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Targeted metagenomics reveals inferior resilience of farm soil resistome compared to soil microbiome after manure application

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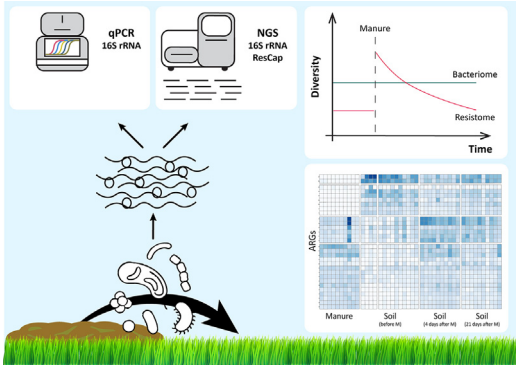
HIGHLIGHTS

- Soil bacteriome and resistome structures were influenced by different forces.
- In time, manure bacteria decreased, but some native soil families were enriched.
- Swift recovery of soil resistome diversity and abundance was observed over 21 days.
- ResCap reliably correlated with standard methods for gene quantification (qPCR).

GRAPHICAL ABSTRACT



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ABSTRACT

Application of animal manure to soils results in the introduction of manure-derived bacteria and their antimicrobial resistance genes (ARGs) into soils. ResCap is a novel targeted-metagenomic approach that allows the detection of minority components of the resistome gene pool without the cost-prohibitive coverage depths and can provide a valuable tool to study the spread of antimicrobial resistance (AMR) in the environment. We used high-throughput sequencing and qPCR for 16S rRNA gene fragments as well as ResCap to explore the dynamics of bacteria, and ARGs introduced to soils and adjacent water ditches, both at community and individual scale, over a period of three weeks. The soil bacteriome and resistome showed strong resilience to the input of manure, as manuring did not impact the overall structure of the bacteriome, and its effects on the resistome were transient. Initially, manure application resulted in a substantial increase of ARGs in soils and adjacent waters, while not affecting the overall bacterial community composition. Still, specific families increased after manure application, either through the input of manure (e.g., *Dysgonomonadaceae*) or through enrichment after manuring (e.g., *Pseudomonadaceae*). Depending on the type of ARG, manure application resulted mostly in an increase (e.g., *aph(6)-Id*), but occasionally also in a decrease (e.g., *dfrB3*) of the absolute abundance of ARG clusters (FPKM/kg or L). This study shows that the structures of the bacteriome and resistome are shaped by different factors, where the bacterial community

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composition could not explain the changes in ARG diversity or abundances. Also, it highlights the potential of applying targeted metagenomic techniques, such as ResCap, to study the fate of AMR in the environment.

1. Introduction

Antimicrobial resistance (AMR) has been considered as one of the major challenges to global public health. Agriculture contributes to the emergence and spread of antibiotic resistance genes (ARGs) and antibiotic residues in the environment (Thanner et al., 2016). Manure from (intensive) livestock production has been widely used as fertilizer because of its nutrient-rich and stable organic carbon composition. However, the application of animal manure also results in the introduction of manure-derived bacteria and their ARGs into the soil, enriching the already occurring set of ARGs (Jechalke et al., 2014b; Udikovic-Kolic et al., 2014).

The gene groups encoding antimicrobial resistance – defined as resistome – in manure and soils are clearly different (Noyes et al., 2016). Depending on the animal source, genes conferring tetracycline and aminoglycoside resistance have been shown to be highly prevalent in animal fecal matter, or manure (Fang et al., 2018; Han et al., 2018; Munk et al., 2018; Xie et al., 2018a), and *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* are the predominant *phyla* in dairy manure (Lopatto et al., 2019; Wichmann et al., 2014). Recently, a strong correlation between the resistome and bacterial taxonomy has been reported in pig and poultry feces in nine European countries (Munk et al., 2018). Soil microbial communities are highly diverse in bacterial species (Fierer, 2017). Among other factors, soil texture strongly influences soil microbial communities (Girvan et al., 2003) and has proven effects on the survivability of fecal bacteria in soils (Franz et al., 2014; van Veen et al., 1997). In grasslands, the bacteriome can be dominated by *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (Kaiser et al., 2016; Lopatto et al., 2019), but through manure fertilization, the community structure may change due to the input of nutrients (Pan et al., 2014), antimicrobial compound residues (Jechalke et al., 2014a), or manure-derived taxa.

Most studies that explored the soil resistome, particularly in manured soils, report the use of ARG-targeted high-throughput qPCR (HT-qPCR) coupled with 16S rRNA gene sequencing. HT-qPCR has the advantage of analyzing significantly more resistance determinants (up to 384 primer sets) than conventional qPCR, providing resistome profile changes with relative abundance to the 16S rRNA gene population structure. However, it still does not target the majority of known ARGs (ResFinder database contains over 2700 gene variants). Metagenomic shotgun sequencing (MGSS) has the potential to sequence all genetic material of a given sample. However, MGSS analyses are constrained by low sensitivity in detecting the "rare biosphere" unless cost-prohibitive deep sequencing strategies are applied. Often the ecologically important taxa are present in concentrations below the "default" MGSS detection limits (Lynch and Neufeld, 2015). In general, the resistome constitutes the minority in the whole gene pool of environmental samples, and is, therefore, challenging to target. Recently, ARG target-enrichment strategies, such as ResCap, have been introduced to overcome this challenge specifically for the resistome (Lanza et al., 2018).

Up to now, a limited number of metagenomic studies targeting both the bacterial community and resistome of manure and soils have been performed, sampling either dairy (Guron et al., 2019; Noyes et al., 2016) or swine farms (Fang et al., 2018; He et al., 2019; Leclercq et al., 2016). However, in these studies, samples were collected only at a single time point, and consequently, do not capture temporal variations in the soil bacteriome and resistome structure induced by the application of manure. Only two studies focused on application of dairy manure on grassland, where typically, no tillage is performed, but did not measure the microbial community (Muurinen et al., 2017; Nõlvak et al., 2016). More knowledge is available for swine or poultry manure incorporated into agricultural fields, but overall, the number of field studies characterizing both the bacteriome and/or the resistome over time is quite limited (Hong et al., 2013; Lopatto et al., 2019; Riber et al., 2014; Udikovic-Kolic et al., 2014). Therefore, changes in the bacterial community and resistome composition in a field approach remain to be thoroughly studied, particularly in a context of application of dairy manure on grassland.

We hypothesized that the application of manure would significantly alter the bacterial community, and that recovery of the community would occur after a period of time. We similarly expected that ARG diversity would also initially increase and then decrease to values similar to the ones found before manure application. The goal of this study was to evaluate the impact of manure application on the bacterial community and resistome of manured soils and nearby watercourses, with emphasis on the resilience of the bacterial community and the resistome diversity changes over time. We used high-throughput sequencing and qPCR for 16S rRNA gene fragments as well as targeted shotgun metagenomics (ResCap) (i) to evaluate the effect of the introduction of manure-derived bacterial and ARG taxa on the bacterial community and its resistome of manured soils and adjacent watercourses, over a period of three weeks; (ii) to explore the fate of individual bacterial and ARG taxa once introduced to soils or water; (iii) to correlate the bacterial community structure with the resistome structure; and finally, (iv) to compare the outcome of ResCap to qPCR results obtained previously. To the best of our knowledge, this work reports the first usage of ARG-targeted metagenomics to analyze the effect of manure application to soils.

2. Methodology

2.1. Sampling locations and sample collection

The soil and water samples used in this study were collected from six dairy farms, with different soil textures (clay, sand, or peat), during the manuring season of 2017 (between February and August). Each farm had similar soil usage (grassland) and fertilization rates (farmers' personal communication) over the five years prior to the sampling campaign.

Details on the manure properties, frequency of manure application, soil physicochemical properties, and sampling procedure have been described previously (Macedo et al., 2020). Briefly, the manure samples were collected shortly before being applied to the field and after the mixing. Manure (liquid slurry) was used as received from manure transportation trucks, without any extra processing, except for mixing, prior to soil application. Throughout the year, manure is collected and stored below the stables until the compartment's holding capacity is full, after which it is transferred to a storage silo until the start of manuring season. The manure is then applied to grassland by injection at approximately 10 cm depth, and no-tillage is performed. The soil and water samples were collected before the application of manure (time point T0) and at defined time intervals after manuring (1, 4, 7, 14, and 21 days; time points T1, T2, T3, T4, and T5, respectively). Samples from nearby gardens, with no history of manure application, but of the same texture were also collected and used as controls (NM). The NM samples were collected from gardens due to the absence of forest areas of the same soil texture close to the farms.

Composite soil samples were collected and prepared according to ISO guidelines (ISO 10381-6:2009). Briefly, over 25 grab samples of topsoil (0–10 cm) were collected every 40 steps with a soil probe, while walking the fields in a "W" pattern (of those, 4–8 samples were collected within the visual manure application bands). After collection, the soil was homogenized and enclosed in plastic zip-lock bags. Water samples were collected with sterile 2-L bottles from the adjacent discharge ditch. Water samples were collected as close as possible to the exit of a soil drainage pipe discharging to the ditch. All samples were kept on ice during transport and stored at −20 °C before DNA extraction.

2.2. DNA extraction and quantification

The total DNA extracts were obtained from 200 mg, 250 mg, and 100 mL of manure, soil, and water samples, respectively. The DNA from manure and soil samples was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN; Hilden, Germany) and the FastDNA® Spin Kit for Soil (MP Biomedicals; Irvine, CA, USA), respectively. The DNA from the water samples was isolated with DNeasy® PowerWater® Kit (QIAGEN) after filtration through 0.22 μm pore PVDF filters (Merck-Millipore; Burlington, MA, USA). The DNA extraction of soil and water samples proceeded according to the manufacturer's instructions, but for manure samples, a bead-beating step with Precellys Evolution (Bertin Instruments; Montigny-le-Bretonneux, France) was added for enhanced cell lysis (Knudsen et al., 2016).

Each DNA extraction was performed in triplicate, after which the DNA from each sample was pooled, containing similar volumes of each original extract. For each extraction kit used, blank extracts were randomly performed as controls to monitor kit and processing contamination. The DNA quantification was performed using Quantus Fluorometer (Promega; Madison, WI, USA) according to the manufacturer's protocol, and checked for purity with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

Ultimately, 140 DNA extracts were sent for 16S rRNA gene amplicon sequencing, which comprised ten from manure, 62 from soil, and 58 from water samples, as well as ten negative extraction controls. These samples covered time points NM, T0, T1, T2, T3, T4, and T5. A subset of the samples ($n = 74$) was sent for ResCap-enriched metagenomic shotgun sequencing. The ResCap samples included ten DNA pools from manure samples, 34 from soils, and 30 from water samples. These samples corresponded to the time points NM, T0, T2, and T5, among which the highest resistome changes were expected.

2.3. qPCR amplification of 16S rRNA gene and selected ARGs

Amplification of the 16S rRNA genes (*rrs*) and selected ARGs (*sul1*, *erm(B)*, and *tet(W)*) has been described previously (Macedo 2020). Briefly, the absolute abundance of these genes was quantified by qPCR in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA), and each qPCR run was tested with a standard curve ranging between 10^3 and 10^7, or 10^{-5}–10^{-9} target copies of standardized gene fragments (gBlocks; IDT technologies, Belgium) for *rrs*, or ARGs, respectively, and non-template controls. Cut-off values were calculated based on the lowest quantity of genes included in the calibration curve of all plates, and all calibration curves had a signal intensity of >2 threshold cycle (C_t) difference to the non-template controls. The average of the highest C_t obtained from this point across all plates was taken, and the standard deviation was added to it.

All assays were performed on the diluted DNA extracts, in duplicate, and the results were inspected to ensure that each duplicate fell within 1 C_t. The amplification efficiencies of all qPCR assays ranged between 88% and 102%, and the melting curves were performed to confirm the amplicon specificity, starting at 65 °C with successive increments of 0.5 °C, up to 95 °C.

The quantifications occurred for each original extract, in duplicates, following the Standard Curve method described elsewhere (Brankatschk et al., 2012), and the final values for each pooled sample were obtained by averaging the results obtained for each original extract. Later, the qPCR results were used to normalize ResCap values (*rrs*) and to compare them with the quantification obtained from ResCap (ARGs).

2.4. Microbiome profiling using rrs gene amplicon sequencing

Sequencing of *rrs* amplicons was performed at MrDNA Molecular Research LP (Shallowater; TX, USA). Sequence libraries of the V4-V5 region were constructed using PCR with primers 515F/926R (Caporaso et al., 2012; Quince et al., 2011) during a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (QIAGEN) under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. The samples were run at 30 cycles to achieve a good signal without hitting the amplification plateau. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Guided by expected microbial diversity, 300 bp paired-end reads were generated using the MiSeq Reagent Kit V3 (Illumina; San Diego, CA, USA) at a depth of 40,000 reads for manure and soil samples (hereafter "high-diversity samples"), and at 20,000 reads for water samples (hereafter "low-diversity samples") and negative controls. These sample sets were purified using Agencourt XP AMPure beads (Beckman Coulter; Brea, CA, USA) prior to Illumina library preparation. Sequencing was performed on an Illumina MiSeq (Illumina).

The QIIME2 workflow (version 2018.11) was used to perform quality control and filtering of sequence data (Bolyen et al., 2019). Raw sequence data were imported into QIIME2, samples were demultiplexed, and primer sequences were trimmed from the sequence reads using *q2-cutadapt* (Martin, 2011). Then, feature tables and representative sequences for unique amplicon sequence variants (ASV) were constructed for each data set after quality filtering of paired-end reads (i.e., denoising, error-correction, and chimera removal) using the *DADA2* q2-plugin (Callahan et al., 2016). Parameters for quality filtering were set as follows: to obtain a median Phred score > 30 at each base position, the first ten and the first three bases were trimmed from reads of high- and low-diversity samples, respectively, and forward and reverse reads were trimmed at position 250 and 220 and at position 265 and 205 from high- and low-diversity samples, respectively. The five feature tables and representative sequence sets were both then merged into two merged data sets. Taxonomy assignment until the genus taxonomic level was performed on representative sequences using the scikit-learn naive Bayesian classifier (Pedregosa et al., 2011) trained on full *rrs* sequences from the SILVA database version 132 (Quast et al., 2013).

Data analysis was performed using *phyloseq* version 1.22.3 (McMurdie and Holmes, 2013) in R statistical software version 3.5.0 (R Core Team, 2020). Prior to calculating bacterial diversity, ASVs were removed that were not assigned to Bacteria ($n = 2869$), and when assigned to Chloroplast ($n = 993$) or Mitochondria ($n = 1417$). Furthermore, using the *decontam* package (Davis et al., 2018), 19 predicted contaminant ASVs which were linked to blank controls were removed, retaining a total of 16,311 ASVs across all samples. Rarefaction curves and library sizes can be found in the Supplementary material (Supplementary File 1, Figs. 1–3). The raw reads are accessible under the NCBI Bioproject number PRJNA665747.

2.5. ResCap workflow and data processing

The ResCap workflow is composed of three main steps, KAPA HyperPlus Library construction (for MGSS) and enrichment, Illumina short-read sequencing, and data annotation. The ResCap method was

initially described by (Lanza et al., 2018), while the followed procedure was according to the manufacturer conditions (Roche SeqCap EZ workflow version 2.3). Briefly, 100 ng of DNA per sample was enzymatically fragmented 20 min to a size between 200 and 500 bp, after end-repair and adapter/barcode ligation using seven cycles amplification. Eight purified libraries were pooled at 125 ng library each and enriched according to the SeqCap EZ Library SR procedure (Roche). After the final ligation-mediated PCR for 14 cycles, the enriched library pools were paired-end 150 bp sequenced on Illumina NovaSeq6000 at a target sequencing depth of 2 million read-pairs (4 M reads) per enriched sample.

After barcode demultiplexing, the raw reads (over 90% ≥ Q30) were adapter-clipped, erroneous-tile filtered, and quality-trimmed at Q20 (PHRED score) using BBduk (Bushnell, 2013). Read-pairs were subsequently mapped against the ResFinder database ((Zankari et al., 2012) version of February 2020) using the default global-alignment algorithm of BBMap (Bushnell, 2013). To normalize for library prep, enrichment, sequencing depth, and gene length, FPKM (Fragments Per Kilobase Million) values for each ResFinder gene were calculated and subsequently converted to FPKM/*rrs* gene copies. Each step of relative volume/concentration adjustment or amplification was taken into account for the relative normalization (this includes the relative volumes during library preparation, the number of samples in each pool during the library preparation, the number of PCR cycles during enrichment, and the equimolar loading of the final pools on the sequencer). Finally, the outcomes were corrected using the qPCR-obtained *rrs* copies per sample and multiplied by 10^9. This final FPKM value represents the normalized ARG count per bacterial community.

To circumvent potential problems with ambiguous mapping of read-pairs to single highly identical ARGs in the ResFinder database, we aggregated the FPKM data per identified ARG at 90% identity clustered ARGs as the lowest level of detail (Munk et al., 2018). Briefly, all ARG variants from ResFinder (accessed 19 February 2020) were clustered using at least 80% coverage and a 90% sequence identity threshold using CD-HIT-EST (v4.8.1) (Huang et al., 2010). Each cluster was manually inspected and optionally renamed to reflect their representant/reference sequence and gene members (Table S7) similar to (Munk et al., 2018). The raw reads are accessible under the NCBI Bioproject number PRJNA665747.

2.6. Alpha and beta diversities

For both bacteriome and resistome data, alpha diversity (Chao1 richness) was estimated after rarefaction. Other richness indexes (observed, Shannon, and Pielou's evenness) can be found in the supplementary information (Supplementary File 1, Figs. 5 and 8).

In the bacteriome dataset, prior to rarefying, samples with low library sizes were excluded (< 5% of max read sum; 24 water samples removed), and then, the data was split into two datasets. The first bacteriome dataset was composed by manure and soil and the other by manure and water samples. The manure-soil dataset consisted of 10,943 ASVs, distributed in 72 samples (1796 and 9620 ASVs for manure and soil samples, respectively), while the manure-water dataset consisted of 4128 ASVs, distributed in 44 samples (1492 and 2675 ASVs for manure and water samples, respectively). Rarefying at 14,480 reads resulted in 5368 ASVs removed from the first dataset while rarefying at 4022 reads resulted in 12,183 ASVs removed from the latter.

The resistome diversity was based on the NCBI accession numbers of the ARG reference sequence from ResFinder and consisted of 424 different genes, distributed in 74 samples (265, 313, and 347 different ARGs for manure, soil, and water samples, respectively). The ARG gene cluster count matrix was rarefied to the number of the lowest sample library size (183,629 hits).

The beta diversity analysis using Bray-Curtis dissimilarities were calculated using the R package *vegan* (Oksanen et al., 2019) for each dataset. The effects of time point, farm, and soil type were determined using permutational multivariate analysis of variance (PERMANOVA) and are depicted in non-metric multidimensional scaling ordination plots based on the Bray-Curtis distances with 999 permutations. For each variable tested, the homogeneity of group dispersion was confirmed by testing for multivariate homogeneity of groups dispersions (PERMDISP2).

2.7. Procrustes analysis

The Procrustes analysis was used to assess the correlation between the bacteriome and the resistome composition. The ordinations were created from the gene cluster FPKM/*rrs* matrix and the ASV count matrix, and NMDS was performed based on Bray–Curtis dissimilarities. The symmetric Procrustes correlation coefficients between the first two axes of the bacteriome and resistome ordinations. Significance was tested using PROTEST, with default 999 permutations, and was calculated separately for manure, soil, and water samples (alpha significance threshold: 0.01).

2.8. Differential abundance analysis

To identify bacteria and ARG that differed in abundance before and after manure application, the raw ASV and ARG cluster counts were analyzed separately, using the DESeq2 package (Love et al., 2014). For each analysis, samples from before manuring (T0) were used as normalization reference. For each ASV and ARG, a Wald test was used to determine whether the fold change between time points was statistically significant (alpha significance threshold: 0.01).

Additionally, manure-associated (M) bacterial ASVs absent before (NM or T0) but present in soil and in water samples after (T1 – T5) manure application were identified. In addition to this exploratory approach, the samples were screened for selected families comprising typical manure-associated pathogens as well as hospital-related pathogens (i.e., *Salmonella*, *Campylobacter*, *Listeria*, *Yersinia*, and *Clostridium*, ESKAPE pathogens).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the bacteria and ARG diversity indexes and in ARG abundance based on qPCR and ResCap data across different sample types and time points. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance threshold of $p < 0.05$ was considered to be statistically relevant for all ANOVA, except when mentioned otherwise. These analyses were performed with R version 3.6.3 (R Core Team, 2020) and RStudio (Version 1.2.5033; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

3. Results

3.1. Diversity of bacterial populations in manure, soil, and water

Chao1 patterns showed that the soil samples had a greater richness of bacterial ASVs than manure (Fig. 1, $p < 0.01$), while water samples showed lower bacterial taxa richness than manure ($p < 0.01$), but higher evenness ($p < 0.01$; Supplementary File 1, Fig. 5). The application of manure did not affect the estimated total number of ASVs found in manured soils ($p = 0.78$; Fig. 1) nor in the adjacent watercourses ($p = 0.15$) as compared to soil and water before manure application, respectively.

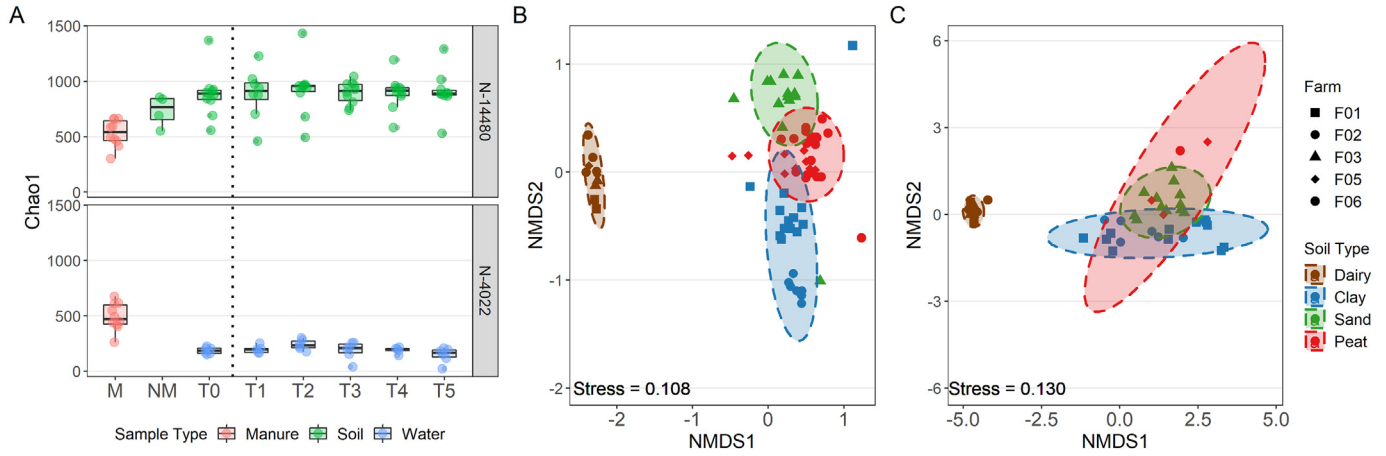

Fig. 1. No changes in microbiome diversity (Chao1) after manure application across sample types (A). Manure samples (M) as well as never-manured soils (NM), and samples before (T0) and after (T1 – T5) manure application are presented under the corresponding rarefaction depth. Non-metric multidimensional scaling (NMDS) plots illustrating Bray-Curtis dissimilarity matrices evidence the clustering of soil (B) and water (C) microbiome samples by farm and soil texture, regardless of having manure applied. Other richness indexes (Observed, Shannon, and Pielou's evenness) can be found in the suppl. Material.

The NMDS ordination based on Bray-Curtis dissimilarity revealed the clustering of samples based on soil textures (PERMANOVA, $p < 0.01$ in both soil and water samples) and farm identity (Fig. 1; PERMANOVA, $p < 0.01$ in both soil and water samples). No significant effect of manuring or time-points after manuring was found. The effect of soil texture and farm explained 36% and 48%, and 16% and 24% of the variation in soil and water samples, respectively.

In soil communities, *Bacteroidetes* (19.9–43.5%), *Proteobacteria* (20.7–38.4%), *Verrucomicrobia* (6.4–17.3%), *Acidobacteria* (3.8–15.8%), *Actinobacteria* (2.8–10.4%) were the most abundant phyla, whereas in manure communities *Bacteroidetes* (37.0–62.4%), *Firmicutes* (18.0–36.3%), *Proteobacteria* (1.5–40.2%), *Spirochaetes* (1.7–15.6%), *Tenericutes* (0.5–8.1%) were dominant (Supplementary File 1, Fig. 4). In water, the abundant bacterial communities were *Proteobacteria* (4.0–68.6%), *Bacteroidetes* (0.0–60.3%), *Cyanobacteria* (0.0–92.1%), *Actinobacteria* (0.0–21.2%), and *Verrucomicrobia* (0.0–20.6%).

3.2. Tracing manure-derived bacteria in farm soils

Although manure application did not result in changes in the overall community composition, the abundance of specific taxa was changed shortly after manure application. The differential abundance analysis showed that from the 30 most abundant families, seven families had their abundances significantly increased after manure application (from T0 to T2; $p < 0.01$; Fig. 2). Typical environmental bacteria were among the most abundant taxa (e.g., *Burkholderiaceae*, *Chitinophagaceae*, and *Flavobacteriaceae*), but some families (e.g., *Dysgonomonadaceae* and *Ruminococcaceae*) were very abundant in manure and in soils directly after manuring (Figs. 2 and 3). Other families, such as *Pseudomonadaceae*, were significantly increased in manured soils, even though they were not abundant in manure (Figs. 2 and 3).

Manure application resulted in the direct introduction of 26–136 ASVs to farm soils, as observed by the overlap of manure and manured-soil

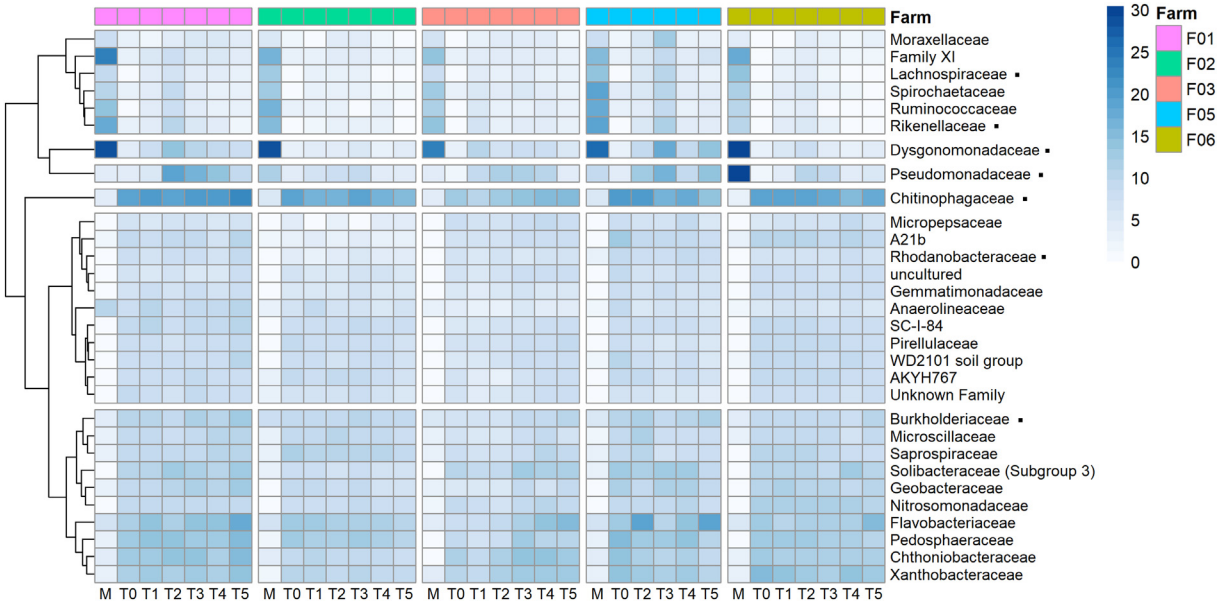

Fig. 2. Heatmap demonstrating the 30 most abundant families in manure and soil samples. Manure resulted in the transfer of manure-derived bacteria, but also in native soil bacteria enrichment. Differential abundance analysis based on the negative binomial distribution (*DESeq2*) showed that marked families (black dot) were significantly changed ($p < 0.01$) between soil before receiving manure (T0) and soil four days after receiving manure (T2). Other time points (T1 – T5) are also displayed. For better visualization, the cubic root of the ASV counts is shown.

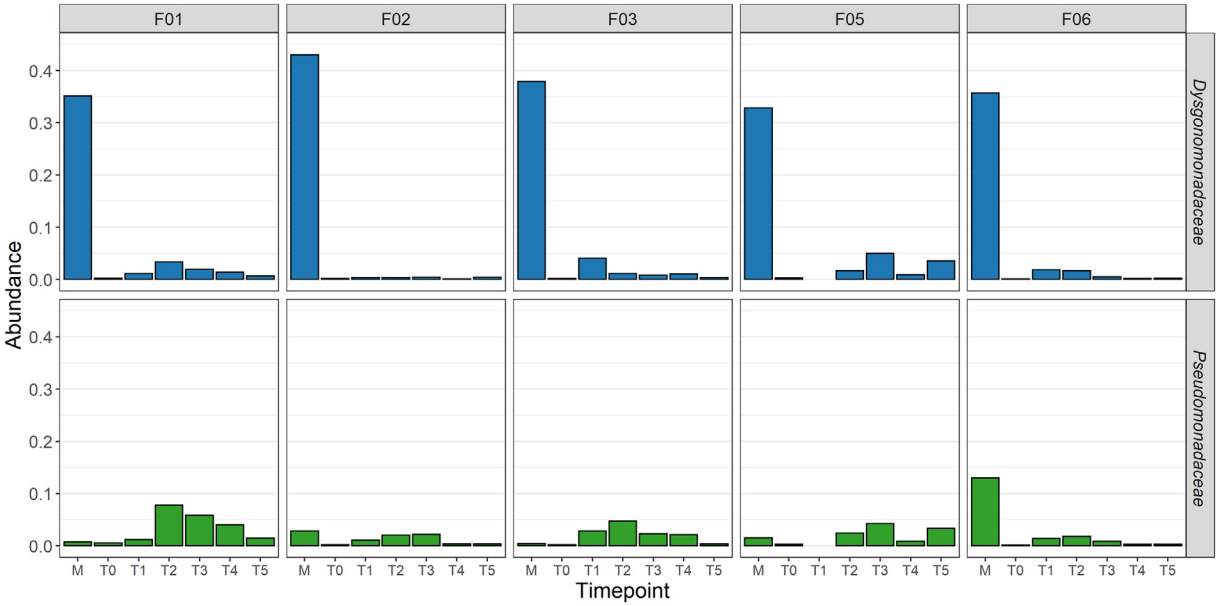

Fig. 3. Bar chart demonstrating the relative abundance of *Dysgonomonadaceae* and *Pseudomonadaceae*, evidencing their abundance peak in soils shortly after manure application, in the different farms. Their relative abundance is shown in manure samples (M), and soil samples before (T0) and after (T1 – T5) manure application.

ASVs, corresponding to eight families (incl. *Dysgonomonadaceae*; Supplementary File 1, Fig. 6). From these, three families were increased shortly after manure application ($p < 0.01$; Figs. 2 and 3) and showed their highest relative abundance within the first week after manure application, regardless of the farm. No manure-derived bacteria were detected in water samples after manure application.

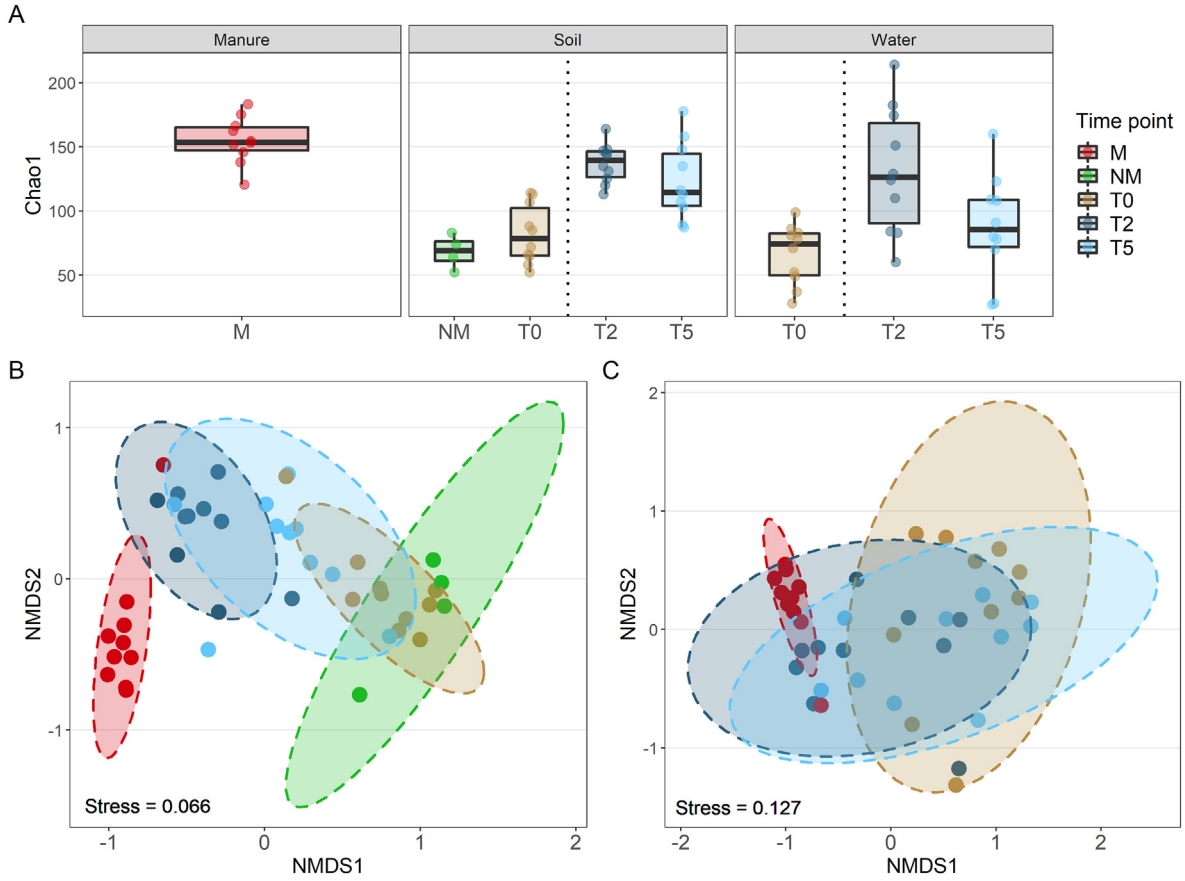

Fig. 4. Increase of resistome diversity (Chao1) after manure application across sample types (A). Non-metric multidimensional scaling (NMDS) plots depicting Bray-Curtis dissimilarity matrices demonstrate the clustering of soil (B) and water (C) resistome samples by time points, regardless of the farm and soil texture. Other richness indexes (Observed, Shannon, and Pielou's evenness) can be found in the suppl. Material (Supplementary File 1, Fig. 8).

3.3. Resistome diversity in manure, soil, and water

Manure application resulted in significantly increased ARG diversity in soil and water samples measured four days after the application of manure (T2) and in soils three weeks after manuring (T5; Fig. 4; $p < 0.01$). Regardless of the soil characteristics of each farm, the resistome diversity of both soil and water samples was increased by the application of manure, with the strongest changes apparent four days after manuring (T2). At T5 (21 days after manuring), diversity was still significantly increased in soils, but not in water.

The NMDS ordination based on Bray-Curtis dissimilarities revealed that the resistome composition was significantly influenced by manure application and days after manuring in soils (PERMANOVA, $p = 0.001$; Fig. 4), but not in water samples ($p = 0.011$). Instead, the resistome profile in water samples was mainly shaped by the soil texture ($p = 0.001$; $r^2 = 0.209$). The effect of manure application was stronger in soils and could explain 51% and 14% of the variation in soil and water samples, respectively.

Among manure samples, resistance to tetracyclines was most abundant (-4.27 ± 0.18 log FPKM/*rrs*; Supplementary File 1, Fig. 7), followed by resistance to aminoglycosides (-4.54 ± 0.27), and macrolides (-4.51 ± 0.19). The resistome of both never-manured soil (NM) and soils before manure application (T0) was quite similar, being dominated by resistance to trimethoprim (-4.29 ± 0.35 log FPKM/*rrs*), quinolones (-4.42 ± 0.16), and macrolides (-4.76 ± 0.17). However, after the application of manure, the soil resistome shifted and became dominated by resistance to aminoglycosides (-4.23 ± 0.29 log FPKM/*rrs*), tetracyclines (-4.59 ± 0.17), and trimethoprim (-4.86 ± 0.36). A similar shift occurred between water samples before and after manure application, with resistance to aminoglycosides and tetracyclines becoming more abundant after manure application (Supplementary File 1, Fig. 7).

3.4. Manure-induced ARG changes

The differential ARG abundance analysis revealed that depending on the type of gene, the abundance of some ARGs increased after manure application, while others decreased (Fig. 5 and S9; $p < 0.01$). For example, genes belonging to the clusters represented by *aph(6)-Ib* or *erm(B)* were among the most abundant ARGs in manure, and their abundance increased after manure application in both soil and water samples. In contrast, *dfrB3* and *oqxB* were among the most abundant ARGs in soils before manure application and decreased in soils after manure application.

In soils, manuring resulted in a significant increase in abundance of *aph(6)-Ib* and *erm(B)* by roughly 1 log, from 8.13 ± 0.77 and 7.71 ± 0.20 log FPKM/kg to 9.13 ± 0.23 and 8.59 ± 0.32 log FPKM/kg respectively ($p < 0.01$; Fig. 6). The abundance of *dfrB3* decreased from 9.24 ± 0.24 to 8.43 ± 0.43 log FPKM/kg ($p < 0.01$; Fig. 6). In water samples, manuring resulted in the significant increase of *aph(6)-Ib* and *erm(B)* abundance by roughly 2 logs, from 4.53 ± 0.7 and 3.85 ± 0.60 log FPKM/L to 6.16 ± 0.67 and 5.75 ± 0.71 log FPKM/L, respectively ($p < 0.01$; Fig. 6), but the abundance of *dfrB3* remained stable at roughly 5 log FPKM/L (Fig. 6).

3.5. Comparison between ARG: qPCR vs. ResCap

In general, when compared to qPCR results, ResCap led to a slight underestimation of the gene abundance, depending mainly on sample type (-2.26 ± 0.22, -0.65 ± 0.47, and -0.58 ± 0.93), but also on the gene (either *sul1*, *erm(B)*, or *tet(W)*; Supplementary File 1, Fig. 12). The gene abundances measured by qPCR were positively correlated to the ones measured with ResCap for *sul1* ($r = 0.83$, $p < 0.01$), *erm(B)* ($r = 0.89$, $p < 0.01$), and *tet(W)* ($r = 0.85$, $p < 0.01$), even though an ideal 1:1 correspondence between the two datasets was not found. The qPCR results used for comparison were obtained from the same DNA extracts described here, prior to pooling, and were previously published elsewhere (Macedo et al., 2020).

3.6. Bacteriome and resistome correlation (Procrustes)

Based on Procrustes and PROTEST significance analysis comparing the ARG composition and bacterial composition for each sample type of the first two axis from NMDS ordinations, the resistome and bacteriome did not correlate in manure ($p = 0.578$, permutations = 999), soil ($p = 0.046$, permutations = 999), or water samples ($p = 0.132$, permutations = 999).

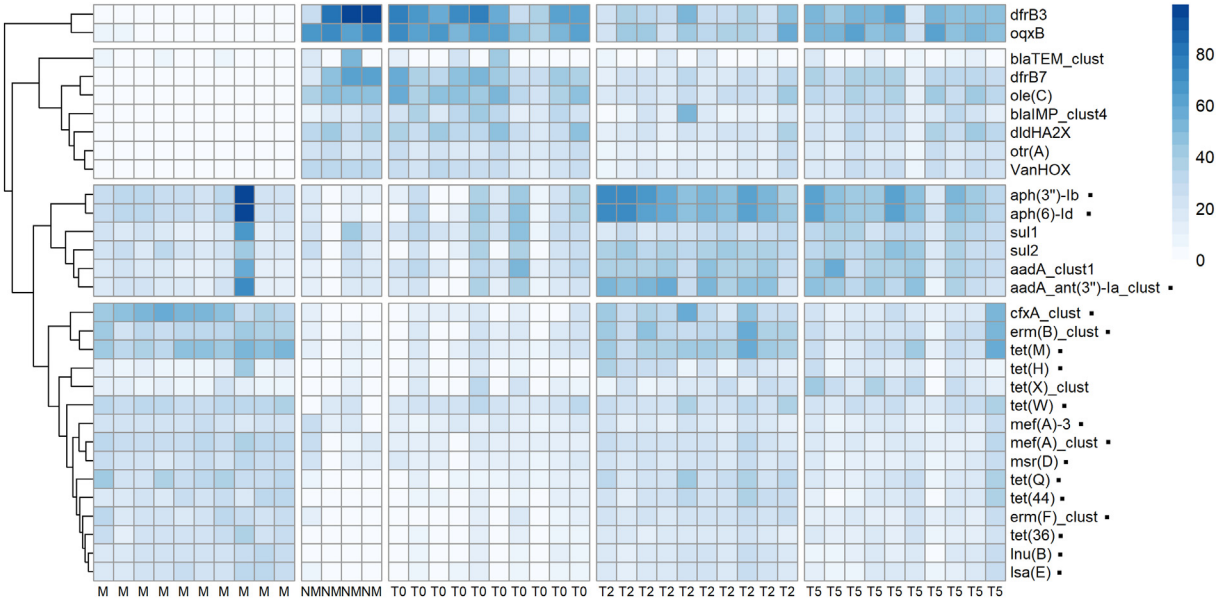

Fig. 5. Heatmap demonstrating the 30 most abundant ARG clusters in soil samples, evidencing different ARG patterns, and how manure (M) resulted in the abundance change of certain ARGs. Differential abundance analysis (*DESeq2*) showed that the marked ARG clusters (black dot) were significantly increased ($p < 0.01$) between soil before receiving manure (T0) and soil four days after receiving manure (T2). Never-manured (NM) and 21 days after receiving manure (T5) are also displayed. For better visualization, the cubic root of ARG abundance is shown. ARG clusters in water samples are depicted in Supplementary File 1, Fig. 9.

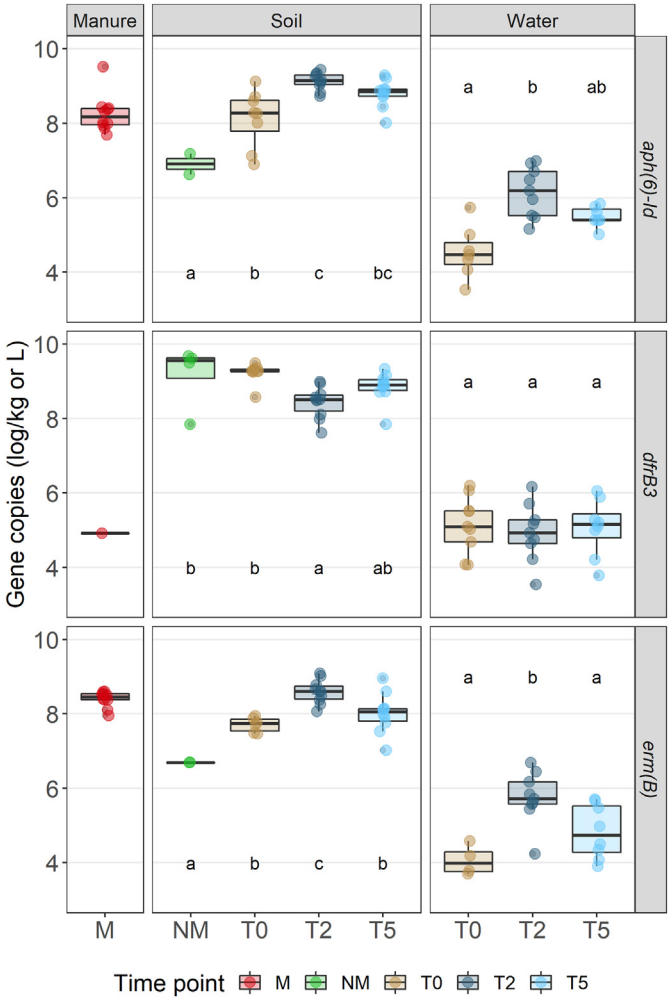

Fig. 6. ResCap-based absolute abundance estimates of *aph(6)-Id*, *dfrB3*, and *erm(B)* gene clusters, representing the main trends found in gene abundance changes. The time points represent manure (M), never-manured soils (NM), before (T0), four-days after (T2), and 21-days after (T5) manure application to soil and water samples. Group differences were determined using ANOVA ($p < 0.05$).

4. Discussion

In this study, we used qPCR (for *rrs*), high-throughput 16S rRNA gene sequencing (for bacteriome), and targeted metagenomics (ResCap, for resistome) to evaluate the impact of manure application on the bacteriome and resistome of manured soils and nearby watercourses. The results confirmed the hypothesis that manure application introduced both bacteria and ARG to soils and that after three weeks, their abundance tended to decrease to levels found before manuring. The effects of manuring were more evident and longer-lasting in the resistome than in the bacteriome of agricultural soil.

4.1. Manure enriches AMR-relevant bacteria in farm soils

In general, the dominant phyla of manure and soil bacterial communities in this study were characteristic of bacteriomes described in other studies (Han et al., 2018; Lopatto et al., 2019; Riber et al., 2014; Wang et al., 2018). Manure, soil, and water alpha diversities and *phylum* composition were constant across the sampling campaign, showing that the application of manure did not affect the overall bacteriome diversity of soil and water samples.

Results similar to the ones described in the present study were reported in other field studies of manured soils where tillage has been performed (Lopatto et al., 2019; Riber et al., 2014; Xie et al., 2018a), but not in manured-soil microcosm studies (Han et al., 2018; Wang et al., 2018). In the latter, soil diversity decreased after receiving manure, regardless of its animal source. Possible reasons for the divergent outcome of these studies lie in differences in the experimental time frame, the diverse inherent soil characteristics, and weather conditions between studies. In this study, the soil had higher bacterial richness than manure, which could explain why manure application did not contribute to an overall diversity increase, and the location where the samples were collected (farm identity), followed by soil texture, were the main drivers shaping the bacterial community.

Soil communities are known to be affected by soil texture (Blau et al., 2018; Girvan et al., 2003) and also by edaphic factors (Lauber et al., 2008). Still, four out of the eight families that were introduced to soils by manure amendment had a significantly higher abundance after manuring ($p < 0.01$), as shown by the overlap of manure and manured-soil ASVs and *DESeq2* analyses. These families belonged either to *Firmicutes* or *Bacteroidetes*, which are the most common phyla found in manure (Ding et al., 2014; Wichmann et al., 2014), and have been recognized as important ARG hosts in soils (Forsberg et al., 2014; Han et al., 2018; Leclercq et al., 2016). Typically, manure-derived bacteria are not well adapted to survive in soils (Bech et al., 2014; Franz et al., 2014; Heuer et al., 2008), and consequently, tend to decrease shortly after introduction in soils. Indeed, in this study, these families were hardly detected three weeks after manure application.

At the same time, *Pseudomonadaceae* and *Moraxellaceae* were presumably enriched after manure application. As manure is commonly applied to soils for its nutrient content, the growth of certain bacterial groups is stimulated (Goldfarb et al., 2011). In general, γ-*Proteobacteria*, particularly *Pseudomonas*, take part in the primary succession after a disturbance in the bacterial community of soil and aquatic environments (Becerra-Castro et al., 2016; Song et al., 2017; Vadstein et al., 2018). It has been recently shown that manure application to soils can enrich antimicrobial-resistant bacteria (Ding et al., 2014; Hu et al., 2016; Udikovic-Kolic et al., 2014), and both *Acinetobacter* and *Pseudomonas* have been associated with ARG persistence in manure-treated soils (Leclercq et al., 2016). Whether and which of the ARG that were enriched in this study were located on Pseudomonas has to be evaluated by further studies. Here, the relative abundance of *Pseudomonadaceae* decreased over time, reaching levels similar to the ones found before manure application, showing that the manure input had only a short-term effect on the relative abundance of this family. The findings of the current study corroborate previous studies reporting manure enrichment of bacteria relevant to the spread of AMR.

In general, water samples were affected to a minor extent by manure application than soils. Depending on the soil NPK requirements and local legislation, 30 m^3/ha of manure (slurry) is typically applied to soils, corresponding to approximately 40 g/kg soil (Ding et al., 2014). From this, and further depending on multiple factors (e.g., climate, soil texture), it would be expected that only a small fraction of manure bacteria would leach into the water ditches (Bech et al., 2014). Despite manure displaying higher bacterial diversity than water, its contribution to the bacteriome structure of water samples was not detectable, which can explain why no manure-derived ASVs were found in water samples.

4.2. Manure provokes an intense short-term increase of ARGs

Overall, resistance to aminoglycosides and tetracyclines were abundant throughout the different samples, but particularly more in manure and manured soils and waters. Resistance to tetracyclines and aminoglycosides is commonly reported in manured soil studies (Chen et al., 2019; Han et al., 2018; Zhang et al., 2019, 2017). As tetracyclines are commonly administered to farm animals, resistance to this antimicrobial class was expected. However, aminoglycosides are not so widely used on a national scale in the Netherlands (de Greef et al., 2019). Previously, (Muurinen et al., 2017) showed that some *aadA* genes were

increased by manure storage. Considering that all manure samples used in this study were stored below the stables before being applied in the fields, this could partly explain the high abundance of aminoglycoside resistance. However, resistance to aminoglycosides has been strongly correlated to mobile genetic elements (i.e., integrases and transposases) (Zhang et al., 2017), and has been positively associated with corresponding antibiotics used in poultry farms (Luiken et al., 2019). Because neither farm antimicrobial consumption data was collected, nor mobile genetic elements were measured, it is not possible to determine which aspect was more important in this case.

In soils, manure application resulted in an intense short-term increase of ARGs (after four days), which could still be observed after 21 days. The combination of results from this study with the previously published findings (Macedo et al., 2020) suggests that most ARGs will probably recover to abundances found before manure application in a period of roughly 40 days. Elsewhere, a similar increase in ARG content immediately after application of cow manure was observed in a field study where tillage was not performed (Muurinen et al., 2017) and in two microcosm studies (Han et al., 2018; Zhang et al., 2017). However, the temporal succession in some of those studies seems to occur slower than in this study, as there the overall number of ARGs after 40 days was still significantly higher than before manure application. Notably, Zhang et al. (2017) reported a period of 20 days for ARG numbers to decline back to numbers before amendment. Furthermore, the present study corroborates the previous findings that the fate of AMR depends extensively on ARG identity, as seen for genes that were very abundant in manure and not in soils before manure application (increased after manuring; e.g., *aph(6)-Id*, *erm(B)*, *cfxA*, *tet(W)*, or *tet(M)*), and for the ones that were abundant in soils without manure but not in manure (decreased after manuring; e.g., *dfrB3*, *oqxB*, *otr(A)*, or *ole(C)*). In water samples, manure application resulted in the increase of ARGs (after four days), after which their diversity was decreased to levels before manure application. There is a lack of studies focusing on the overall ARG diversity in surface water streams adjacent to manured fields, over time. The findings of this study are consistent with the ones shown by Muurinen et al. (2017), where the number of ARGs in ditch waters two and six weeks after manure application was similar to the numbers found prior to manuring.

Moreover, in this study, manure application was the main variable driving resistome diversity in soils, explaining 51% of the NMDS distribution. Previously, in a multivariate analysis study, Muurinen et al. (2017) also found that the manure amendment was among the variables that significantly explained ARG variation in soils. However, long-term studies indicate that bacterial composition and soil properties have the strongest role in shaping ARG profiles in manured soils (Guo et al., 2018; Xie et al., 2018b), which contradict the observations from the current study. Not only did soil properties (here represented by the soil texture) not show a significant correlation with the resistome structure, but also no statistically relevant association between the resistome and bacterial composition was found. Although not common, the lack of correlation between resistome and bacteriome and soil properties has also been reported in a long-term manured-soil study (Cheng et al., 2019). While soil environments are known for being a source of ARG diversity, regardless of having a history of manure application (D'Costa et al., 2011; Nesme and Simonet, 2015), here, the ARG diversity observed in never-manured soil and in samples before receiving manure was well below the ARG diversity observed in manure. The input of ARGs from manure was intense both in abundance and diversity and led to a steep increase of ARG levels, thus shaping the overall resistome structure. While manure strongly affected the resistome, the direct contribution to the bacterial community was rather low, thus explaining why no correlation was found between the resistome and the bacteriome. Compared to the existing microbiota, the manure amendment only added a fraction of additional bacteria to soil, as *rrs* abundance was constant before and after manuring in soil and water samples (Supplementary File 1, Fig. 10). Considering the amount of manure applied, it corresponded roughly to adding 1–10% bacteria to the ones already present in soils, apparently not enough to shift the overall community composition.

4.3. ResCap is a promising tool to determine the ARG fate in the environment

Generally, the data obtained from ResCap showed a strong positive correlation with the qPCR-obtained quantifications (Supplementary File 1, Fig. 11 and 12). However, differences in the absolute agreement were apparent between sample types. Manure samples showed a stronger underestimation when compared to qPCR (roughly −2 log units), and the water samples showed high variability in abundance differences between ResCap and qPCR data (standard deviation varied 0.874–0.914 log units). The reasons for these differences are not entirely clear, and one can only speculate. As metagenome shotgun uses longer intact DNA and qPCR uses short fragments for detection, the extent of DNA fragmentation might contribute to the observed difference between values. Additionally, different hybridization or PCR efficiencies between sample types could have interfered.

ResCap is reported to achieve better recovery of target genes than MGSS and to greatly enhance the sensitivity and specificity of metagenomic methods (Lanza et al., 2018), increasing the number of mapped reads from 1 in 1000 read-pairs (Munk et al., 2018) up to 200-fold (1 in 5). Here, a total of 475 ARG clusters were found in the 74 samples analyzed, including 79, 65, and 47 gene clusters that confer resistance primarily to aminoglycosides, beta-lactams, and tetracyclines, respectively. By introducing a probe-hybridization step, data quality and reproducibility increase as it reduces the variance in target coverage, delivering a better cost-effective approach (Mamanova et al., 2010). However, low efficiencies in the hybridization step, or signal overloads, may affect the outcome. Additionally, as it requires enzyme-based steps as does qPCR (e.g., amplification during library preparation), inhibition might also bias the results. At the same time, qPCR is considered the method of choice for gene quantification but is not free from potential pitfalls. Final quantification is affected by protocol-related issues (e.g., choice of primers, and their concentration, mastermix), equipment used, among others (Bustin et al., 2009; Rocha et al., 2018; Travis et al., 2011). Additionally, it can only measure a limited number of genes, even in a high-throughput setup, never delivering a complete picture of the sample ARG landscape. In terms of errors, a variability of <3-fold in absolute units, corresponding to circa 0.5 log units, was found in an inter-laboratory comparison using qPCR data (Travis et al., 2011), which the authors considered "small in biological terms."

The choice to evaluate the impact of anthropogenic activities, namely manure application, in the environment is challenging and requires compromising between accuracy and amount of information. When compared to qPCR (Supplementary File 1, Fig. 12), the results obtained with ResCap showed an average difference of <10-fold (1 log unit) in overall soil and water samples. Although this difference is considered biologically relevant in absolute terms, when placed in a context of highly polluted environments, such as manured soils (roughly 10^{10} total ARG copies/g; (Han et al., 2018)), or wastewater streams (roughly 10^{8} total ARG copies/mL; (Quintela-Baluja et al., 2019)), it may not be so relevant because the background ARG level in those scenarios is already relatively high. The semi-quantitative aspect of metagenomics, which ResCap also features, is of great value. It should not be disregarded solely based on the loss of accuracy but rather be validated in further methodological studies. ResCap has been previously validated and shown to provide better results than metagenomic shotgun sequencing (Lanza et al., 2018). Here, we applied this technology to a new context and concluded that ResCap can simultaneously generate results on many resistance genes while still giving at least semi-quantitative results. Therefore, one should be careful in presenting the results, as factors such as sample ARG abundance and presence of impurities in the DNA extracts may significantly affect the ResCap-based quantification.

4.4. Implications on-farm management

Traditional farming practices were followed while this study was being conducted. Manure was stored below the stables or in silos, during winter, and it was applied during manuring season (spring/summer) multiple times, as farmers commonly do. While the effects of manuring in the resistome of agricultural soil were transient, an intense increase of ARGs occurred simultaneously with the enrichment of native soil bacteria. Therefore, solutions to minimize the input of ARGs into soil should be further investigated. The application of anaerobic digestion treatments of animal waste holds potential because it allows farmers to recover the nutrients and produce biogas. While manure treatment is efficient in removing fecal indicator pathogens and some ARG-carrying bacteria (Iwasaki et al., 2019; Pandey et al., 2015), some ARGs persist after the treatment (Huang et al., 2019). Nevertheless, because of the costs of implementing and maintaining treatment structures, the majority of the farms applies manure directly in soils, without any treatment.

5. Conclusions

In this field study, both soil bacteriome and resistome showed strong resilience to the input of manure over a timeframe of 21 days. Manure application resulted in the input of manure-derived bacteria that did not affect the overall community composition. After being introduced to soils, these bacteria decreased, in a period of three weeks. However, manure application also resulted in the enrichment of fast-growing bacteria, namely *Pseudomonadaceae*. On the other hand, the resistome displayed a temporal shift with an intense increase of ARG diversity and abundance after manure application, but these effects were temporal. It was shown that the fate of ARG depends on the gene identity, and that both bacteriome and resistome structures were shaped by different factors, where the bacterial community did not show a significant relationship with ARG abundance.

This study also highlighted that targeted-metagenomic techniques, such as ResCap to enrich the resistome, provide an excellent tool to explore and assess the fate of AMR in the environment, as they provide the complete picture of the resistome landscape at an affordable price. Additionally, when coupled with 16S rRNA gene quantifications, it reliably correlated with standard methods used for gene quantification (qPCR). However, caution is advised when analyzing the results as sample-related bias may be prone to occur (e.g., inhibition).

CRediT authorship contribution statement

Gonçalo Macedo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **H. Pieter J. van Veelen:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Lucia Hernandez-Leal:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Peter van der Maas:** Writing – review & editing, Funding acquisition. **Dick Heederik:** Conceptualization, Writing – review & editing. **Dik Mevius:** Conceptualization, Writing – review & editing. **Alex Bossers:** Conceptualization, Methodology, Validation, Data curation, Writing – review & editing. **Heike Schmitt:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

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