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Consequences of Manure and Fertilizers over Time on Resistance to Antibiotics in Soil

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Abstract

Manure and chemical fertilizer applications significantly affect bacterial community populations in soil, also it is even elusive that such changes will affect resistance to antibiotics in the soil and the underlying processes. In the research, bacterial populations with ARGs (Antibiotic Resistance Genes) soils from one lengthy field research (25 years) were described using Illumina HiSeq sequencing and high-throughput quantitative PCR, respectively. There were five distinct treatments: Pig manure (M), chemical fertilizers (NPK), NPK with straw return (NPKS), no fertilizers (control), and all of the above (NPKM). NPK and NPKS both reducesoil pH and caused a large change in bacterial populations, but they only found a little impact on the range and abundance of the ARG. The strong influence of NPK hampered the effect of straw return. Comparatively, increases in pig manure (M and NPKM) preserved every variety of the bacterial population dramatically changed both profiles of the ARG adding more Pig manure-derived ARGs and a rise in every number of native ARG members. ARGs and transposase genes co-occur strongly in soil bacteria in manured soils, which may be the result of horizontal gene transfers (HGT). Their findings point to the significance of HGT in preserving the composition of ARG in agricultural soils, particularly the above-mentioned that receive regular fertilizer applications.

Keywords: Manure, Bacterial community, Antibiotic resistance genes, NPK

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1. Introduction

The concentration of antibiotic residue in manure put the native microbial communities under selective pressure after manure was applied to the soil, and bacteria may generate antibiotic resistance genes (ARGs) by spontaneous horizontal gene transfer or mutation, which would cause resistant microbes to increase. Antibiotics also genes for antibiotic resistance are naturally found in soil (ARGs). Environmental resistance called a key factor in determining the human pathogen resistome, includes ARGs in the soil as significant components. Without any anthropogenic effect, native ARG groups may be detected in soil bacterial populations. In addition to the native inhabitants, soil bacteria can also pick up exogenous sources of resistance, such as in every case with the frequently observed transfer of ARG into agricultural soils from animal manure [1]. Reusing animal manure in agricultural soils is a significant method of managing nutrients that can increase soil fertility. However, because antibiotics are so widely used in animal industries for the diagnosis, treatment, and prevention of illness as well as growth-promoting feed additives, significant quantities of antibiotics are not absorbed and the associated ARGs have unintentionallstand released after manure treatments, into the soil environment [2]. Many commensal or pathogenic microorganisms in the surroundings host the different ARGs. As a function, likely, changes to the bacterial community might potentially affect the ARG profile. Previous research has demonstrated a strong correlation between resistome composition in sludge composts and soils and bacterial phylogeny and taxonomic organization [3]. A soil's bacterial diversity, composition, and structure can be impacted by the application of animal manure. There is some proof that manure has a long-lasting impact on soil resistance mostly via changing the bacterial composition rather than by introducing bacteria directly from the manure. Chemical fertilizer applications over an extended period can significantly affect the soil's characteristics and bacterial ecology. Nevertheless, there is limited evidence on how alterations in ARG resistome in the soil are affected by the bacterial population when animal manure and chemical fertilizers are applied over an extended time [4]. The bacterial and soil resistome population responds to repeated use of chemical fertilizers and animal manure, as well as how these two factors interact. That ARG- and perhaps antibiotic-containing

organic matter-rich animal dung has distinct effects on chemical fertilizers have on the bacterial population and resistome of the soil. In soils treated with varied quantities of manure, they analyzed the bacterial populations with ARGs and chemical fertilizers for 25 years to test this hypothesis. Their results revealed that, despite substantial links between the soil ARGs' composition and the taxonomic structures of bacteria, the key elements influencing ARG profiles in manured soils were exogenous ARG introduction and the HGT procedure [5]. The paper [6] was designed to ascertain the impact of years of dairy manure on the quantity and presence of antibiotic resistance genes (ARGs) in farming soil for a four-year agricultural production period. The application of various fertilizers during the pakchoi plant's growth and after harvest allowed Antibiotic Resistance Genes (ARGs) and their behavior in the soil will be investigated [7]. The paper [8] spread in the soil environment of agricultural areas when livestock manure is applied. Yet, it is still unclear how ARGs migrate vertically and what causes them to do so in manured soil when swine manure is applied. Real-time qPCR was used to monitor and analyze the methods of bacterial populations, Mobile Genetic Elements (MGEs), and ARGs after long-term swine manure treatment, at various soil depths (0–80 cm). The paper [9] intended to consolidate and critically assess research on the elimination of Mobile Genetic Elements (MGEs), Antibiotic Resistance Genes (ARGs), and Veterinary Antibiotics (VAs) through Anaerobic Digestion (AD) of manure and identify regions that require attention for more effective removal. The paper [10] suggested soil types may impact the ARG profiles in greenhouse soil, comparing research on the fate of ARGs in various manured soil kinds tested in the field are currently lacking. The paper [11] used two approaches to explore agricultural soils and dairy manure-derived amendments: shotgun metagenomics resistome profiling and the qPCR measurement of anthropogenic ARG markers. Applications of long-term manure to paddy soil were particularly attentive to the impacts due to the presence of antibiotics and genes associated with antibiotic resistance on denitrification (ARGs) on anaerobic ammonium oxidation (anammox) [12]. The paper [13] hypothesized that the roots of plants may significantly impact every destiny of certain antibiotics in fields treated with less manure, but impact genes related to mobility and antibiotic resistance. The paper [14] dissolved organic carbon (DOC) and concentrations of antibiotics in the aqueous phase of soil have been found to positively correlate with the incubation period. Except for chlortetracycline and lincomycin, adding composted adding manure to the soil improved the apparent sorption coefficients of these antibiotics. The paper [15] suggested that different soil types get an impact on the event and growth of antibiotic resistance as well as the expansion of Antibiotic Resistant Bacteria (ARB) and Antibiotic Resistance Genes (ARGs), it is crucial to comprehend these effects occur.

The Quantitative PCR at high throughput with Illumina HiSeq sequencing was used to characterize bacterial populations in soils from antibiotic resistance genes (ARGs) a protracted field experiment.

2. Materials and methods

2.1 Methods for taking soil samples and performing tests

Examinations were started in 1990 and are now ongoing at the Assam Province of India's (111˚53′E, 26°45′N) Qiyang Red Soil Experimental Station. The soil is a kind of quaternary red clay known as ferric cambisol. The property has a standard rotational farming system of maize and wheat and is situated in a subtropical climate. The experimental setup and fertilization plan were well-reported. Table 1 displays the rates for fertilizers, manure, and straw.

Before crops continue being sown, 70% of fertilizers were used in the maize season, with the remaining 30% used during the wheat season. Each treatment consisted of two plots, each 20m ×10m in size, divided by cement plates that were a depth of 100 cm. For the purpose of sampling; however, only three of these sub-plots were chosen at random to serve as duplicates for the additional analysis. Each sub-plot had six cores (0–20 cm) that were augured out and bulked together as one duplicate. All samples were carried as quickly as possible to the lab, maintained on ice, transported, and stored in sterile plastic bags. Subsamples were dried by air, at 4 °C and passed through a 0.15-mm filter to determine their chemical characteristics. The 2.0-mm filter was used to filter DNA extraction subsamples, which were then freeze-dried and maintained at 80˚C.

2.2 Identifying soil qualities

A pH meter was used to determine the pH of the soil in a suspension of 1:5 dirt to water (Germany's Sartorius, PB-10). Soil Organic Carbon (SOC) and Total Nitrogen (TN) were calculated using a CN analyzer. After digesting soils with aqua regia, P, K, Ca, Mg, Cu, and Zn were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.3 DNA purification and extraction

A Power soil of around 0.25 g of DNA was extracted using a DNA Isolation Kit of freeze-dried soil. The DNA solutions from each sample's two extractions were then mixed. To get rid of PCR inhibitors, unpurified DNA samples were processed. In a short, the isolated DNA was overnight treated at 20 °C with 2.5 volume of ethanol, 3 M sodium acetate in 0.1 volume (pH = 5.2), and 0.1 volume. Centrifugation on 16,000 g for one hour at 4 °C produced every DNA pellet. The pellet was cleaned with ethanol at 70% before being redissolved in the Isolation Kit's elution solution (10 mM Tris). A spectrophotometer called the NanoDrop 2000C was used to evaluate the DNA's quality. Quant-iTPicoGreen test kit concentrations were used to identify double-stranded DNA (dsDNA), which was then for further analysis, kept at 20˚C.

2.4 Bacterial community characterization

To amplify a gene, the primer sets 515 F (5′-barcode-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) were utilized 16S

rRNA gene's V4-V5 regions. As previously mentioned, the procedure was completed using a DNA thermal cycler, such as the ABI GeneAmp PCR System 9700. The AxyPrep DNA Gel Extraction Kit was used to purify and quantify the amplicons after they were cut from 2% agarose gels with Qubit 3.0, paired-end sequencing (2 x 250) was performed on the data using the Illumina HiSeq platform after the samples were mixed in equal amounts (equimolar ratios). The adapter, as well as any unclear nucleotides, and barcodes, were eliminated from the raw data, as was previously detailed. This was followed by read assembly using QIIME 1.17. Clean data that had been created were examined using Operational Taxonomy Units (OTU) as an open reference, QIIME 1.9 selection was made following online instructions and default settings. Using UCLUST and RDP classifiers, respectively, the OTUs were selected (97% similarity) and the taxonomy was assigned (80% confidence threshold). Using filter_otus_from_out_table_py, deleted from the OTU table were sequences that are chimeric or that are present in less than three samples. Filter_taxa_from_otu_table.py was used to exclude Sequencing which had classified as c_Chloroplast, K_Archaea, f_Mitochondria, and those without an assignment (Unassigned). They retrieved totaling 897,652 sequences (averaging 47,822–67,409), which were organized through 7958 OTUs. Using a random sampling of 47,822 sequences, QIIME estimated the Shannon index and based on the OTU table, a Bray-Curtis dissimilarity. The NCBI Sequence Read Archive (SRA) database received the raw reads (Accession Number: SRP111302).

2.5 Quantitative PCR with high throughput using ARGs (HT-qPCR)

The Real-time qPCR Wafergen Smart Chip system platform was used to investigate six transposase genes (*tnp*A), 213 ARGs (285 primers), and one 16S rRNA gene on nanochips using the previously reported methods. The 213 ARGs included several with multiple primer sets. The acceptable amplification efficiency range is between (80%-20%) and the detection limit (Ct = 31) was adjusted in accordance. On a single nanochip, three technical replicas of each sample were inserted. Randomly placed on three separate nanochips were three biological duplicates. Further analysis was conducted on all detections in each treatment's technical and biological replicas. The findings from several same gene primer sets were aggregated and also considered just as originating from a single gene to compute abundance, diversity, Procrustes, and variance partitioning analyses. The 16S rRNA gene's relative copy number normalized served as the unit of measurement for the ARG abundances.

The calculation of absolute ARG abundance followed the preceding instructions. Beta-lactam, tetracycline, vancomycin, sulfonamide, and other macrolide-lincosamide streptogramins B (MLSB), aminoglycosides, chloramphenicol, and antibiotic classes were included in the classification of ARGs. In addition, ARGs were classified into the following four categories: inactivation of antibiotics, cellular defence, efflux pump, and maybe other or unknown mechanisms. The Ct values of the primer sets were utilized in the computation of the fold changes representing the improvements of ARGs that took place in soils that received fertilizers or manure in contrast to the soil that served as the control. This was done in order to determine if the improvements of ARGs were caused by the addition of fertilizers or manure.

2.6 Data analysis

Using SPSS 18.0 and a combination of other statistical programs, multiple comparisons of the treatments were carried out with the analysis of variance (ANOVA) and the Duncan test set at the 0.01 probability level. The ARG profiles' diversity indices and diversity Principal Coordinates Analysis (PCoA) were computed using the vegan package in R (version 3.1.3). Canonical Correspondence Analysis (CCA) and Redundancy Analysis (RDA) were carried out in R using vegan. The Spearman correlation coefficients were produced using R and used to build networks in Cytoscape 3.4.0. Two distinct control groups against manured soils or control versus fertilized soils underwent analyses of VPA and networks.

3. Results and Discussions

3.1 Soil properties

The control soil's pH value was 5.69. Table 1. Chemical fertilizer (NPK) use over an extended time has significantly acidified the soil, lowering its pH by 1.5 units. The addition of straw did not lessen the acidity of the soil (NPKS). Manure treatments alone raised soil pH by 0.95 units; however, NPK applications with manure (NPKM) avoided soil acidification. In comparison to the control soil, Soil Organic Carbon (SOC) levels rose in response to the NPK, NPKS, M, and NPKM treatments by 23%, 26%, 92%, and 69%, respectively. In comparison to every treatment about NPKM, M got more manure Table 1. In comparison to unfertilized and NPK and NPKS-fertilized soils, manured soils had substantially greater higher (all P 0.01) quantities with P, Ca, Mg, Cu, and Zn than the control soils.

3.2 ARG and bacterial community diversity

The five soils had combined pairs of ARG primer sets altogether, or 101 different ARGs. NPKM, M, NPKS, and NPK are every therapy used in the control group, there were 24, 41, 36, 94, and 98 distinct ARGs found, respectively. The other four soils contained all of the ARGs found in the control soils. Applications of manure or chemical fertilizers considerably improved the αdiversity of ARG profiles as compared to the control, with manure treatments having a bigger impact as shown the Figure 1a. In the first coordinate, M and NPKM manured soils and the control showed a significant difference in PCoA analysis, which explained 89.9% of the difference as shown the Figure 1b. While mostly on the second coordinate, which accounts for only 6.5% of the difference, a substantial variation from the control was seen in the ARG profile in soil that had received NPK or NPKS fertilization. There was no discernible difference both between NPK and NPKS and between M and NPKM. Potential explanatory factors for soil pH and N, SOC, P, and Mg contents, and structure of the ARG profiles' variance included these variables. Extreme Collinearity was present (variance inflation factor > 10) observed in every concentration of Cu and Zn. SOC had a significant role in explaining the differences between

manured soils and non-manured soils (M and NPKM) (control, NPK, and NPKS).

Application of manured (M and NPKM) did not appreciably impact every bacterial community's α-diversity. Figure 1c shows that the bacterial community's -diversity was dramatically reduced as a result of the NPKS and NPK treatment. The bacteria community's -diversity and soil pH exhibited a substantial and favorable connection (P = 0.001; Spearman, r = 0.76). On every first axis, the organization compared to the (P = 0.015, ANOSIM) control, as well as individuals in M and NPKM (P = 0.002, ANOSIM), was substantially different in NPK and NPKS, which explained 63.5% of the variance. The remedies for NPKM and M differed significantly from every control group as well (P = 0.011, ANOSIM), although the main change mainly affected the second axis and only partially explained the variance, at 18.0%. NPK and NPKS are grouped in the PCoA figure, while M and NPKM are grouped in Figure 1d.

3.3 ARG and tnpA distribution and composition

In the control soil, 0.04 copies of the 16S rRNA gene were discovered to be ARGs' total abundance. Manure amendment dramatically raised the abundance of the 16S rRNA gene rose by 8.9 and 7.9 times, respectively, in M and NPKM, to 0.37 and 0.33 copies/gene. There was little distinction between the two manure treatments. Figure 2a shows that the NPK and NPKS treatments had no discernible impact on the overall ARG abundance.

The antibiotic efflux pump was absent from the control soil the most common form of resistance (92.5%). The proportion of the antibiotic deactivation mechanism rose as a result of the use of chemical fertilizers or manure, rising from 6.7% in the control to 34.9%-43.1%. Sulfonamide and tetracycline resistance weren't discovered in the control group based on every type of resistance, however, all seven other ARG types were found in Figure 2b. Genes for multiple drug resistance were prevalent in the control group, making for 83.5% of the overall abundance. Chloramphenicol, beta-lactam, and MLSB resistance genes made up 7.3%, 5.5%, and 2.9%, respectively, of overall abundance. Chemical and manure fertilizer applications had a considerable influence on the soil ARG composition. Beta-lactam, vancomycin, and pyrazinamide-associated ARG frequencies were considerably higher after treatments with both NPK and NPKS. The frequency and prevalence rate of ARGs that confer Vancomycin, pyrazinamide, aminoglycoside, tetracycline, and beta-lactam resistance were both markedly enhanced by treatments with M and NPKM. Sulfonamide resistance genes were noticeably found in manure-treated soils.

For the ARG types, no appreciable change was found between the NPKM and M treatments Figure 2b. Every two categories of resistance genes that were most prevalent in manured or fertilized soils were beta-lactam gene resistance and genes as multiple drug resistance. Genes for multidrug resistance were present in 38.2%, 55.2%, 41.8%, and 35.0% of the total population respectively, for every procedure for treating NPK, NPKS, M, and NPKM. Figure 2b shows that in the four soils, regarding beta-lactam resistance genes, the corresponding percentages were 40.9%, 32.2%, 26.7%, and 31.3%.

3.4 ARG augmentation

The 137 ARG primer set pairs that were discovered in any soil might be broken down into 10 categories based on their distribution (Groups A to J). Group A had 26 sets of ARG primers in all soils, including the control, and they most possibly represented every native soil resistome member. Every median fold change in NPK and NPKS was 1.8 and 1.0, respectively, but there was little to no increase in ARG enrichment in soils that had received chemical fertilizers. The greatest enrichment values for individual ARGs were 28.6, 9.6, 167, and 126, NPKS, M, and NPKM, respectively. Genes shared by M but not the control, NPKM, NPK, or NPKS made comprised Group D, E, and F.

3.5 Variables that affect the soil ARG profiles

The primary contributions to the design of ARG profiles were identified using every Variation Partitioning Analysis (VPA). These contributors included every soil characteristic, a bacterial population, and mobile genetic elements (MGEs). Figure 1d shows the primary coordinates analysis of the soil bacterial population using the OTU table, which uses PCo1 and PCo2 to identify the bacterial community's composition. Genes for transposase (*tnp*A) were added to the publication as major components of MGEs to denote the potential for gene movement. VPA demonstrated certain t*np*A genes, bacterial community structure, and soil characteristics could all be used to account for heterogeneity in the ARG profile structure. The three different types of variables' individual effects, meanwhile, paled in comparison to their combined impacts Figure 3. The characteristics of the soil and the bacterial community were related to the most significant contributor (36.3%) between the various soils treated with NPK, NPKS, also control, as shown in Figure 3a. In the paper using control, M, and NPKM interactions between bacterial populations, genes*tnpA, and* soil factors generated 66.1% of ARG profile structural variation, as shown in Figure 3b. In the two groups of studies, the overall advantages of the bacterial community's composition and soil attributes were equivalent. The *tnp*A genes had an (84.3%) control, M, and NPKM rate significantly larger compared to the paper with the (53.3%) control, NPK, and NPKS, as shown in Figure 3.

3.6 The community of soil bacteria and ARGs

The bacterial community and the ARG profile were investigated using the Mantel test and the Procrustes analysis. The findings demonstrated a certain, in general, the structure of ARG profiles matched well with that of bacterial composition in response to various fertilizer or manure treatments.

3.7 The soil contains native ARGs

The presence of 24 ARGs in the control soil, which hasn't gotten any manure or fertilizer in 25 years, highlights how commonplace ARGs are in the soil environment. These ARGs, which were also found in the soils that had been fertilized or manured, are thought to reflect the local resistome existing in the soil at the experiment location. The

majority of native ARGs are connected to MLSB, chloramphenicol, and other medication resistance.

Every control soil had some ARGs that were anti-beta-lactam resistant, chloramphenicol, and both MLSB, but their abundance and frequency are both substantially lower than those of across all soil types, and multidrug resistance genes are much lower. These efficiencies be a result of various soil types supporting various bacterial populations, as well as various background amounts of metals and antibiotics.

3.8 Manure elevated ARG concentrations while retaining the diversity of the bacterial community

Permanent pig dung treatments greatly enhanced the quantity and soil rich in AGRs, quadrupling their number of ARGs also doubling dozens to hundreds of times the number of native ARGs. Table 1 shows that while pig manure treatments raised SOC and decreased the acidity of the soil caused by chemical fertilizers, significantly increasing ARG abundance poses a threat to the ecosystem that needs to be addressed properly. By killing their bacterial hosts, techniques like thermophilic composting may be able to lower the number of ARGs in manure, however, complete ARG eradication is challenging due to the genes' transferability and repeatability.

3.9 The ARG profile had less of an effect on the bacterial ecology than chemical fertilizers

The diversity and structure of the bacterial population were more significantly impacted by the addition of chemical fertilizers (NPK & NPKS) than were those of the ARG profile. As shown the figure 1 nitrogen applied continuously (urea) dramatically reduced soil Table 1, the bacterial diversity and markedly altered every composition of the bacterial community in soil, as shown the Figures 1c and d. The most significant element influencing the diversity and community structure of soil microbes is understood to be soil pH. Chemical fertilizers had a significant impact on the bacterial population, but they had little impact on the soil's ARG diversity and abundance, as shown in Figure 2a. Given that chemical fertilizers don't include exogenous ARGs, this is not surprising. Nonetheless, there was still evidence of ARG composition change, as shown in Figures 1b and 2b. It has been claimed that the soil's microbial population and chemical composition are key factors determining the antibiotic resistance of various matrixes.

3.10 ARG composition and the bacterial community of the soil

There have demonstrated every harbored ARGs have a close association with the organization of bacterial communities in various settings. The compositional pattern of the ARG resistome and the bacterial composition's structure was shown to strongly correspond in the current investigation (Figure 5). Nevertheless, chemical fertilizer treatments drastically changed the makeup of the bacterial population while having no impact on the ARG profile. Manure treatments had a greater impact on the quantity and variety of soil ARGs than on the bacterial population, as shown in Figure 1.

3.11HGTs and resistance clusters in manure-exposed soil

Localization of ARGs on nearby genetic elements can result via HGT using MGEs such as plasmids, integrons, and transposons. Every upkeep about ARG traces in manure-treated soils may have been significantly aided by HGT in the current investigation. In manured soils, there is evidence that ARGs cluster prominently among themselves rather than with the taxonomy of the bacteria. This implies that certain genes might be restricted to certain bacterial lineages and that their co-occurrence may have been caused by ARG mobilizations.

Table 1: Various therapies' fertilization plans

Control	0	0	0	0	0	5.69 (0.26) b	0.86 (0.01) d
NPK	651	997	200	0	0	4.13 (0.13) c	1.06 (0.03) c
NPKS	651	997	200	0	Half straw return	4.09 (0.10) c	1.08 (0.01) c
M	0	0	0	60.0	0	6.64 (0.13) a	1.65 (0.04) a
NPKM	195	997	200	41.7	0	5.84 (0.09) b	1.45 (0.03) b

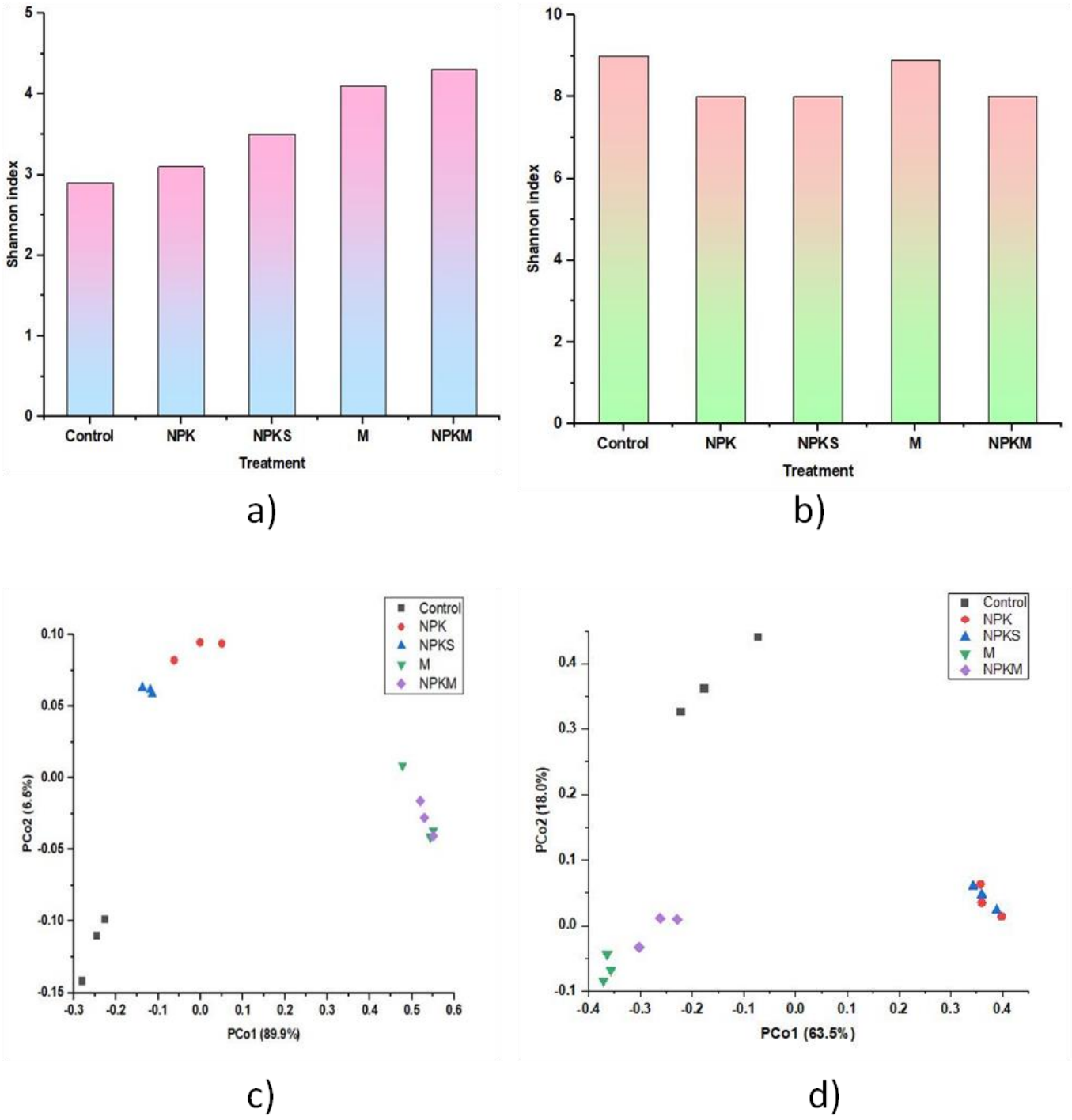

Figure 1: Bacterial community diversity and ARG diversity in soils; (a) The soil ARG Shannon index; (b) The ARGs' principal coordinates analysis; (c) Shannon index of the microbial community in soil; (d) The soil bacterial community is coordinated by the principal.

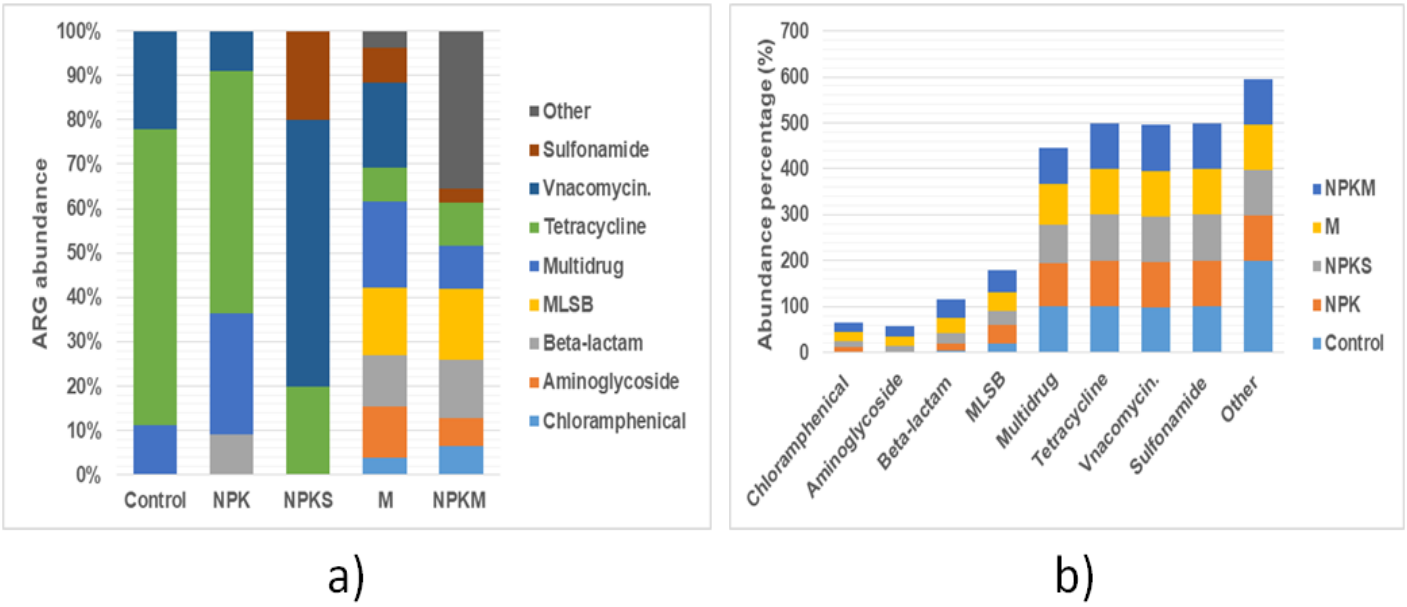

Figure 2:ARG distribution and composition across various treatments; (a) ARG abundance serves as a measure for the composition; (b) The proportion of each type of resistance

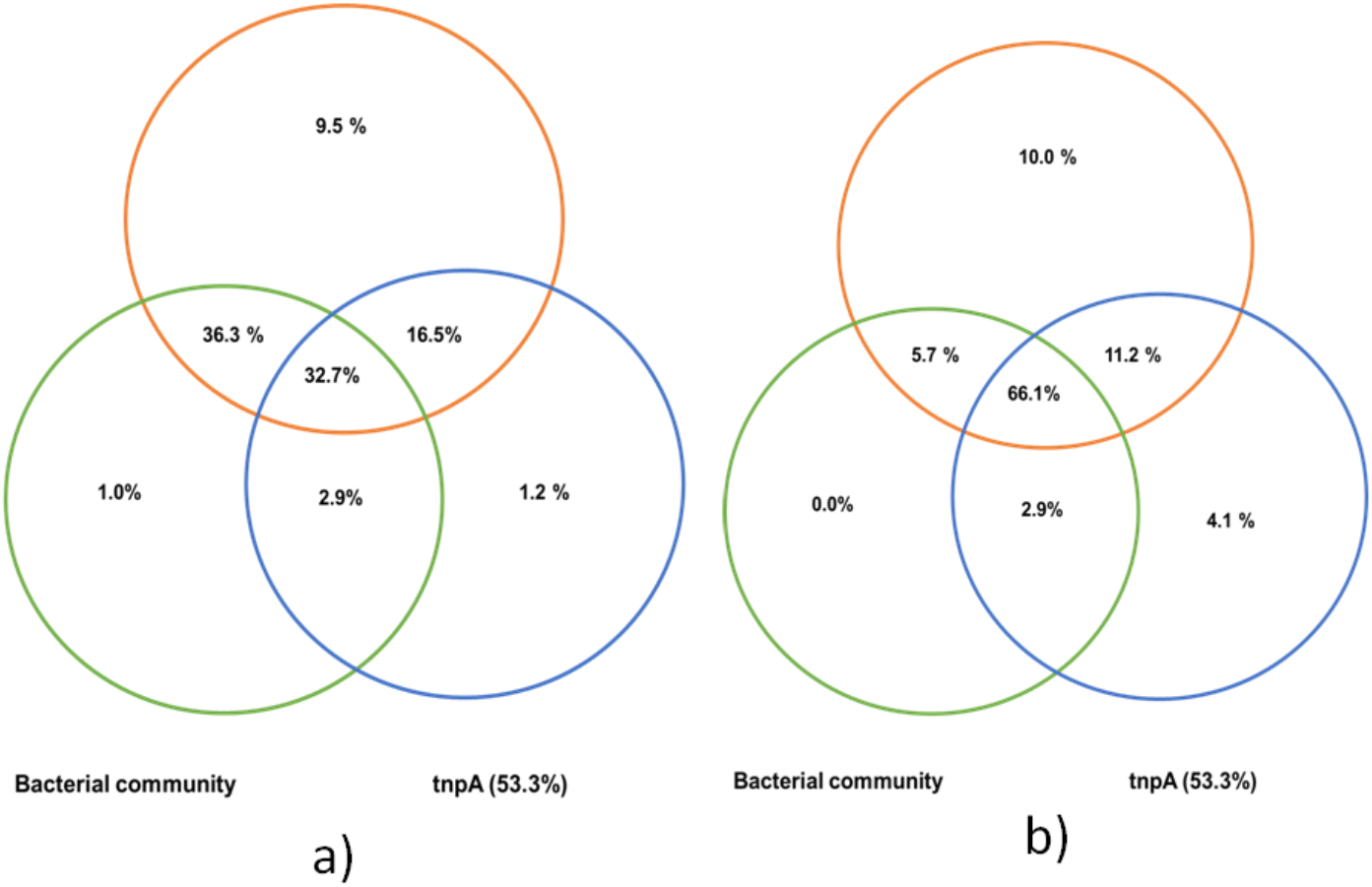

Figure 3: The soil's ARG profile structure; (a) Treatments for the control, NPK, and NPKS analyses; (b) Analysed using the control, M, and NPKM treatments

4. Conclusions

The present paper's findings demonstrated such variety also Antibiotic Resistance Genes (ARGs) were substantially more prevalent in soil than boosted by repeated applications of pig dung. Applications of manure both increased the native ARGs in the soil and introduced foreign ARGs. In manured soils, clusters of ARGs that are mostly independent of bacterial phylogeny point to the predominance of ARG horizontal gene transfer that was prompted by manure additions. Chemical fertilizer use for an extended time changed the bacterial population in the soil but had no impact on the ARG profile.

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