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Resilience of agricultural soils to antibiotic resistance genes introduced by agricultural management practices

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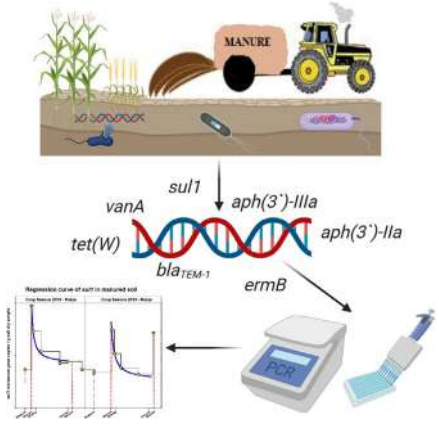
HIGHLIGHTS

- Pig manure application increases the abundance of *sul1*, *ermB*, *tet(W)*, bla_{TEM-1} and *aph(3')-IIIa* in agricultural soil
- Agricultural soil shows a high resilience capacity: ARG concentrations reach baseline levels within a crop season
- Pesticide application raised the absolute and relative abundances of *ermB*, *tet(W)* and *aph(3')-IIIa* in non-manured soil
- *VanA* concentration remains stable for two vegetation crop seasons in agricultural and forest soils
- Concentrations of *aph(3')-IIIa* and *tet(W)* remain rather stable before (fresh pig faeces) and after (pig manure) maturation

GRAPHICAL ABSTRACT



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ABSTRACT

Antimicrobial resistance (AR) represents a global threat in human and veterinary medicine. In that regard, AR proliferation and dissemination in agricultural soils after manure application raises concerns on the enrichment of endogenous soil bacterial population with allochthonous antibiotic resistance genes (ARGs). Natural resilience of agricultural soils and background concentrations of ARGs play key roles in the mitigation of AR propagation in natural environments. In the present study, we carried out a longitudinal sampling campaign for two crop vegetation periods to monitor spatial and temporal changes in the abundance of seven clinically relevant ARGs (*sul1*, *ermB*, *vanA*, *aph(3')-IIa*, *aph(3')-IIIa*, bla_{TEM-1} and *tet(W)*) and ribosomal 16S RNA. The absolute and relative abundances of the selected ARGs were quantified in total community DNA extracted from agricultural (manured and non-manured) and forest soils, fresh pig faeces and manure slurry. We observed that ARG concentrations return to background levels after manure-induced exposure within a crop growing season, highlighting the resilience capacity of soil. Naturally occurring high background concentrations of ARGs can be found in forest soil in due distance under low anthropogenic influences. It was observed that pesticide application increases the concentrations of three out of seven ARGs tested (*ermB*, *aph(3')-IIIa* and *tet(W)*). Moreover, we noticed that the absolute abundances of *sul1*, *vanA*, *ermB* and bla_{TEM-1} resistance genes show an increase by 100- to 10,000- fold,

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from maturation of fresh pig faeces to manure. Outcomes of our study suggest that agricultural soil environments show a strong capacity to alleviate externally induced disturbances in endogenous ARG concentrations. Naturally occurring high concentrations of ARGs are present also in low human impacted environments represented by the indigenous resistome.

1. Introduction

Soil environments provide the natural habitat for complex and dynamic microbial populations (Bahram et al., 2018; Bender et al., 2016; Daniel, 2005). Soil is well known to give shelter to a large diversity of soil microbes which can produce antibiotics (Perry et al., 2016). The soil resistome represents a reservoir of indigenous antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) as well as pathogenic bacteria carrying antibiotic resistance genes deriving from soil amendments with animal manure and sewage sludge (Surette and Wright, 2017; Wright, 2010; Xie et al., 2016). In the past 20 years, an improved understanding of the environmental dimension of antibiotic resistance has shed light on its dissemination in long term amended agricultural soils (Cheng et al., 2016; Guo et al., 2018; Hu et al., 2016; Xie et al., 2018). Agricultural management practices play a key role in shaping the microbial biodiversity in soil environments (Armalyte et al., 2019; Wolters et al., 2018). One of the most common conventional farming practices is fertilization of soil with animal manure, leading to ARB&ARGs accumulation and dissemination in natural environments. The conventional agricultural practices using inorganic fertilizers and pesticides are drivers influencing the soil microbial communities (Hartmann et al., 2015). Findings regarding the application of pesticides and their effects on ARB&ARG abundances and microbial diversity were reported in previous studies (Kalia and Gosal, 2011; Kurenbach et al., 2018; Newman et al., 2016). Little is known about how these specific compounds modulate the microbial community pattern and their direct influences on the prevalence and the abundancies of different ARGs in agricultural soils.

In terms of ecological significance of antibiotic resistance in livestock and agriculture, manure represents a reservoir and an allochthonous source of ARB&ARGs and therefore soil is pictured as a 'sink' for the large microbial and chemical cocktail input (Guo et al., 2018; Nesme and Simonet, 2015). It is generally known that soil amendments with organic fertilizers boost the prevalence and abundance of ARB&ARGs in agricultural systems (Cheng et al., 2016; Xie et al., 2018), natural resilience capacity of soil environments playing a crucial role in the accumulation and spread of ARB&ARGs and chemical compounds. Recent studies have shown that a good soil drainage could minimize the effect of animal manure application on agricultural soils in field scale (Cheng et al., 2016) as well as in microcosms experiments (Hu et al., 2016). In contrast, Peng et al. (2017) reported that ARGs persist in a soil environment at least for one year, but the pathogenic bacteria found in manure samples could not establish themselves in soil environments (Peng et al., 2017). An increase and accumulation of specific ARGs after repeated animal manure applications was also observed over the years (Heuer et al., 2011b). The soil resistome is certainly impacted by different agricultural management practices, but nonetheless the results should be interpreted based on the naturally occurring background levels of ARGs (ARG concentrations found in low human impacted areas, in due distance) and reported to the baseline concentrations (ARG concentrations that are found in agroecosystems, at the beginning of the study) (Rothrock et al., 2016). In the last decade, most of the studies reported an increase of ARB&ARG concentrations after manure application (Heuer et al., 2011a; Xie et al., 2018; Zhao et al., 2017). Tetracycline (*tet*) and sulfonamide (*sul*) resistances genes are the most frequently observed resistance determinants in livestock waste and manured agricultural soils (Ben et al., 2019; He et al., 2020), followed by macrolide (*erm*) and β-lactam (*bla*) ARGs (Qian et al., 2018). The absolute abundance of different ARGs in untreated livestock waste and manured soil ranges from 10^6 to 10^{11} copies/g dry weight (He et al., 2020). Peng et al. (2017) outlines a comparative analysis of 38 ARGs in pig manure and soils. Average absolute abundance records of *ermB* and bla_{TEM-1} in pig manure are 10^{10} and 10^7 copies/g respectively (Peng et al., 2017). Sun et al. (2020) reported in a recent study that similar ARG concentration patterns for *sul1*, *sul2*, *tet(A)*, *tet(M)*, *tet(O)* and *tet(W)* in agricultural soils are observed on a global scale in China, Europe and North America. The sums of the relative abundance for above mentioned *sul* and *tet* resistance genes (mean value) show similar levels and present no statistical difference (Sun et al., 2020). The relative abundance of *sul1* genes in Chinese manured soil ranges from 3.9×10^{-3} to 1×10^3 (Zhao et al., 2017). Zhou et al. (2017) found relative abundances of *sul1* resistance gene in agricultural soils in China from 10^{-6} to 10^{-2} copies/16S rRNA (Zhou et al., 2017). In a study carried out in swine manured agricultural soil in Canada by Marti et al. (2014), the relative abundance of *sul1* and *ermB* was ranging from 10^{-2} to 10^{-4} copies/16S rRNA for both resistance genes (Marti et al., 2014). It is well known that *aph(3′)-IIIa* is highly prevalent, with usually high concentrations, and *aph(3′)-IIa* is hardly detectable in agricultural soils, their maximum absolute concentrations in maize and potato fields in Austria being 6.2×10^4 and 8.6×10^2 copies/g soil respectively. Of all tested fields, 6% were positive for *aph(3′)-IIa* (median: 150 copies/g soil; range 31–856) and 85% for *aph(3′)-IIIa* (1190 copies/g soil; 13–61,600) (Woegerbauer et al., 2015a). A comprehensive understanding of the molecular factors that shape the microbial diversity in livestock waste and soil environments after manure amendments will be of vital significance for further risk assessment approaches.

In the present study we aimed to obtain information regarding ARG concentrations in agricultural and adjacent forest soils and their development over a prolonged timeline under usually applied land management techniques by the farmers. To achieve this goal a longitudinal sampling campaign was carried out over two years in agricultural fields (manured with pig manure slurry and non-manured) with similar soil characteristics, forest soil, fresh pig faeces and manure. The monitoring campaign shows spatial and temporal changes in the abundance of seven clinically relevant ARGs (*sul1*, *ermB*, *vanA*, *aph(3′)-IIa*, *aph(3′)-IIIa*, *tet(W)* and bla_{TEM-1}). The seven ARGs selected are major representatives of resistance determinants providing tolerance to six different classes of critically and highly important antimicrobials for human and veterinary medicine (WHO, 2019; Scott et al., 2019) and encompass four different resistance mechanisms (antibiotic inactivation, ribosomal protection, antibiotic target replacement and antibiotic target alteration) (Anonymous, 2019). The hypothesis of the study is that manure exposure of soil increases the concentrations of the corresponding endogenously present resistance determinants and after challenge, the ARG loads return to their baseline levels in agricultural soils within a crop growing season. The naturally occurring background level of the tested ARGs was determined in associated environments, under lower anthropogenic impact, such as forest soil in due distance to the agricultural fields. We present baseline concentrations of clinically relevant ARGs in agricultural soils under conventional farming practices and report temporal variations of ARG levels for two consecutive crop growing periods.

2. Materials and methods

2.1. Description of the catchment area

The experimental fields are located in Petzenkirchen, Lower Austria (48°9′N 15°9′E) in the HOAL (Hydrological Open Air Laboratory) catchment, established for the analysis of water flow processes in agricultural fields and surrounding areas (Fig. S1 – Supplementary Information) (Blöschl et al., 2016). The agricultural fields within the catchment are cultivated by the co-operating farmers using the same land management techniques as in their conventional non-HOAL fields which allows hypothesis testing under naturally occurring conditions. The farmers grant access to their HOAL fields and allow soil sample extractions but a randomized plot design is not possible as they exclusively decide about field use and cultivated crop. Agricultural management practices were performed according to the code of good agricultural practices in Austria. A local weather station and sensors installed under the soil subsurface monitor continuously important environmental parameters: precipitation, air temperature, humidity, wind speed, atmospheric pressure, raindrop size, snow depth, soil temperature, surface water/runoff discharge. These metadata are valuable elements for correlation with ARG prevalences and concentrations in soil cultivated under usually applied land management techniques.

In the past 5 years, the catchment has been subject to crop rotation (maize, winter wheat, barley and rapeseed) and over the study period, maize and winter wheat were the major crops. A small-scale rural pig farm, situated in the catchment area, provides manure as organic fertilizer for the agricultural manured fields. The farm is equipped with a storage/maturation tank for animal waste and the manure is spread onto the agricultural fields two times per year. For our longitudinal study, a manure-amended and a non-manured field (that receives also compost depending on the soil characteristics for the specific crop) with similar soil characteristics (Table 2) were selected (Fig. S1). As it is a rain fed area, the agricultural catchment has never been irrigated and both fields are under conventional farming, additionally receiving inorganic fertilizer based on NPK (nitrogen, phosphorous, potassium) minerals. The average annual precipitation is about 747 mm and the mean temperatures during the study period for the maize (April–October) and winter wheat (October – July) seasons were 17 °C and 9.0 °C respectively. Detailed information regarding average weather parameters recorded during the two crop vegetation seasons is presented in Table 1. During the first vegetation period, maize was grown on both experimental fields. During the second crop-growing period, maize was cultivated on the manured field and winter wheat on the non-manured field, respectively. The mean amounts of fertilizers and pesticides applied for the two vegetation periods are as follow: the manured test field received 25 m^3/ha pig manure slurry applied by spraying, 300 kg ha^{-1} inorganic fertilizer and 1.5 l ha^{-1} pesticide, the non-manured field received 12 m^3/ha compost, 600 kg ha^{-1} inorganic fertilizer and 0.5 l ha^{-1} pesticide (Table 2). Soil from a deciduous forest was also sampled. The forest is situated within the HOAL catchment. It was selected based on its lower anthropogenic impact. Due to its minor human interference, the observed ARG concentrations in the forest soil are considered as naturally occurring background in the environment. Sampling sites and time points are listed in Table S1.

Table 1
Average values weather data measured for each crop season during the study period.

Weather parameters	Vegetation crop season		
	2018	2019	
	Maize (Apr – Sept)[a]	Maize (Apr – Sept)[a]	Winter wheat (Oct – July)[b]
Rain (mm)	176.44	339.73	246.11
Soil temperature (°C)	15.8	15.1	9.9
Air temperature (°C)	18	16.7	9.4
Wind (m/s)	1.2	1.4	1.8
Atmospheric pressure (hPa)	982.37	982.16	983.31
Relative humidity (%)	74.6	75.5	77.1

[a] Maize growth season.
[b] Winter wheat growth season.

2.2. Soil characterization (physico-chemical properties)

The present study was conducted in a silty-clay soil, where the predominant soil type is Cambisol with medium to poor infiltration capacities (Blöschl et al., 2016). The main soil characteristics for each experimental field (manured, non-manured and forest soils) are presented in Table 2.

2.3. Experimental design and soil sampling procedure

Ten single soil extractions drawn randomly along an X-pattern of the field under consideration were taken from top 30 cm (Jahn et al., 2006) using a Pürckhauer core driller and combined to a composite soil sample, representative for the experimental unit „field". Soil core samples were collected at seven different time points during the study period: baseline level (T_0 - one month before the new crop season started and the agricultural fields were free from any crop/fertilizers/pesticides application) followed by sampling campaigns at defined time intervals after the organic or inorganic fertilization time point: 1 day (T_1), 1 week (T_2), 6 weeks (T_3), 18 weeks (T_4) and after harvest (T_5). Additionally, one soil sampling campaign took place in between the two crop seasons (T_6). The agricultural lands were monitored for a period of approximately 600 days for the two crop seasons and a total 120 single soil cores were collected per tested field over the study period. The soil was placed in sterile sealable plastic bags, thoroughly mixed and transported in a cooling box at 4–10 °C. In the laboratory, the ten collected soil core samples for each selected agricultural field were pulled together and mixed to achieve a representative composite sample. An aliquot of the composite soil sample was used directly for the determination of the corresponding moisture content and after sieving (mesh size: 2 mm), 50 g of soil were stored at −20 °C for molecular analyses. The remaining soil was air-dried for further characterization. Soil from a deciduous forest with minimal anthropogenic influences, was also sampled over the study period, at seven sampling time points covering all weather seasons (winter/spring/summer/autumn). Fresh pig faeces and manure samples were collected from the pigsty and the slurry tanker before application to the agricultural field, stored on ice, transported to the laboratory and processed on the same day. Pig manure samples were centrifuged at 10000 *g* for 10 min at 4 °C and stored at −20 °C for further analysis.

2.4. DNA extraction and quantitative analysis of ARGs by qPCR

Total DNA was extracted from four 0.25 g aliquots (wet weight) of the sieved composite soil sample using the PowerSoil kit (Qiagen) following the manufacturer's instructions. The resulting four eluates were combined and vacuum centrifuged (Eppendorf) in order to finally obtain a single tube with concentrated DNA extracted from a total of 1 g soil. The DNA was purified (PowerCleanup kit, Qiagen), checked for purity (ND 1000 spectrophotometer; NanoDrop Technologies) and stored at −20 °C for further downstream analysis. Temporal and spatial concentrations of seven ARGs were analyzed by real time TaqMan PCR in total community DNA extracted from agricultural and forest soils, fresh pig faeces and manure slurry. The selected ARGs are major representatives of resistance determinants, providing tolerance to six different classes of critically and highly important antimicrobials for human and veterinary medicine (WHO, 2019), based upon four different modes of action (Anonymous, 2019): sulfonamides (*sul1*; antibiotic target replacement), macrolides – (*ermB*; antibiotic target alteration),

Table 2
Soil characteristics and mean amounts of fertilizers/pesticides applied over the study period.

Field ID	pH	Humus %	Sand %	Silt %	Clay %	Total nitrogen %	Total organic carbon %	Organic fertilizer m^3/ha	Inorganic fertilizer kg ha^{-1}	Compost m^3/ha	Pesticides l ha^{-1}
Manured field	6.5	1.9	7.7	69.8	22.5	0.128	1.10	25	300	–	1.5
Non-manured field	7.0	2.2	8.5	74.5	17	0.136	1.28	–	600	12	0.5
Forest	7.0	3.6	10.8	69.0	20.2	0.248	2.12	–	–	–	–

glycopeptides (*vanA*; antibiotic target alteration), aminoglycosides – (*aph(3′)-IIa*, *aph(3′)-IIIa*; antibiotic inactivation), β-lactams – (bla_{TEM-1}; antibiotic inactivation) and tetracyclines (*tet(W)*; ribosomal protection). The 16S ribosomal RNA was targeted to estimate the number of bacterial cells in the samples. The qPCR runs were performed on LightCycler480 (Roche, Austria) using hydrolysis probes. The 10 μl qPCR reaction mixture consisted of 2.5 μl molecular biological grade water (Sigma Aldrich, Austria), 0.5 μl TaqMan assay (Ingenetix, Austria), 5 μl Master Mix (LightCycler 480 Probes Master 2×, Roche, Austria) and 2 μl pure DNA template. The thermal cycling conditions were as follow: initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10s, 60 °C for 30s and 72 °C for 10s, and a final cooling step at 40 °C for 10s (Table S2). All qPCR reactions were performed in three technical replicates and biological molecular grade water was used as template for the negative control. For absolute quantification, a logarithmic dilution series of the amplification standard (represented by a linear amplicon of the respective ARG target cloned separately into the plasmid pCR2.1) were pipetted in triplicates per ARG and the second derivative maximum algorithm of the LC480 software version 1.5 was used. All qPCR results are reported per dry weight of soil, pig manure and fresh faeces, respectively.

2.5. Data analysis and statistical evaluations

Data analysis was done using Sigma Plot and R software. For the identification of soil type induced effects on ARG concentrations, a variance analyses using the Tukey post hoc test and ANOVA based on the decimal logarithm of the ARG abundances was performed. For the correlation with environmental parameters (air pressure, precipitation, wind soil temperature, air temperature, relative humidity, snowfall) Spearman rank correlations were established.

The regression curve analysis for decay rates, based on a 3 parameter exponential decay formula (Eq. (1)), was used successfully to explain the temporal ARG decrease rate for the two crop vegetation seasons in the manured soil after organic amendment application. The formula follows a kinetic half-life time approach in exponential decay, "a" relating to the baseline level and "b" strongly influencing the decay rate. As half-life times usually are characteristic for first order kinetics, the die off process observed could directly be explained as a natural occurring process (as e.g. a die off during disinfection) suggesting no further factors (as e.g. selection) are relevant.

$$y = a * \exp(b/(x + c)) \quad (1)$$

2.6. Validation of the qPCR TaqMan assays

For the determination of the 95% limit of detection (LOD) and the intra- and inter-assay variabilities of the applied TaqMan assays, a semi-logarithmic dilution series consisting of 10 dilution steps (10^6–0,51 copies/assay) were prepared for each ARG and the 16S targets. The positive control supplied by the manufacturer (Ingenetix, Austria) of the TaqMan assays was used as template for the dilutions. For the validation of the qPCR systems, the data of 24 replicates per dilution step were acquired. In total a minimum number of 240 measurements points (8 replicates per dilution step × 10 dilutions × 3 independent PCR runs = 240) per target were used for statistical evaluation of the 95% LOD determined by logit regression analysis. Precision and repeatability of the assays were determined by calculating the variation coefficient of the intra- and inter-assay variabilities. Detailed information of TaqMan assays data validation is provided in Table S3 from the Supplementary Information file.

3. Results

The absolute concentrations of *sul1*, *ermB*, *vanA*, *aph(3′)-IIa*, *aph(3′)-IIIa*, *tet(W)*, bla_{TEM-1} and 16S rRNA were determined in fresh pig faeces and manure slurry used for organic fertilization of the tested fields, and were monitored over two crop growing seasons in agricultural manured, non-manured and forest soils.

3.1. ARG profiles during the maturation period from fresh pig faeces to manure slurry

Related to dry weight, the average absolute abundance of *sul1* was 1.8×10^6 copies/g in pig faeces and 2×10^{10} copies/g in manure, indicating a 10,000-fold ARG concentration increase during manure storage. The concentrations of *vanA*, *ermB* and bla_{TEM-1} increased during this period by 100- to 1000-fold (Fig. 1). Manure maturation had no significant effect on *aph(3′)-IIIa* and *tet(W)* copy numbers for the two crop seasons (Table S4). In the pig manure samples, a high relative abundance was observed for the gene targets associated with resistance to sulfamethoxazole (*sul1*), macrolides (*ermB*), tetracyclines (*tet(W)*) and aminoglycosides (*aph(3′)-IIIa*) ranging from 5.44×10^{-1} to 1.18×10^{-2} copies normalized to ribosomal 16S RNA gene numbers. The resistance gene with the lowest abundance in the pig manure was represented by *aph(3′)-IIa* with an average absolute abundance of 2.70×10^4 copies/g manure dry weight. *Aph(3′)-IIa* was below the limit of detection in faeces. Although, *sul1* concentration in pig manure sample was high (mean value: 2×10^{10} copies/g dry weight manure), its average absolute abundance in fresh pig faeces and agricultural soils was four to five orders of magnitude lower (9×10^5 and 1.8×10^6 copies/g soil/faeces dry weight on average; Table S4).

3.2. Temporal changes of the ARG profiles in soil

During the experimental period, all selected resistance genes were detected in the tested samples for all sampling points, except *aph(3′)-IIa*, which was observed only in 4 out of 12 sampling points in the manured soil and 1 out of 12 soil samples in the non-manured field respectively. *Aph(3′)-IIa* was not detected in forest soil samples in none of the sampling time points (data not shown in the manuscript; absolute concentrations can be found in Table S4).

In all examined soils, *tet(W)* and *sul1* were the most abundant resistance genes, followed closely by *ermB*. The ampicillin resistance gene bla_{TEM-1} was the ARG with the lowest concentration in the tested agricultural soils. In the forest soil samples, the resistance gene with the lowest abundance was *aph(3′)-IIIa*. Absolute concentrations for all tested ARGs in the forest soil are listed in Table S4. After manure application in spring, the absolute abundances of all ARGs – except for *vanA* - increased with at least one log unit, followed by a decrease and a return to baseline levels within the same crop growing season (Fig. 2).

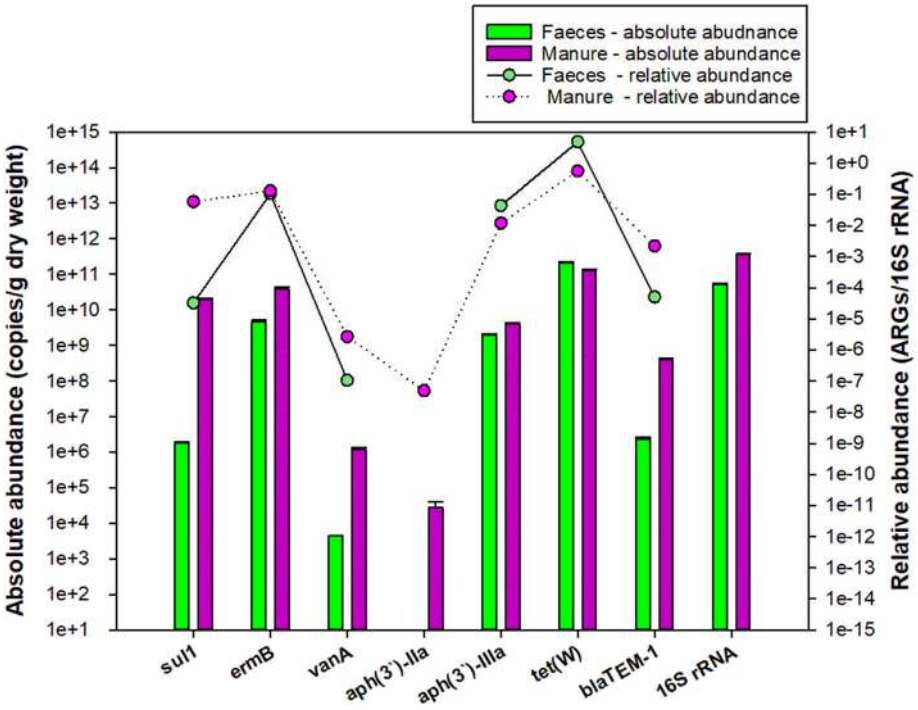

Fig. 1. Absolute and relative concentrations of the selected ARGs and 16S rRNA in fresh pig faeces (before maturation) and manure (after maturation) (error bars represent the standard deviation).

3.3. Removal rates analysis for sul1, ermB and tet(W) in manured agricultural soil

Calculations of the removal percentages in manured agricultural soil (all calculations are reported to the concentration present in soil at T1–1 day after manure application) for *sul1* showed identical patterns for the first and second vegetation seasons for 50% and 75% removal rates. Half of its initial concentration was removed after 4.5 days. 75% of *sul1* concentration in manured soil disappeared in less than 2 weeks in both crop vegetation seasons. For the 90% removal rate of *sul1*, the timeframe is different for the two crop seasons: for the first vegetation period the number of days necessary to reach this percentage are almost double than for the second season. For the first crop season, *tet(W)* presents the fastest removal rates, in 2.5 days almost 50% was removed, in contrast with *sul1*, the ARG most persistent in agricultural manured soil. *ErmB* and *tet(W)* concentrations present same removal behavior, 90% had been removed in 11 days during the first crop season. Removal rates for the three resistance genes and the respective number of days are presented in Table S5. Decay regression curve analysis results, following a three parameter exponential decay function (Eq. 1), are exemplarily displayed for *sul1* (Fig. 3).

3.4. Assessment of vanA and 16S rRNA concentrations in agricultural and forest soils

Manure amendment on agricultural fields boosted the concentrations of *sul1*, *ermB*, *aph(3′)-IIa*, *aph(3′)-IIIa*, *tet(W) and* bla_{TEM-1}. *VanA* concentration remained rather stable over the study period in manured and non-manured agricultural fields as well as in forest soil (Fig. 4). Using Student's *t*-test, *vanA* concentrations in manured soil were

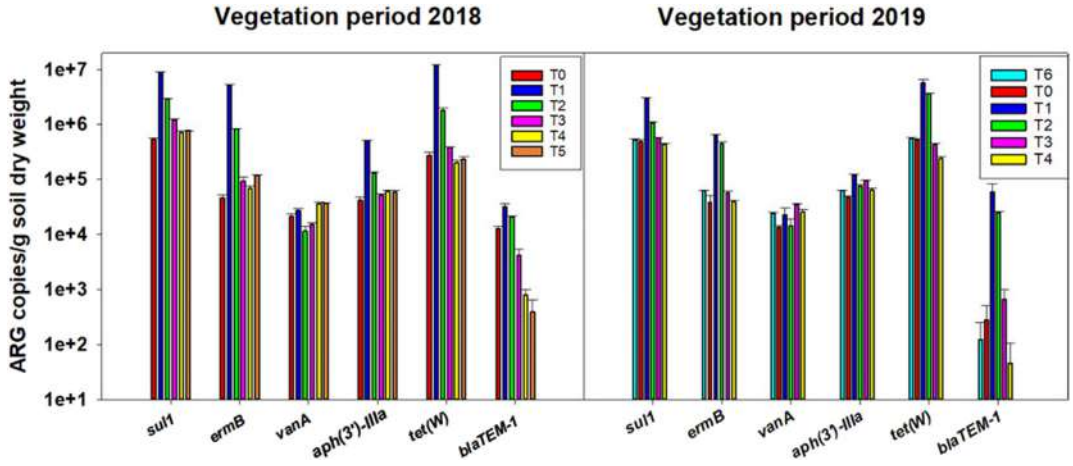

Fig. 2. *Temporal changes of the tested ARG concentrations in manured agricultural soil for two vegetation seasons* (error bars represent the standard deviation; T0 – Baseline, T1–1 day after manuring, T2–1 week after manuring, T3–6 weeks after manuring, T4–18 Weeks after manuring, T5 – After harvest, T6–2 months after harvest).

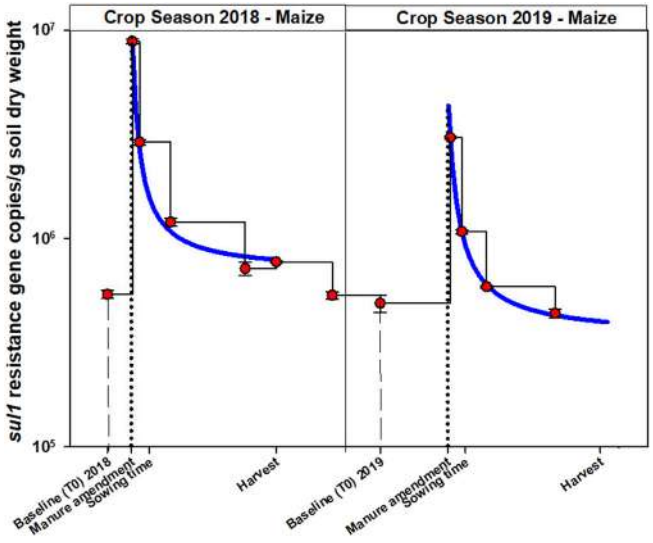

Fig. 3. Sul1 decay patterns after manure application for two crop vegetation seasons (fitted regression curves are based on Eq. (1); error bars represent the standard deviation).

significantly different ($p < 0.001$) compared with non-manured soil. This significant difference correlates positively with the manure amendment, taking into account its high concentration in pig manure samples (average value: 1.18×10^6 copies/g manure dry weight). No statistically significant differences between the two crop seasons in both, manured ($p = 0.726$) and non-manured ($p = 0.596$) soils could be observed. *VanA* absolute concentrations ranged from 1.14×10^4 to 3.68×10^4 copies/g in manured soil, 1.2×10^3 to 1.89×10^4 copies/g in non-manured soil and 1.08×10^4 to 2.56×10^4 copies/g in forest soil, respectively.

No significant fluctuations of the total number of bacterial cells have also been observed over two vegetation periods (estimated by 16S copy number as surrogate) in the tested soils: manured, non-manured and forest. The abundance of the ribosomal 16S RNA gene ranged from 4.65×10^8 to 5.55×10^{11} copies/g soil/faeces/manure dry weight, with the lowest value in the non-manured soil and the highest in the pig manure samples (Fig. 5). 16S rRNA absolute abundance does not show a statistically significant difference ($p = 0.175$) between the two agricultural fields, manured and non-manured respectively. Moreover, there is not a statistically significant difference in the estimated total bacteria cells in the non-manured soil between the first crop season (maize) and the second one (winter wheat) (Mann-Whitney Rank Sum test; $p = 0.132$). On the other hand, although in the manured soil, the crop and the conventional farming practices were similar for both vegetation seasons, the ribosomal 16S RNA concentration presents a statistically significant difference ($p < 0.001$).

Absolute abundance of *sul1* in the baseline sampling time point for the non-manured agricultural soil is corresponding with its concentrations in forest soil. Differently, in the manured soil the baseline concentration of *sul1* shows two orders of magnitude increase. The remaining ARGs tested (*ermB*, *aph(3′)-IIIa*, *tet(W)* and bla_{TEM-1}) present the same pattern: baseline concentration levels in manured and non-manured soils are comparable with their concentrations in forest soil for the first vegetation crop season and for the second season only the non-manured soil has a similar concentration for baseline and background concentrations (Table S4).

3.5. Correlation between abiotic factors (weather parameters) and ARG concentrations

The Tukey Post hoc test showed a difference between the two crop seasons between the two agricultural fields, mainly due to distinct agricultural management practices. One of the factors that influenced the ARG concentrations in the non-manured soil is represented by crop rotation (different crops and their specific agricultural practices - inorganic/organic fertilization; pesticide application etc.). *Sul1*, *ermB*, *aph(3′)-IIIa and tet(W)* concentrations are significantly correlated with different tested soils (manured, non-manured and forest) but not with changing weather conditions over the study period. In contrast, *vanA*, bla_{TEM-1} and 16S rRNA were not influenced by the selected soils, but rather by different weather parameters: *vanA* - soil and air temperature; bla_{TEM-1} - rainfall events and wind; 16S rRNA - rainfall events and atmospheric pressure. Statistical data rank correlation (Spearman) showed a

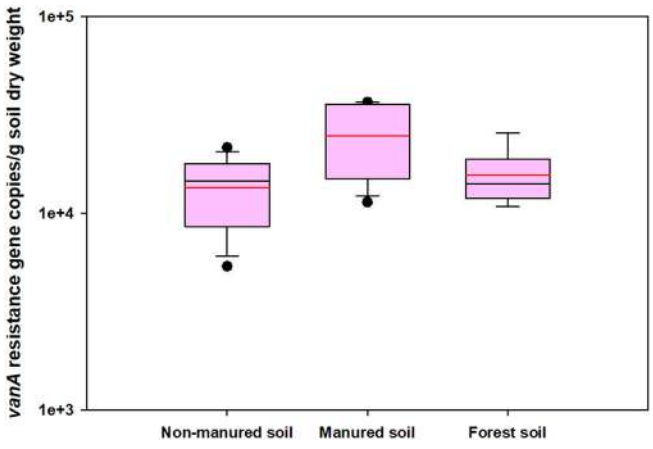

Fig. 4. VanA absolute abundance in agricultural manured, non-manured and forest soils – box plots represent all data for two crop vegetation seasons. Significant difference between manured and non-manured soils. No difference between non-manured and forest soil. In the box plots, the boundary of the box closest to zero indicates the 25th percentile, the black line within the box marks the median and the red line the mean value, the boundary farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th percentile (Q1 – quartile 1) and 90th percentile (Q3 – quartile 3). Data falling outside the Q1-Q3 range are plotted as outliers of the data.

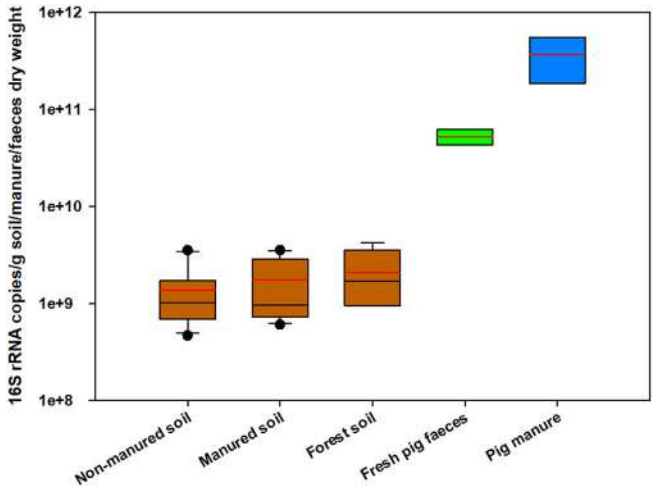

Fig. 5. 16S rRNA absolute concentrations in manured, non-manured and forest soils, fresh pig faces and manure slurry reported to the dry weight – box plots represent all data for two crop vegetation seasons. Fresh pig faeces and manure samples present identical values for mean and median values. In the box plots, the boundary of the box closest to zero indicates the 25th percentile, the black line within the box marks the median and the red line the mean value, the boundary farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th percentile (Q1 – quartile 1) and 90th percentile (Q3 – quartile 3). Data falling outside the Q1-Q3 range are plotted as outliers of the data.

positive correlation (>0.3) between *vanA* resistance gene and ribosomal 16S RNA with soil and air temperature. Negative rank correlations (<−0.3) were found for *vanA* with wind velocity, *tet(W)* with soil and air temperature and 16S rRNA with rainfall events.

Based on a linear model for logarithmic ARG concentrations per field, ribosomal 16S RNA concentration substantially fluctuated according to monthly variations of the weather conditions, especially rainfall events – increased amount of precipitation leads to lower 16S rRNA concentrations in the tested soil samples.

3.6. Effect of pesticide application on the abundance of ARGs

Addition of pesticide did not reveal an increase of the ARG concentrations in the manured field. In contrast, in the non-manured field, different agricultural management practices, especially due to different crops per season, obviously play a key role on the prevalence and abundance of certain ARGs. For instance, during the first crop season, application of pesticide increased the absolute and relative concentrations of three out of seven ARGs tested. Pesticide application exerted a selection pressure on *ermB*, *aph(3′)-IIIa* and *tet(W)* carriers, raising their relative abundance with at least one order of magnitude (Fig. 6). The concentrations of *sul1*, *vanA*, *aph(3′)-IIa* and bla_{TEM-1} were not influenced (Fig. S2).

During the second vegetation period in the non-manured soil, with winter wheat cultivation crop, the abundance of the ARGs was rather stable, with slight fluctuations during the longitudinal study. The concentration of the analyzed resistance genes was not affected by the application of inorganic fertilizer or pesticides.

4. Discussion

In the presented longitudinal study, the prevalence and abundance of selected gene targets associated with antibiotic resistance in agricultural environments were monitored over two vegetation periods. A total of seven ARGs (*sul1*, *ermB*, *vanA*, *aph(3′)-IIa*, *aph(3′)-IIIa*, *tet(W)* and bla_{TEM-1},) and the ribosomal 16S rRNA gene were quantified in manured and non-manured agricultural soils, forest soil, fresh pig faeces and manure slurry, following a spatial and temporal sampling pattern over two crop-growing seasons. Soil environments are known to harbor high concentrations of ARB and ARGs due to a native indigenous resistome (Xie et al., 2018). This naturally occurring soil resistome may serve as an important source for clinically relevant ARGs (Forsberg et al., 2014; Forsberg et al., 2012; Wright, 2010). If assumed that the tested ARGs are single copy genes, our data indicate that up to one out of 10,000 soil bacteria might be carriers for clinically relevant resistance genes (Fig. 2).

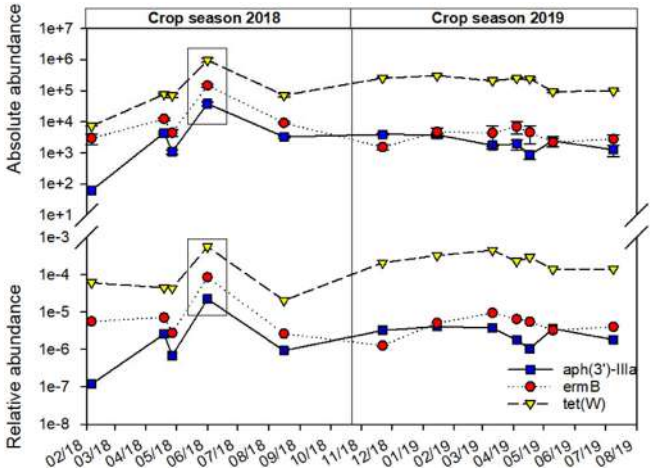

Fig. 6. Pesticide application increases the relative abundance of tet(W), ermB, aph(3')-IIIa in the non-manured soil in the first crop vegetation season (4th sampling time point highlighted inside the square)(error bars represent the standard deviation).

Different conventional agricultural management practices, especially animal manure amendment are known to interfere with ARG prevalences and abundances in agricultural fields (Ben et al., 2019; Cheng et al., 2016; Hu et al., 2016). Animal manure is recognized as one of the most important carriers of externally introduced ARB&ARGs in natural environments (Binh et al., 2008; Liu et al., 2017) and it is not surprising that soil amendment boosts the ARB&ARG concentrations. We identified differences in ARG concentrations between manured and non-manured soils. The main factor that influences the temporal changes of ARG concentrations in the manured soil is represented by the manure amendment (Fig. 2), which is driven by the manure-borne antibiotic resistant bacteria carrying ARGs and extracellular free DNA, whose decay is higher in soil environments in comparison with animal manure (Fahrenfeld et al., 2014; Udikovic-Kolic et al., 2014). We showed that the abundance of *sul1*, *ermB*, *aph(3′)-IIIa*, *tet(W)* and bla_{TEM-1} increased significantly and returned to baseline level within a crop growing season (Fig. 2), results that are in line with previous studies (Cheng et al., 2016; Gao et al., 2020; Heuer et al., 2011a). In contrast, Hong et al. (2013) reported that tetracycline ARG abundances increased after soil manure application and remained at high levels up to 16 months (P.-Y., H, 2013). In our study, based on Eq. (1), the regression curves for *sul1* concentration for two crop seasons in manured agricultural soil were obtained (Fig. 3). An increase of more than one order of magnitude of *sul1* concentration in terms of absolute abundance compared to the baseline level can be observed 1 day after manure application. Measured values and *sul1* regression line clearly demonstrate the soil resilience ability to come back to baseline levels within the same vegetation season. Moreover, similar pattern can be seen for the second crop vegetation season, reinforcing the concept described also by Peng et al. that the die off rate of allochthonous ARB harboring different ARGs in the agricultural soil is greater than its capacity to establish themselves in the new environment (Peng et al., 2017).

It is worth noting that the baseline levels of the tested ARGs in manured and non-manured agricultural soils were the same as in forest soil, with low anthropogenic influences, in the same catchment, but in due distance, underlining that background concentrations of ARGs represent a key factor in data interpretation (Demanèche et al., 2008). Agricultural management practices in human impacted environments may pose a great impact on the soil natural microbial communities (Bevivino et al., 2014). However, the soil natural resilience capacity plays a key role in the persistence and dissemination of ARGs in the environments (Martínez et al., 2015).

The interdependent relationship between soil ARG enrichment after animal manure application, the background soil ARG levels and soil characteristics was also described by Munier and Xagoraraki (Munir and Xagoraraki, 2011). ARGs conferring resistance to different classes of antibiotics were found in pristine soil environments, with low anthropogenic impact, such as forest soil (Hu et al., 2016), highlighting the fact that high concentrations of ARGs reside naturally in these habitats (Forsberg et al., 2014; Forsberg et al., 2012). Our findings are in accordance with the study of Hu et al. (2016) that indicates a stable concentration of the tested ARGs in forest soil for *tet(W)*, *sul1* and *vanA* over the study period. *VanA* shows very similar concentrations in agricultural and forest soils, suggesting that agricultural management practices do not pose a great influence on its abundance and persistence (Fig. 4). Notable, the total bacterial abundance presents the same pattern as *vanA* indicating that the total microbial number remains rather stable (Fig. 5), meanwhile the relative abundance of different ARGs changed, being influenced by environmental factors (rainfall events, soil and air temperature, relative humidity, wind and atmospheric pressure) and agricultural management practices.

The ARG with the lowest abundance in all tested samples, except forest soil and fresh pig faeces, is represented by the *aph(3′)-IIa* resistance gene. Considering the association of *aph(3′)-IIa* with genetically modified organisms (GMOs) and its very low abundance in agricultural

soils in Austria, the results are consistent with the earlier findings (Woegerbauer et al., 2015b). Surprisingly, a high concentration of *aph(3′)-IIa* was detected in the pig manure slurry samples whereas the resistance gene was not detected in the fresh pig faeces before the storage period. It was shown that the storage period increases the abundance and persistence of different ARGs, from fresh manure to stored manure, up to five folds (Ruuskanen et al., 2016). In our study, a higher abundance was detected for five out of seven resistance genes (*sul1*, *ermB*, *vanA*, *aph(3′)-IIa* and bla_{TEM-1}) ranging from 1 to 4 log units after storage period (Fig. 1). Interestingly, for *tet(W)* and *aph(3′)-IIIa* the concentrations remain rather similar before and after storage period, thus reflecting the impact of gene localization (plasmid or chromosomal), the mechanism and conditions of transmission (vertical or horizontal gene transfer) on the gene proliferation and dissemination.

A key finding of the present study is that selected ARGs were influenced by pesticide application in the non-manured field. Our data indicate that the applied pesticide (herbicide) exerted a selection pressure on the prevalence and abundance of three out of seven tested ARGs (*ermB*, *aph(3′)-IIIa* and *tet(W)*) (Fig. 6). Consistent with our preliminary results, Kurenbach et al. (2018) reported the complex effect of herbicide on the evolution of antibiotic resistance, emphasizing that the application of pesticides does not have the same impact on different ARB carrying specific ARGs (Kurenbach et al., 2018). Noteworthy, no effect on the prevalence and abundance of the targeted ARGs was observed during the two vegetation periods in the manured soil after pesticide application.

5. Conclusion

This study supports the affirmation that agricultural soil environments show a strong capacity to alleviate externally disturbances induced by amendments with fertilizers and pesticides in endogenous ARG concentrations. After manure amendment, ARG concentrations return to baseline levels within a crop growing season. Pesticide application raised the relative abundances of *ermB*, *aph(3′)-IIIa*, and *tet(W)* in non-manured soil for a short period of time. The results showed that high prevalence and abundance of naturally occurring ARGs, considered as background concentrations, can be found in forest soil. Background ARG concentrations in soil with low human impact are key factors in data interpretation. *VanA* concentrations remain rather stable in agricultural as well as in forest soils over the two years longitudinal study, suggesting that agricultural management practices do not have the same impact on different ARB carrying specific ARGs. Overall, the agricultural management practices influence the indigenous soil resistome, but background and baseline ARG concentrations are to be carefully considered in accurate experimental design, data interpretation and ARG risk assessment.

CRediT authorship contribution statement

Elena Radu: Investigation, Writing - original draft. **Markus Wögerbauer:** Conceptualization, Writing - review & editing. **Gerhard Rab:** Resources, Data curation. **Matthias Oismüller:** Data curation. **Peter Strauss:** Resources, Writing - review & editing. **Peter Hufnagl:** Methodology, Resources. **Richard A. Gottsberger:** Methodology, Resources. **Jörg Krampe:** Writing - review & editing. **Karin Weyermair:** Formal analysis. **Norbert Kreuzinger:** Supervision, Funding acquisition, Writing - review & editing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.143699.

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