor adaptability and survival of manure bacteria in soil (Hammesfahr *et al.* 2008; Heuer *et al.* 2008), more recent studies based on amplicon sequencing revealed a richness increase following manure application in soils under long-term fertilization legacy (Hartmann *et al.* 2015; Francioli *et al.* 2016), with an estimated survival of fecal-related bacteria up to several weeks (Yao *et al.* 2015).

Microbial community compositions determined for both organic fertilizers used in the present study were coherent with previous reports (Schlüter *et al.* 2008, Kröber *et al.* 2009; Wirth *et al.* 2012 Sundberg *et al.* 2013; Lu, Lu and Zhang 2014; Cho, Hwang and Park 2015; Moset *et al.* 2015; Stolze *et al.* 2015; Wolters *et al.* 2016a). Organic fertilizer-associated differences were mainly due to enriched Clostridiales and Bacteroidetes in soils (Fig. 4). In contrast to other studies, the bacterial community composition of the bulk soil had very low relative abundance of Acidobacteria (Lundberg *et al.* 2012; Ding *et al.* 2014; Schreiter *et al.* 2014) and higher proportion of Gemmatimonadetes (File S3, Supporting Information; DeBruyn *et al.* 2011). This reflects again the fertilization legacy effect of the area, as Gemmatimonadetes were previously observed in long-term organic fertilization studies (Chaudhry *et al.* 2012), while Acidobacteria were found to be negatively correlated with high nitrogen amounts brought by repeated manure application (Ding *et al.* 2014; Yao *et al.* 2014; Eo and Park 2016; Francioli *et al.* 2016). Furthermore, a legacy effect can also be seen at the genetic level, as long-term fertilization of soils was shown to enrich for specific RGs (e.g. with wastewater, Dalkmann *et al.* 2012). Altogether, our results confirm that long-term fertilization has important consequences on soils, impacting several components from antibiotic residues, RGs prevalence to deep restructuring of microbial communities composition.

Fertilization effects on microbial communities, RG and MGE prevalence.

Compared to inorganic fertilization, initial effects of manure application in bulk soil were more pronounced in terms of RG, MGE and OTU richness increase and responding taxa than digestate (Tables >1 and 2, Figs 2 and 4). Furthermore, RDA revealed many target genes as strongly correlated to the microbial community harbored in manure (Fig. 5a and b), indicating the importance of manure bacteria as a potential source in bulk soil shortly after fertilization (t_1). The increased relative abundances of RGs and MGEs in manure treated soils observed in this study are coherent with published reports on manure application to bulk soils (Hu *et al.* 2016; Nõlvak *et al.* 2016; Sandberg and LaPara 2016). However, while the observed increase of RGs could be caused by co-introduction *via* organic fertilizers, the possibility of a bloom of soil indigenous resistant bacteria due to nutrient

Table 2. Responding genera in bulk soil for the experiment. Genera were extracted using negative binomial distribution of the raw counts and generalized linear modeling with the package *EdgeR* (Likelihood ratio test, FDR-adjusted *p* -value <0.05; *uncl*: unclassified).

Treatment	Phylum	Genus	Before (t_0)	After (t_1)	Harvest(t_2)
Manure	Acidobacteria	*Geothrix*	0.03 ± <0.01	0.06 ± 0.02	0.16 ± 0.04*
	Actinobacteria	*Dietzia*	0	0.09 ± 0.07*	0
	Actinobacteria	*Streptosporangium*	0.05 ± 0.01*	0.01 ± <0.01	0.01 ± <0.01
	Bacteroidetes	*Mangroviflexus*	0	0.04 ± <0.01*	0
	Bacteroidetes	*Petrimonas*	0	0.07 ± 0.01*	0
	Bacteroidetes	*Proteiniphilum*	0	0.08 ± 0.01*	0
	Bacteroidetes	*uncl. Bacteroidetes*	0.01 ± <0.01	0.01 ± <0.01	0.15 ± 0.13*
	Euryarchaeota	*Methanosaeta*	0	0.19 ± 0.11*	0
	Firmicutes	*Guggenheimella*	0	0.01 ± <0.01*	0
	Firmicutes	*Lactobacillus*	0	0.15 ± 0.03*	0
	Firmicutes	*Proteiniclasticum*	0	0.03 ± 0.01*	0
	Firmicutes	*Saccharofermentans*	0	0.2 ± 0.01*	0
	Proteobacteria	*Acidovorax*	0	0.01 ± <0.01*	0
	Proteobacteria	*Acinetobacter*	0	0.15 ± 0.1*	0.01 ± <0.01
	Proteobacteria	*Alkanindiges*	0	0.04 ± 0.01*	0
	Proteobacteria	*Brevundimonas*	0.03 ± 0.01	0.13 ± 0.06*	0.01 ± <0.01
	Synergistetes	*Cloacibacillus*	0	0.02 ± 0.01*	0
Digestate	Acidobacteria	*Geothrix*	0.03 ± <0.01	0.03 ± 0.01	0.16 ± 0.04*
	Bacteroidetes	*Pedobacter*	0.07 ± 0.01[b]	0.2 ± 0.04[a]	0.12 ± 0.04
	Bacteroidetes	*uncl. Bacteroidetes*	0.01 ± <0.01[b]	0.02 ± 0.01	0.09 ± 0.03[a]
	Firmicutes	*Clostridium XlVa*	0.07 ± 0.01[c]	0.12 ± 0.03[b]	0.34 ± 0.13[a]
	Firmicutes	*Syntrophaceticus*	0	0.03 ± 0.01*	0
	Proteobacteria	*Acidovorax*	0	0.02 ± 0.01[a]	0.01 ± <0.01
	Proteobacteria	*Luteimonas*	0	0	0.01 ± <0.01*
	Proteobacteria	*Stenotrophomonas*	0	0.01 ± <0.01	0.05 ± 0.04*
Inorganic	Proteobacteria	*Acinetobacter*	0	0.02 ± 0.01*	0
	Bacteroidetes	*Alkalitalea*	0	0.02 ± 0.01*	0
	Bacteroidetes	*Pedobacter*	0.07 ± 0.01[b]	0.42 ± 0.32[a]	0.13 ± 0.09
	Bacteroidetes	*Proteiniphilum*	0	0.04 ± 0.02*	0
	Firmicutes	*Saccharofermentans*	0	0.09 ± 0.04*	0
	Proteobacteria	*Sideroxydans*	0	0.02 ± 0.01*	0
	Proteobacteria	*Stenotrophomonas*	0*	0.04 ± 0.04	0.03 ± 0.02
	Proteobacteria	*Arenimonas*	0.03 ± 0.01[c]	0.06 ± 0.02[b]	0.16 ± 0.04[a]
	Acidobacteria	*Geothrix*	0.03 ± <0.01[c]	0.07 ± 0.01[b]	0.26 ± 0.12[a]
	Proteobacteria	*Lysobacter*	0.05 ± 0.01[c]	0.1 ± 0.03[b]	0.24 ± 0.05[a]
	Proteobacteria	*Sphaerotilus*	0.01 ± <0.01[b]	0.03 ± <0.01	0.05 ± 0.01[a]
	Bacteroidetes	*uncl. Bacteroidetes*	0.01 ± <0.01	0.01 ± <0.01	0.06 ± 0.01*

*: value significantly different from the others. In case several values significantly differed, letters 'a','b' and 'c' were used to indicate the highest, intermediate, and lowest means, respectively. Values with no indications did not significantly differ from the others. Values below 0.005% relative abundance are indicated as '0'.

Table 3. Responding genera in the bulk soil right after fertilizer treatment, harvest time and in the rhizosphere according to each fertilizer applied. Genera were extracted using negative binomial distribution of the raw counts and generalized linear modeling with the package *EdgeR* (Likelihood Ratio Test, FDR-adjusted *p* -value <0.05).

Time/compartment	Phylum	Genus	Inorganic	Manure	Digestate
After fertilization (t_1)	Actinobacteria	*Dietzia*	0	0.09 ± 0.07*	0
	Bacteroidetes	*Mangroviflexus*	0	0.04 ± <0.01*	0
	Bacteroidetes	*Petrimonas*	0	0.07 ± 0.01*	0
	Bacteroidetes	*Proteiniphilum*	0.04 ± 0.02[b]	0.08 ± 0.01[a]	0.01 ± <0.01[c]
	Euryarchaeota	*Methanosaeta*	0.01 ± <0.01[b]	0.19 ± 0.11[a]	0[c]
	Firmicutes	*Lactobacillus*	0.01 ± <0.01[b]	0.15 ± 0.03[a]	0[c]
	Firmicutes	*Proteiniclasticum*	0	0.03 ± 0.01*	0
	Proteobacteria	*Acinetobacter*	0.02 ± 0.01[b]	0.15 ± 0.1[a]	0[c]
Harvest (t_2)	Firmicutes	*Clostridium XlVa*	0.09 ± 0.01[b]	0.05 ± 0.01[c]	0.34 ± 0.13[a]
Rhizosphere	Proteobacteria	*Acinetobacter*	0.59 ± 0.31	0.32 ± 0.19	0.05 ± 0.03*
	Proteobacteria	*Yersinia*	0.38 ± 0.37	0.18 ± 0.12	0.02 ± 0.01*
	Firmicutes	*Clostridium_XlVa*	0.02 ± 0.01	0.01 ± <0.01	0.28 ± 0.08*
	Actinobacteria	*Arthrobacter*	1.79 ± 0.18[b]	5.14 ± 2.03[a]	2.36 ± 0.76

*: value significantly different from the others. In case several values significantly differed, letters 'a', 'b' and 'c' were used to indicate the highest, intermediate, and lowest means respectively. Values with no indications did not significantly differ from the others. Values below 0.005% relative abundance are indicated as '0'.

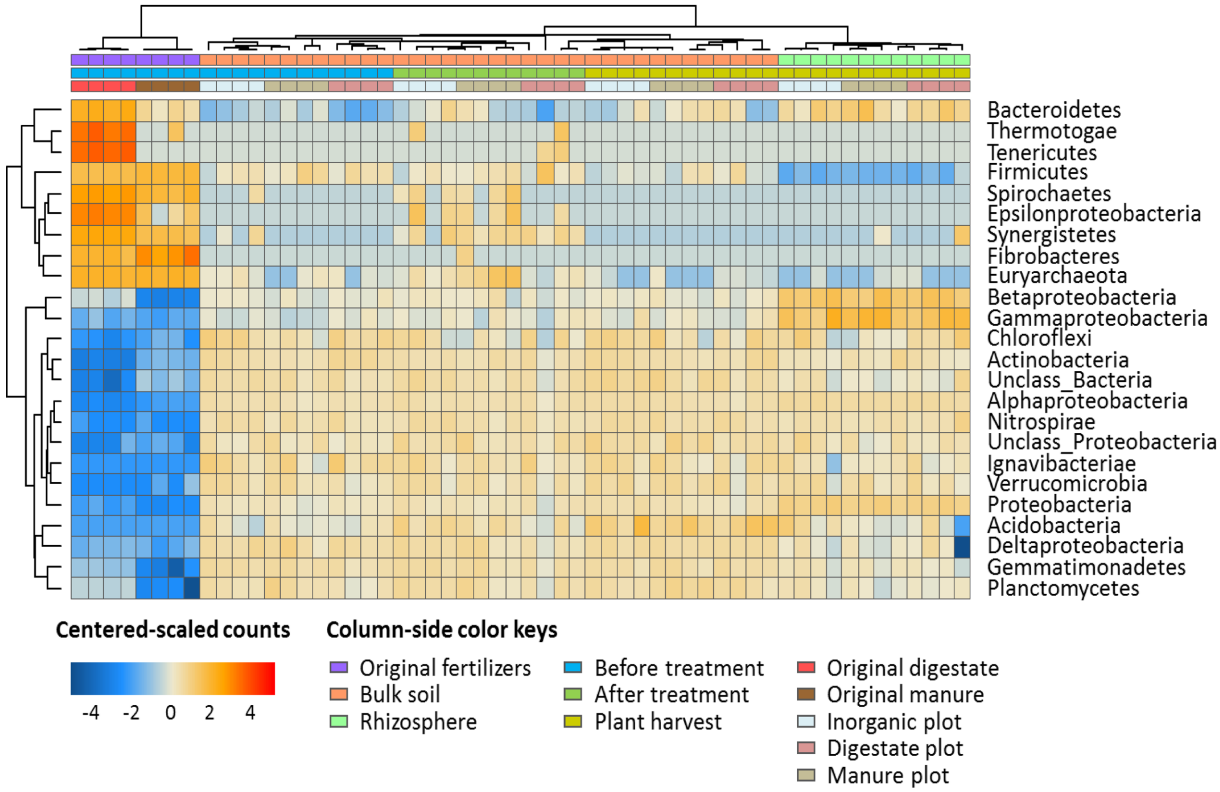

Figure 3. Generalized heatmap summarizing the major significant differences found in this study (nbGLM, Likelihood Ratio Test, FDR-corrected p -value <0.05). The heatmap was generated at the phylum level, except for Proteobacteria that are displayed at class level. The column-side color keys are indicating the sample types, the time and the corresponding fertilization treatment. The side dendrogram shows the clustering of microbial response groups showing similar abundance patterns accross the whole experiment.

input from fertilization is also conceivable, as previously indicated (Ding *et al.* 2014; Udikovic-Kolic *et al.* 2014; Hu *et al.* 2016).

The observed increased abundance of RGs and integrons in soil after application of both organic fertilizers compared to inorganic fertilization was also reported by Nõlvak *et al.* (2016), comparing impacts of mineral fertilizer, cattle slurry and cattle slurry-based digestate in agricultural grassland soil. Thus, our study further confirms digestate as a rich source of RGs and integrons in soil. In contrast to the present study, Nõlvak *et al.* (2016) observed elevated relative abundances of *tet*(A) in soils that received mineral fertilizer and increases of *sul1* and *intI1* after digestate treatment compared to non-fertilized soil. In the present study, non-fertilized plots were not included as the experiment was performed under farm conditions and lack of fertilization would have severely reduced maize plant growth. Other reports revealed enhanced levels of monitored RGs compared to background levels until the vegetation periods end (Nõlvak *et al.* 2016). Observations indicate that other factors than applied fertilizers are contributing to these deviations, such as for instance compositions of indigenous soil bacterial communities, fertilization frequency (Heuer *et al.* 2011) or agricultural management practices during and/or prior to studies. For instance, in soils under legacy of long-term untreated wastewater applications, no clear trend occurred for relative abundance of *sul1* genes, while total copy numbers continuously increased over the years compared to non-irrigated soils, probably due to enhanced biomass and facilitated survival of wastewater-associated bacteria (Dalkmann *et al.* 2012).

At harvest, abundances of RGs and integrons analyzed in this study as well as OTU richness receded in bulk soil, sometimes slightly below levels prior to treatment. In addition, the microbial community composition in the bulk soil seemed to be only temporarily affected by the introduction of non-indigenous bacteria and different types of fertilizer as, at harvest, no treatment-related differences were detectable except for enriched *Clostridium XIVa*-related sequences in digestate plots (Table 3). *Clostridium* representatives are well-known for their dormancy/latent capacities via sporulation under adverse conditions (Pellegrino, Fell and Gillespie 2002), which might have been an advantageous trait explaining their specific enhanced presence at a later stage of the experiment. Thus, it might be speculated that the sudden change of environmental conditions posed during the passage through the different processing steps of the BGP, and the following fertilization procedure was potentially causing stress, possibly explaining why sporulation might have been triggered. Apart from this observation, the results gained in the present study imply a transient nature of observed effects, as previously reported for manure applications (Kopmann *et al.* 2013; Hu *et al.* 2016; Sandberg and LaPara 2016). Similarly, Odelare *et al.* (2011) compared bacterial communities of differently fertilized sandy soils after harvest *via* terminal restriction fragment length polymorphism (T-RFLP), revealing only minor differences between treatments. However, Abubaker *et al.* (2013) (also using T-RFLP) highlighted the importance of soil types in conditioning the resilience of bacterial community structures in bulk soil towards fertilizer application (cattle slurry, biogas residues and compost). Authors speculated that bacterial communities resident in nutrient-rich soils might be more resilient than in nutrient-limited soil (Griffith and Philippot 2013; Soliveres *et al.* 2016).

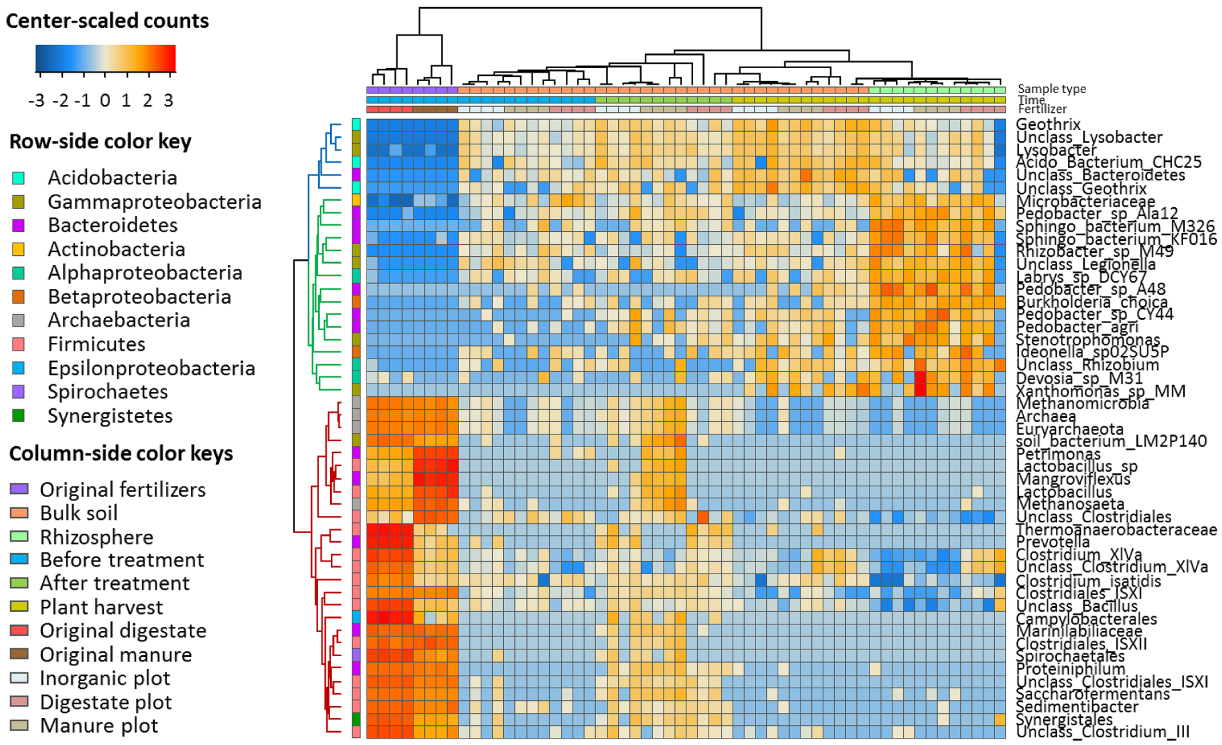

Figure 4. Generalized heatmap summarizing the taxonomic groups responding significantly to the fertilizer treatments overtime (nbGLM, Likelihood Ratio Test, FDR-corrected *p* -value <0.05). The column-side color keys are indicating the sample types, the time and the corresponding fertilization treatment, while the rowside color key is giving the phylogenetic affiliation of each responding group. The branch color on the left side dendrogram is showing the three main response groups observed, respectively, being (1) the bulk soil enrichment overtime (blue), (2) the case-specific enrichment for particular conditions (green) and (3) the enrichment after manure and digestate application (red).

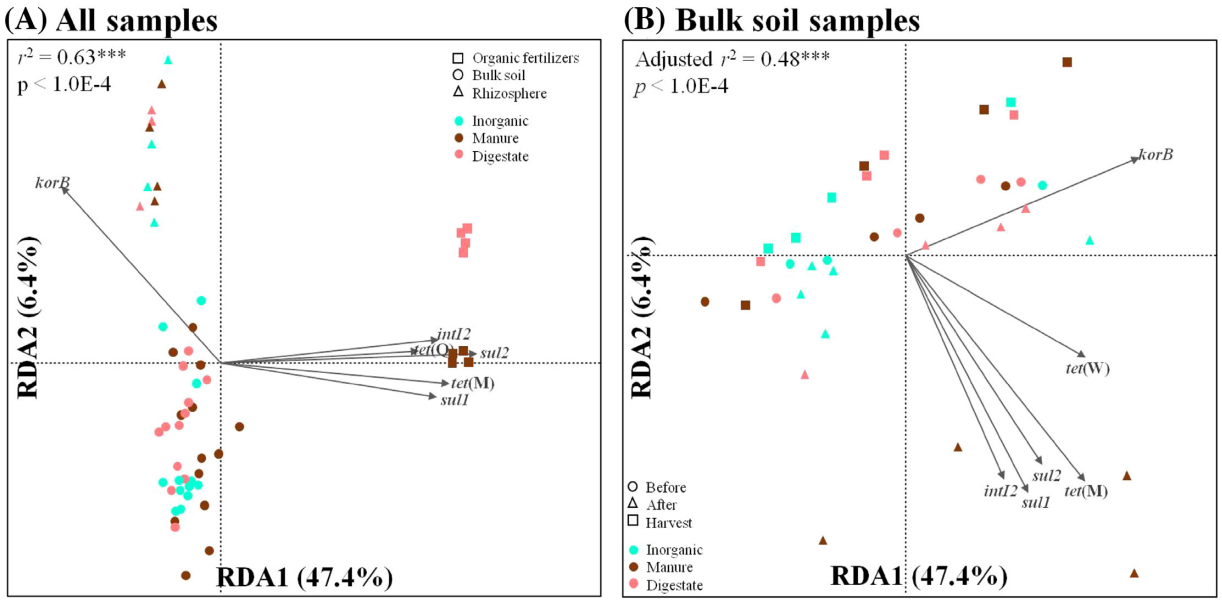

Figure 5. Redundancy Analysis (RDA) performed on 16S rRNA gene amplicon sequencing profiles at genus level using qPCR data on RGs and MGEs as explanatory variables. Panel a) shows a global RDA done with all samples, while panel b) displays an RDA focused on bulk soil samples only. Explanatory variables significantly contributing to the model are shown as vectors (*p* -value <0.05). Robustness of the models were inferred using 1000 permutations. The RDAs were done using the Rgui package vegan.

Additionally, and as discussed before, the past long-term fertilization legacy is expected to have an influence (Francioli *et al.* 2016). For instance, the uptake potential of soil bacteria for particular plasmids might be enhanced in soils treated with manure in the long-term as reported by Musovic *et al.* (2014).

Eighty-eight unique OTUs accounted for the significant richness increase after manure application, of which the major part (48/88) was consisting of OTUs not coming from the original manure. These OTUs were mostly consisting of typical copiotrophs like Proteobacteria (Alpha-, Beta- and Gamma) and Bacteroidetes (Sphingobacteriales) members (Philippot *et al.* 2010), which supports the idea that soil bacteria have been stimulated by nutrient addition. On the other hand, 40/88 OTUs were coming from the manure, mostly from Firmicutes (Clostridiales), Proteobacteria and Bacteroidetes (Bacteroidales) as well as unique phyla (Spirochaetes, Synergistetes, Fibrobacteres and Euryarchaeota). These observations with the concomitant increase in RGs and MGEs reinforce the idea of a simultaneous encounter of soil microbes enriched by manure addition together with manure bacteria, potentially representing a favorable environment for HGT and spreading of harmful genes. Indeed, the build-up of a RG reservoir transferable to soil bacteria may provide fitness advantages in the presence of antibiotic residues from organic fertilizers (Wolters *et al.* 2016b).

The maize rhizosphere as a survival hotspot?

Contrasting the transient nature of observed effects on bulk soil microbiome, fertilization consequences partially persisted in maize rhizospheres until harvest. Microbial communities resident in bulk soil and in the rhizosphere are known to differ (Lundberg *et al.* 2012, Peiffer *et al.* 2013; Schreiter *et al.* 2014), which is coherent with our observations (Figs 3 and 4; File S3, Supporting Information). Although already very abundant in bulk soil, Proteobacteria had even higher proportion and diversity in the rhizosphere (File S3, Supporting Information) confirming previous reports (Schreiter *et al.* 2014; Shi *et al.* 2016). Although this could be related to abiotic parameters fluctuating over time, enrichment of typical Proteobacterial copiotrophs (*Xanthomonas*, *Lysobacter* and *Stenotrophomonas*) likely occurred in response to exudates from the growing maize plants (Shi *et al.* 2016). Besides the fact that Proteobacteria are potential hosts of a large number of MGEs (Suzuki *et al.* 2010; Shintani, Sanchez and Kimbara 2015), the rhizosphere is also a favorable place for HGT (Lopes *et al.* 2016). Differences in relative abundances of RGs and MGEs in bulk soil and rhizosphere soil after manure spread were previously observed by Kopmann *et al.* (2013) and Jechalke *et al.* (2013), who also reported lower relative abundances of *sul1*, *sul2* and *traN* (specific for LowGC plasmids). Transfer frequencies of captured MGEs were found to be enhanced in the rhizosphere compared to bulk soil (Jechalke *et al.* 2013; Kopmann *et al.* 2013). Furthermore, exudates available in the rhizosphere increase the metabolic activity and stimulate HGT processes (Pukall, Tschäpe and Smalla 1996; Kroer *et al.* 1998; Mølbak, Molin and Kroer 2007) and in addition, the presence of biofilms may favor the survival of non-indigenous bacteria and subsequent HGT events (Ren *et al.* 2015, Lopes *et al.* 2016, Madsen *et al.* 2016). Thus, the rhizosphere is assumed to be a favorable hotspot environment hosting a microbiome prone to be engaged in HGT, where RGs associated with MGEs do occur. These observations might explain the long-lasting effects of the organic fertilizers detected in the rhizosphere compared to bulk soil. Thus, the strong correlation of IncP-1 plasmids to the microbial communities in the maize rhizosphere observed in the present study (Fig. 5a) might, on the one hand, be due to the high abundance of suitable host bacteria and might, on the other hand, be further fostered by enhanced HGT in this compartment. Similarly, as observed in the present study also Jechalke *et al.* (2014) reported on higher relative abundances of IncP-1 plasmids (*korB*) in the rhizosphere of lettuce as compared to bulk soil. In digestate fertilized plots, the relative abundance of *tet*(W) (frequently associated with anaerobic bacteria including *Clostridium* (Roberts 2016) was significantly higher compared to other treatments (Fig. 2). In parallel, OTUs assigned to Clostridia (especially *Clostridium* XIVa) were significantly enriched (Table 3, Fig. 4), which might indicate that *Clostridium* representatives are potential *tet*(W) hosts in the rhizosphere. Clostridia typically have a high relative abundance in digestate bacterial communities (Schlüter *et al.* 2008; Wolters *et al.* 2016a). Thus, higher *tet*(W) abundance and enriched *Clostridium* sequences observed in digestate treated rhizosphere samples might represent a risk deserving more attention.

In maize plant rhizosphere samples from plots treated with both organic fertilizers, *Arthrobacter* (Actinobacteria) were enriched compared to the inorganic condition (Table 3, Fig. 4), as previously reported (Jenkins *et al.* 2009), although this difference in abundance was only significant comparing plots treated with manure and inorganic fertilizer.

New knowledge on BGP digestate application

Despite increasing volumes of digestates applied as soil fertilizers in agriculture due to growing BGP implementations (Biogaz Barometer, EurObserv'ER 2014), present knowledge of their microbial communities and resistomes is still limited. BGP digestates are considered a safe source of organic fertilizer, as the mesophilic anaerobic digestion is known to remove potential microbial pathogens and weed seeds (Al Seadi and Lukehurst 2012; Orzi *et al.* 2015). However, little is known about effects of digestate microbes on the soil microbiome, their survival, and the qualitative/quantitative aspects of MGEs/RGs present (Wolters *et al.* 2016a,b).

A recent survey on digestate and manure samples taken from various farms in Germany revealed that digestates tended to have slightly lower relative abundance of RGs than manures except for *tet*(W). Although the mesophilic biogas process leads to a reduction of RGs and antibiotic residues in the digestates compared to manure, the levels might vary and depend on the in-put material (Wolters *et al.* 2016a,b). The in-put material of full-scale BGPs often encompasses different biomass sources (Biogaz Barometer, EurObserv'ER 2014; Wolters *et al.* 2015) and hence different microbial communities and their respective resistomes are mixed. These observations relate to the ecological concept of microbial coalescence (Rillig *et al.* 2015), where microbiomes from different origins may intermix, sometimes resulting in higher diversity (Jacquiod *et al.* 2017b). Thus, the mixed fermenter contents potentially harbors a larger diversity of RGs and MGEs than single input sources and might represent a hotspot for HGT. In addition, bacterial communities of digestate and manure were shown to be clearly distinct, although both being dominated by different OTUs affiliated to Firmicutes and Bacteroidetes (Wolters *et al.* 2016a). Most importantly, both types of organic fertilizers were harboring transferable broad host range plasmids conferring multiple resistances (Binh *et al.* 2008; Heuer *et al.* 2012, Jechalke *et al.* 2013, Wolters *et al.* 2015). Thus, in view of the increasing threat of multiresistant pathogens, the possible transmission of transferable antibiotic RGs from bacteria residing in applied organic fertilizers to

soil bacteria and maybe even further to vegetables eaten raw deserves more attention. One limitation of the procedures used in the present study is that they require a sufficiently high abundance for detection and thus mainly target the dominant taxa, RGs and MGEs in the samples. Hence, other important aspects, such as the presence of fecal bacteria and the analysis of RGs and MGEs potentially harbored in those might have been overlooked due to low abundance. Here, the application of enrichment procedures and selective media could be helpful to broaden our knowledge and improve risk assessments, especially of digestate spread onto soil.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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