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Abstract

Post-digestate treatments may reduce the risk linked to Antibiotic Resistant Genes (ARGs) release with digestate direct land application. Thus, this study aimed to evaluate post-digestate composting and co-composting with biogas production feedstock (maize silage, food processing waste, and poultry litter) effect on abundance of selected ARGs: *erm* (B), *tet* (K), *tet* (M), *tet* (O), and *tet* (S) genes. More than 80% of all ARGs were removed after 90 days of composting but removals from co-composting were lower. Bacteroidetes, Firmicutes, and Proteobacteria dominated fresh digestate, but a network analysis indicated only a few genera were potential hosts of ARGs. Canonical correspondence analysis showed more than 90% variations in ARGs abundance were explained by water extractable trace elements, indicating a strong relationship. The study illustrates the potential of post-digestate composting to mitigate ARGs in the environment.

Keywords: ARGs removal; biogas feedstock; co-composting; poultry litter; trace elements

1. Introduction

Antibiotic resistance genes (ARGs) have been threatening the global health sector and some claim inappropriate use of antibiotics are the main drivers (He et al., 2020). Efforts made to reduce or mitigate the phenomenon of antibiotic resistance include banning growth promoters (Callens et al., 2018), avoiding inappropriate use of drugs (Holmes et al., 2016; Llor and Bjerrum, 2014), and developing novel drugs from natural products (Jackson et al., 2018). Moreover, mitigating the spread of ARGs in the environment is essential to reduce potential health risks. There are several pathways through which ARGs can be released to the environment such as manure land application (Tien et al., 2017), disposal of poorly treated pharmaceutical sludge (Tong et al., 2018), and digestate land application (Derongs et al., 2020).

Anaerobic digestion (AD) of organic wastes is widely reported to mitigate the spread of ARGs despite the rate of removal varying not only by the type of ARGs but also the type and scale of the reactor, retention time, temperature, feedstock type, etc (Gurmessa et al., 2020). The ultimate goal of AD is energy production, but it has been widely reported that changes in the biological and physicochemical processes could remove ARGs (Couch et al., 2019; Sun et al., 2018; Zhang et al., 2018). However, AD does not always help remove ARGs. It has been reported that AD plants can also be a hub for the emergence of new or enrichment of the existing ARGs, promoted by the favorable conditions in the reactors, such as the possible accumulation of antibiotic residues and microbial community dynamics (Ma et al., 2011; Wallace et al., 2018). There is therefore a concern related to the possible spread of ARGs with direct use of digestate as a fertilizer, although this is largely not been studied.

Where AD could suppress abundance of ARGs in organic wastes, inefficiency is possible, thus post-digestate treatments have been suggested to reduce the release of ARGs with digestate use.

Post-digestate composting is one of such strategies (Zhang et al., 2019), and it has been reported to remove about 75% of selected tetracycline resistance genes *tet*(G), *tet*(C) and *tet*(Q) from cattle manure (Qian et al., 2018). ARGs removal with composting is affected by changes in temperature (thermophilic and mesophilic), dynamics of chemical compositions (heavy metals, organic matter content, etc.), and shifts in microbial composition (Oliver et al., 2020). Variation in removal rate could also be due to waste material being composted and composting time length.

Solid digestate has poor nutrient and low total solids (TS) content to effectively support air movement and functionality of microbial activities during composting, which could negatively influence final compost quality and time to maturity (Gurmessa et al., 2021b). On the other hand, co-composting could have better advantages over composting a sole material, including improving ARGs removal efficiency (Chen et al., 2021). Thus, co-composting digestate with fresh and locally available inexpensive material having a better TS content and greater nutrient supply for microbes could be sought for economically feasible effective industrial level composting.

The current study aimed at evaluating post-digestate composting and co-composting with biogas production feedstock effect on the removal of ARGs encoding the resistance to antibiotics conventionally used in animal husbandry and clinical practice, such as macrolide-lincosamide-streptogramin B (MLS_B) [*erm*(B)] and tetracyclines [*tet*(K), *tet*(M), *tet*(O), *tet*(S)] using quantitative PCR (qPCR) assays. The study hypothesized composting solid digestate or co-composting of the same with inputs for biogas production further removes significant proportions of ARGs and alters microbial composition.

2. Materials and Methods

2.1. Experimental setup and sampling

Digestate (fresh) was obtained from a biogas plant located in Marche Region, Italy. It is a byproduct of anaerobic digestion consisting of about 10% poultry litter (composed of chicken manure and wheat straw in a rough ratio of 85 and 15%, respectively) and 90% of mix of other biomass such as maize silage, food processing waste, and fruit processing byproducts. Pilot level composting and co-composting piles were set up as described in Table 1, each amounting to 300 kg (on wet basis) and comprising of digestate and locally available materials in a ratio of 4:1 (w/w). The experiment was set up in completely randomized design with sub-sampling. The composting materials were stacked inside high-density polyethylene boxes, each having volume of 1 m^3. The boxes were modified to allow air movement, and the piles were turned weekly during the thermophilic phase (≈49 days), every two weeks during the mesophilic phase (≈40 days), and every three weeks during the maturity phase. Composting lasted for 90 days, and samples were collected from each box at 0, 7, 35, 70, and 90 days of composting. Three samples were collected at about 5, 15, and 20 cm depth, after turning and thorough mixing. Then, 200 g of each sample was immediately stored at -20°C in a plastic bottle until analyzed for ARGs and microbial composition, whereas about 1 kg was dried at 40°C for chemical analysis.

2.2. Chemical analysis

Samples dried at 40 ^{0}C (about 1kg each) were ground, passed through a 2 mm sieve, and used to measure pH and total C and N content. The pH was determined potentiometrically in H_2O (1:8 w/v). Total C and N were determined by dry combustion method using a CHNS analyzer (EA-1110, Carlo Erba Instruments, Milan, Italy). Total solid content (TS) was estimated as the fraction of the dry mass after samples were dried at 105 ^{0}C for 24 h. Organic matter (OM) was

determined as the loss on ignition (Heiri et al., 2001) at 550 ^{0}C until constant weight was obtained. Water extractable Al, Ca, Ba, Cd, Cu, Fe, Mg, Mn, Na, Ni, P, Pb, S, and Zn were determined by using 10 g of ground compost samples that were added to 100 ml of distilled water (1:10 w/v) and shaken for about 1 h at 10 rpm. The suspension was then centrifuged for 10 minutes at 300 *g* and the solution filtered using Whatman 42 filter. The extract was used to determine the concentration of the elements on an ICP-MS (Pröfrock and Prange, 2012).

2.3. DNA extraction and qPCR quantification of AR genes

DNA was extracted from 0.25 g of each compost sample using E.Z.N.A. ®Soil DNA Kit (OMEGA Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's guidelines. Prior to qPCR analysis, the extracted DNA was checked for quantity and purity using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Moreover, the effective extraction of the bacterial DNA was checked by end-point PCR using the universal procaryotic primer pair 27f-1495r (Weisburg et al., 1991) targeting the bacterial 16S rRNA gene. DNA extracted from five reference bacteria strains, each carrying one of the ARGs under study were used as positive controls in the qPCR reactions as well as for the construction of qPCR standard curves as previously described by Vandeweyer et al. (2019). Each qPCR mixture was composed of 4 µL of extract, 5 µL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany), 900 nm of forward and reverse primers for each AR gene (Table 2), and nuclease-free water to reach the final reaction volume of 10 µL. The qPCR reactions were performed in a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany) with an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 95 °C for 15 s. The qPCR correlation coefficients (R^2) and amplification efficiencies were calculated automatically by Mastercycler® ep realplex software

from the slopes of the standard curves. The qPCR detection limit for each AR gene in study was estimated from standard curves created in the range from ~10^0 to 10^7 gene copies per reaction. For absolute quantification of the five ARGs in study, each extract from the compost samples was run in triplicate along with tenfold serial dilutions of the standards. Each gene copy number detected in the analyzed samples was determined from the slope of the corresponding standard curve. The blank (nuclease-free water instead of DNA extract) and the negative control [DNA extracted from *Enterococcus faecalis* JH2-2 strain (Jacob and Hobbs, 1974)] were run together with the samples. The melt curve analysis with the temperature gradually increasing from 60 to 95°C by 0.4°C/s was performed to check amplification specificity. The results are presented as: *i*) the log of average gene copy number per gram of dry matter of each sample ± standard deviation and *ii*) the fold change, which was estimated to evaluate the reduction of ARGs abundance during the composting period. The latter was estimated as log of the quotient of ARGs copy number in composted sample divided by that in initial digestate sample as previously described by Qian et al. (2018).

2.4. 16S rRNA gene amplicon target sequencing and bioinformatics analysis

DNA was used as a template for the amplification of the V3-V4 region of the 16S rRNA gene using primers and PCR condition described by Klindworth et al. (2013). PCR amplicons were then purified, tagged, and pooled following the Illumina metagenomic flow. Sequencing (paired end mode 2X250bp) was performed on a MiSeq Illumina platform according to the manufacturing instructions. After sequencing, reads were assembled by using the FLASH software. Joined reads were quality filtered with QIIME software and USEARCH was then used for removing chimeric sequencing. Sequences were clustered into operational taxonomic units (OTUs) at 97% of similarity and after the picking step of each centroids sequence taxonomy was

performed against the greengenes database by means the RDP classifier. OTUs table generated by QIIME was rarefied at the lowest number of sequences for sample.

2.5. Statistical analysis

Analysis of variance (ANOVA) conducted on log10 of ARGs abundance in digestate and final composts using Agricolae package in R, and least significance difference (LSD) tests were conducted to separate means when statistically significant results were obtained in the general ANOVA model. To understand the relationship between ARGs abundance and the environmental variables (chemical compositions and enzyme activities), Canonical correspondence analysis (CCA) was conducted using the vegan package in R (Oksanen et al., 2019). Non-metric multidimensional scaling (NMDS) was analyzed and plotted on the OTUs using the Bray-Curtis dissimilarity distances using ecodist package in R to evaluate differences in bacterial community structure among the different piles or shifts in the same from the fresh digestate to thermophilic and mesophilic phases of the composts. Multivariate analysis of variance (MANOVA) test was performed to evaluate the significance of the factors (piles or phases) on the OTUs (response variable). Both the CCA were conducted using and plotted using ggplot2 in R. Additionally, network analysis was conducted to understand co-occurrence of the ARGs and bacteria at phylum and genus level using R (for generating the weight values) and Gephi (for visualization). The weight values were positive and significant ($P<0.05$) coefficient values of Pearson's correlation.

3. Results and discussions

3.1. Physicochemical characteristics of composts

Temperature, pH, C/N ratio, organic matter (OM) content, water extractable macro nutrients, and trace elements were analyzed for digestate and the co-composting materials, and these were monitored over the composting period. Thermophilic temperature lasted for about 49 days. Organic matter declined over the composting period. While major macro nutrients, except Ca and S, increased, trace elements, except Cd and Pb, reduced over composting period. Details of the dynamics of these parameters were reported in Gurmessa et al. (2021b).

3.2. ARGs abundance in digestate and co-composting materials

qPCR standard curves created for each of the five ARGs were characterized by good amplification efficiencies, comprised between 0.91 and 0.99 for *tet*(K) and *tet*(S) genes, respectively, whereas R^2 values were 0.99 for all reactions. The lowest gene copy number per reaction in which the linearity was maintained (detection limit) was $< 10^1$ for the genes *erm*(B) and *tet*(O), and 10^2 for *tet*(K), *tet*(M), and *tet*(S).

All the five ARGs were detected in the digestate and co-composting materials (Fig. 1), but with great variations in number of copies among the types and co-composting materials, ranging from 3.6 log *tet*(O) in food processing waste to 11.3 log *tet*(M) in poultry litter. Poultry litter contained the greatest copies ($p<0.05$) of all ARGs followed by digestate, whereas maize silage and food processing waste had the lowest copies of ARGs. Generally, *tet*(M) was the most abundant gene ($p<0.001$) in digestate and co-composting materials followed by *tet*(K) and *erm*(B), whereas *tet*(O) and *tet*(S) were the least abundant gene in all the materials. The abundance of *erm*(B),

tet(O), and *tet*(S) in poultry litter was about two-fold log higher than that of maize silage and food processing waste.

Similarly, Agga et al. (2020) reported *tet*(M) as the most abundant ARG in digestates of three manure types suggesting possible differences in ARGs persistence under anaerobic digestion process.

3.3. Dynamics of ARGs during post-digestate composting and co-composting

In Fig. 2, the fold change illustrates dynamics of ARGs abundance during the 90 days of composting. ARGs abundance was affected both by the composition of the piles and composting time. However, the change in the abundance of most of the ARGs was not consistent during the thermophilic and mesophilic phases. During the thermophilic phase, the abundance of all genes but *erm*(B) decreased. The abundance of *tet*(K) gene increased during the mesophilic phase although it was reduced at maturity. In contrast, *tet*(S) copies were potentially reduced after a week of composting and, unlike other ARGs, enrichment was rarely observed. E-supplementary data of this work can be found in online version of the paper. This implies temperature dynamics over the composting period does not influence ARGs similarly. Compared to the digestate only pile (D00), ARGs abundance in the other piles was greater, and it was even the greatest in the DPL at the initial period, implying that the co-composting materials could be potential source of ARGs, particularly poultry litter (Fig.1). ARGs abundance in poultry litter, however, largely relies on how it has been handled or managed before use (Gurmessa et al., 2021a).

Relative to the fresh digestate, *tet*(O) was reduced by the greatest fold compared to the other genes investigated (Fig. 2), which was -6.8, -7.6, -8.7, and -10.9 log in the final composts of DPL, DMS, DCB, and D00, respectively. In contrast, the least reduction was found for *erm*(B), indicating the relatively higher persistence of this gene compared to the tetracycline resistant

genes, despite its relative abundance was lower than the other ARGs in the starting materials. Interestingly, ARGs abundance in D00 was reduced by greater fold than in the other piles. In the final composts, the lowest abundance of ARGs was found in D00 whereas the greatest was found in DPL. E-supplementary data of this work can be found in online version of the paper. These results suggest that the materials, especially poultry litter, used for co-composting might have promoted or be the source of the ARGs. This can be further explained by the positive log change in the DPL (Fig. 2), showing enrichment in ARGs with the addition of poultry litter to digestate.

It must be noted that fold change results discussed here were only in reference to the initial content in the piles. Compared to the copies number in the digestate, the benefit of using co-composting materials was not satisfactory, as *tet*(K) and *tet*(M) copies were enriched in DCB and DMS piles.

3.4. Bacteria community dynamics based on the 16S rRNA gene amplicon target sequencing

Three phyla dominated the bacteria community of fresh digestate: Firmicutes were the most abundant (73%), followed by Proteobacteria (18%), and Bacteroidetes (9%) (Fig. 3A). During composting, succession of Actinobacteria, Planctomycetes, and Verrucomicrobia formed a distinct bacteria community structure compared to that of fresh digestate. The emergence and presence of Actinobacteria in high proportion over the composting period indicates the well-functioning of the composts (Sundberg et al., 2013). At genus level, *Clostridium* (38%), *Sporosarcina* (33%), *Bacillus* (7%), and *Pseudomonas* (7%) were the top four dominant OTUs (Fig.3B).

Non-metric multidimensional scaling (NDMS) of the Bray-Curtis dissimilarity analysis was conducted to understand the effect of co-composting or temperature regime on bacterial community structure. The influence of piles on bacterial community was significant (p<0.01) (Fig.4A), with significant effect on abundance of Firmicutes (p<0.001) and Bacteroidetes (p <0.05) as MANOVA test revealed. The change in bacteria community structure was further elaborated with the non-metric multidimensional scaling (NDMS) of the Bray-Curtis dissimilarity analysis, which showed a significant (p<0.05) shift in the bacterial community structure between the digestate and the two phases of composting (Fig. 4B). MANOVA test showed Firmicutes were significantly (p<0.001) affected, and their relative abundance was reduced during the shift from initial phase to the thermophilic and mesophilic phases. The shift in the abundance of these phylum and the succession of new phyla or genera over the composting period may be indictor of the compost status.

Verrucomicrobia was not found in DCB during the thermophilic and mesophilic phases, nor in DPL during the mesophilic phase. This phylum is ubiquitous in soils, but its habitat is influenced by temperature, pH, and chemical composition (Freitas et al., 2012). Since it appeared during the composting period, it could be an indicator of a well-functioning compost.

Planctomycetes were observed in DCB, DMS, and DPL only during the mesophilic phase. The presence of this bacteria phyla could be an indicator of the transitioning from thermophilic to mesophilic phase. In contrast, its absence in the D00 might be due to the lack of carbohydrates in this pile, unlike the other piles which contained fresh materials (Buckley et al., 2006).

3.5. Co-occurrence of ARGs and bacteria

The co-occurrence of ARGs and bacteria (OTUs at genus level) was studied by Network analysis conducted on Pearson correlation coefficients (P<0.05) (Fig. 5). Despite more than 100

significant connections observed among the different genera, the results showed the exclusive co-occurrence of ARGs and the dominant bacterial phyla from the digestate such as Bacteroidetes (*Arenibacter* and *Fluvicola*), Proteobacteria (*Steroidobacter* and *Cellvibrio*) and Firmicutes (*Symbiobacterium*) (Fig. 3A). *Arenibacter* showed a significantly strong correlation with all the ARGs, thus implying this genus could be their potential host. In addition, *Cellvibrio* displayed co-occurrence with all ARGs except for *tet*(O). Furthermore, *Fluviicola*, *Steroidobacter*, and *Symbiobacterium* co-occurred with *tet*(M) gene, which implies that *tet*(M) might have a broader host range compared to the other ARGs. Unlike what was previously reported during the composting of poultry litter by Cui et al. (2016), Actinobacteria, Verrucomicrobia, or Planctomycetes, which emerged during the composting period, did not show co-occurrence with any of the ARGs showing other bacteria in the digestate were the potential hosts, and the shift in the composition during the composting was responsible for the degradation of ARGs.

Arenibacter is ubiquitous in various ecosystems and known for its capability of degrading high molecular weight substances including organic matter and pollutants (Roy et al., 2020; Stiborova et al., 2020). However, its potential as a host of ARGs has rarely been reported. In contrast, *Cellvibrio* was reported to be host of multiple ARGs in animal waste (Gou et al., 2021; Han et al., 2018), despite its significant functional role in lignocellulose degradation during composting (Raut et al., 2021). It also constitutes significant proportion of the bacteria communities in DPL and DMS, possibly due to the presence of lignocellulosic substrate (wheat straw and maize silage components, respectively). Within the Proteobacteria, *Steroidobacter* was the other potential host of *tet*(M). Previously, Wan et al. (2017) reported *Steroidobacter* as a potential host for selected ARGs in pig waste water. In the current study, it was found that the maize silage component

might promote the abundance of *Steroidobacter*, as it was only found in the DMS pile without being affected by both the thermophilic and mesophilic temperature regimes. *Fluviicola* and *Symbiobacterium* belonging to Bacteroidetes and Firmicutes, respectively, co-existed and were found to be potential co-host for *tet*(M).

3.6. Relationship between ARGs abundance and chemical compositions

Canonical Correspondence Analysis (CCA) results showed variations in abundance of ARGs, except *erm*(B), could be well explained by the dynamics of chemical compositions over the composting period. It was interesting that abundance *erm*(B) showed little relationships with the chemical compositions, and this gene was also least affected by composting and had the lowest removal rate.

The CCA results were visualized in Fig. 6. DCB formed distinct cluster, while there were potential overlaps among the other piles. In Fig. 6A, dynamics of OM, pH, and extractable Ca, Mg, and K explained about 64% of the total variations in the ARGs abundance. However, both *tet*(M) and *erm*(B) had little relationship with these environmental variables. In contrast, *tet*(O) positively related to C/N ratio, OM, Ca, and Mg contents, and negatively related with pH. Both *tet*(S) and *tet*(K) had positive relationship with pH, K, and S. OM potentially contributed to the total variance explained, showing ARGs degradation could rely on the OM stability of the composts over the composting period.

As reported in Fig. 6B, trace elements (Al, Ba, Cd, Cu, Fe, Mn, Ni, Pb, and Zn) explained 90% of the total variations in ARGs abundance. Particularly, Al, Cd, and Cu had positive relationships (co-occurrence) with *tet*(K) and *tet*(S), whereas *tet*(M) had positive relationships with Fe, Mn, and Zn. Interestingly, *erm*(B) had no relationship with any of the trace elements, whereas *tet*(O) showed positive relationship with Cd. The greater contents of trace elements

(Gurmessa et al., 2021b) and abundance of ARGs during the first phase and the significant reductions during the maturity period suggests co-occurrences. The weak relationship between trace elements and *erm*(B) could be linked to its weak response to composting as evidenced in Fig.2. Similar to the these findings, Cui et al. (2016) reported a strong co-occurrence of bioavailable trace elements and ARGs during composting of poultry litter. These findings may further give an insight into the significance of composting on stabilizing organic substance that could lead to both immobilization of trace elements and reduction of ARGs.

3. Conclusions

Post-digestate composting removed more than 80% of ARGs from digestate, and it was at least as effective as co-composting with maize silage and food processing waste. Despite the strong co-occurrence among the several bacteria groups, only a few genera were potential hosts of ARGs, and none of these belonged to those phyla (Actinobacteria, Planctomycetes, and Verrucomicrobia) that succeeded following composting. Results suggest the shift in microbial structure due to composting had little link with degradation of ARGs but could be an indicator of a well-functioning compost, rather, the physicochemical dynamics during composting or co-composting may be responsible for ARGs removal.

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Figure captions

Fig. 1. Abundance of ARGs in fresh digestate and co-composting materials. Bars indicated with different letters are statistically significant ($p<0.05$, n = 3).

Fig. 2. Dynamics of ARGs in compost piles, expressed as log10 of fold change, during the composting period. Negative and positive results of the log fold change indicate reduction and enrichment, respectively.

D00 = Digestate, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter.

Fig. 3. Relative abundance of bacteria in the different piles (D00 = Solid digestate compost, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter) and fresh solid digestate (DIG) at phylum (A) and genus (B) level. DIG = Fresh solid digestate.

Fig. 4. Differences in the composition of bacterial community among the piles (D00 = Solid digestate compost, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter) and fresh solid digestate (DIG) (A) and at the different compost phases (B), defined by non-metric multidimensional scaling (NMDS) analysis**.**

Fig. 5. Co-occurrence of ARGs and bacteria groups based on significant ($P<0.05$) Pearson's correlation.

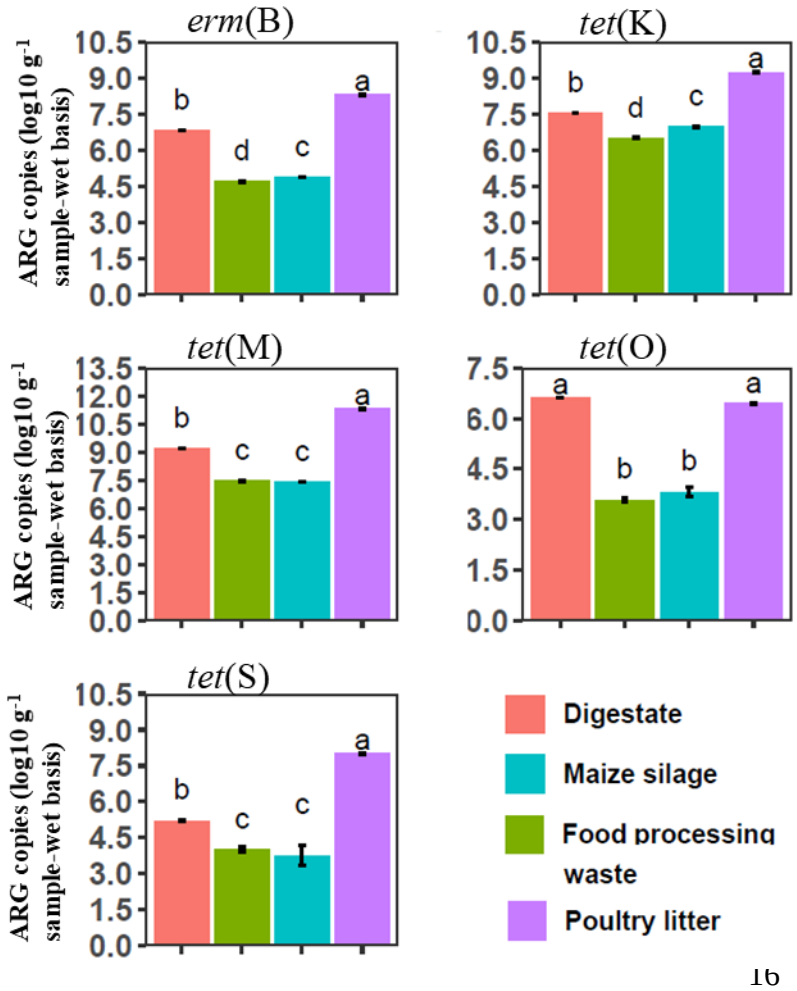
Fig. 6. Canonical coordinate analysis (CCA) of ARGs (response variables) and chemical composition (environmental variables) of composts. Two environments: A(C/N ratio, OM, pH, and macronutrients) and B(trace elements). D00 = Digestate, DCB = Digestate + Food processing waste, DMS = Digestate + Maize silage, DPL = Digestate + Poultry litter.

Table 1 Composition of the compost piles.

Pile	Composition	Mix ratio (w/w)
D00	Solid digestate	
DCB	Solid digestate + Food processing waste	4:1
DMS	Solid digestate + Maize silage	4:1
DPL	Solid digestate + Poultry litter	4:1

Table 2 Primers used in the qPCR reactions targeting the five ARGs of interest (Flórez et al., 2014).

ARG	Primer sequence (5'-3')	Product size (bp)
erm(B)	F- GGATTCTACAAGCGTACCTTGGA	69
	R- AATCGAGACTTGAGTGTGCAAGAG	
tet(K)	F- TGCTGCATTCCCTTCACTGA	69
	R- GCTTTGCCTTGTTTTTTTCTTGTAA	
tet(M)	F- CAGAATTAGGAAGCGTGGACAA	67
	R- CCTCTCTGACGTTCTAAAAGCGTAT	
tet(O)	F- AATGTCAGAACTGGAACAGGAAGAA	59
	R- CGTGATAAACGGGAAATAACGTT	
tet(S)	F- CGAGGTCATTCTCATTGGTGAA	84
	R- CAGACACTGCGTCCATTTGTAAA	



16

17

18 **Fig. 1**

19

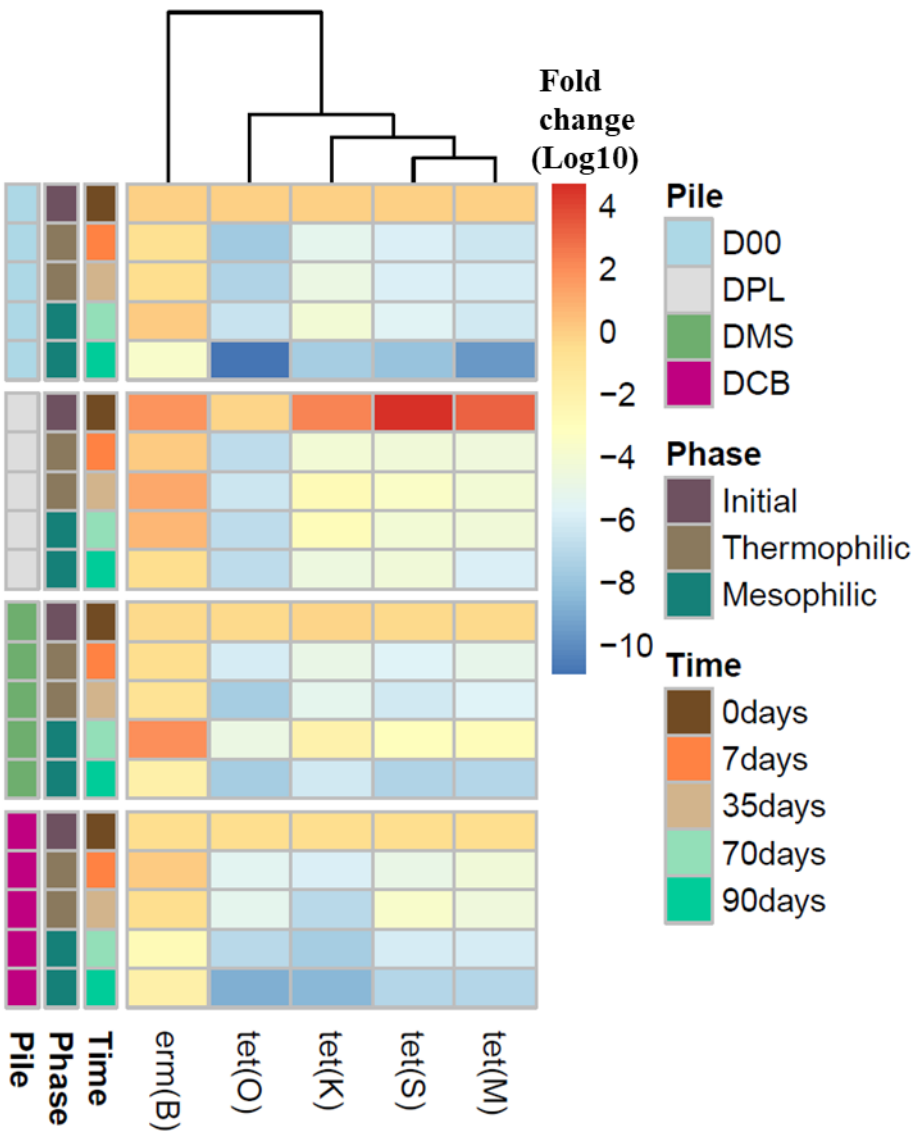

Fold change (Log10)
4
2
0
−2
−4
−6
−8
−10
Pile
D00
DPL
DMS
DCB
Phase
Initial
Thermophilic
Mesophilic
Time
0days
7days
35days
70days
90days
Pile
Phase
Time
erm(B)
tet(O)
tet(K)
tet(S)
tet(M)

Fig. 2

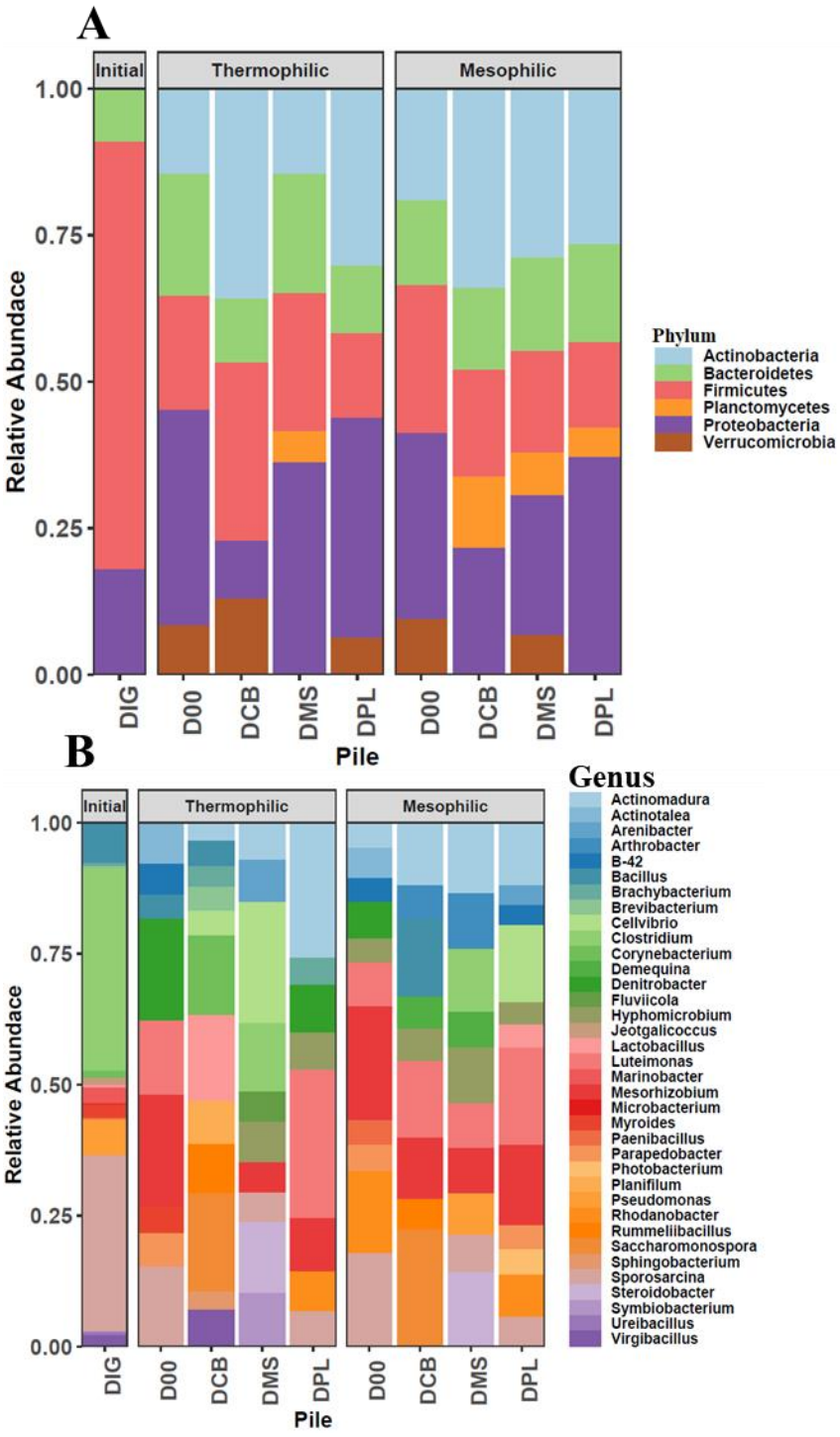

A
Initial
Thermophilic
Mesophilic
1.00
0.75
0.50
0.25
0.00
Relative Abundace
DIG
D00
DCB
DMS
DPL
D00
DCB
DMS
DPL
Pile
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Planctomycetes
Proteobacteria
Verrucomicrobia
B
Initial
Thermophilic
Mesophilic
1.00
0.75
0.50
0.25
0.00
Relative Abundace
DIG
D00
DCB
DMS
DPL
D00
DCB
DMS
DPL
Pile
Genus
Actinomadura
Actinotalea
Arenibacter
Arthrobacter
B-42
Bacillus
Brachybacterium
Brevibacterium
Cellvibrio
Clostridium
Corynebacterium
Demequina
Denitrobacter
Fluviicola
Hyphomicrobium
Jeotgalicoccus
Lactobacillus
Luteimonas
Marinobacter
Mesorhizobium
Microbacterium
Myroides
Paenibacillus
Parapedobacter
Photobacterium
Planifilum
Pseudomonas
Rhodanobacter
Rummelibacillus
Saccharomonospora
Sphingobacterium
Sporosarcina
Steroidobacter
Symbiobacterium
Ureibacillus
Virgibacillus

Fig. 3

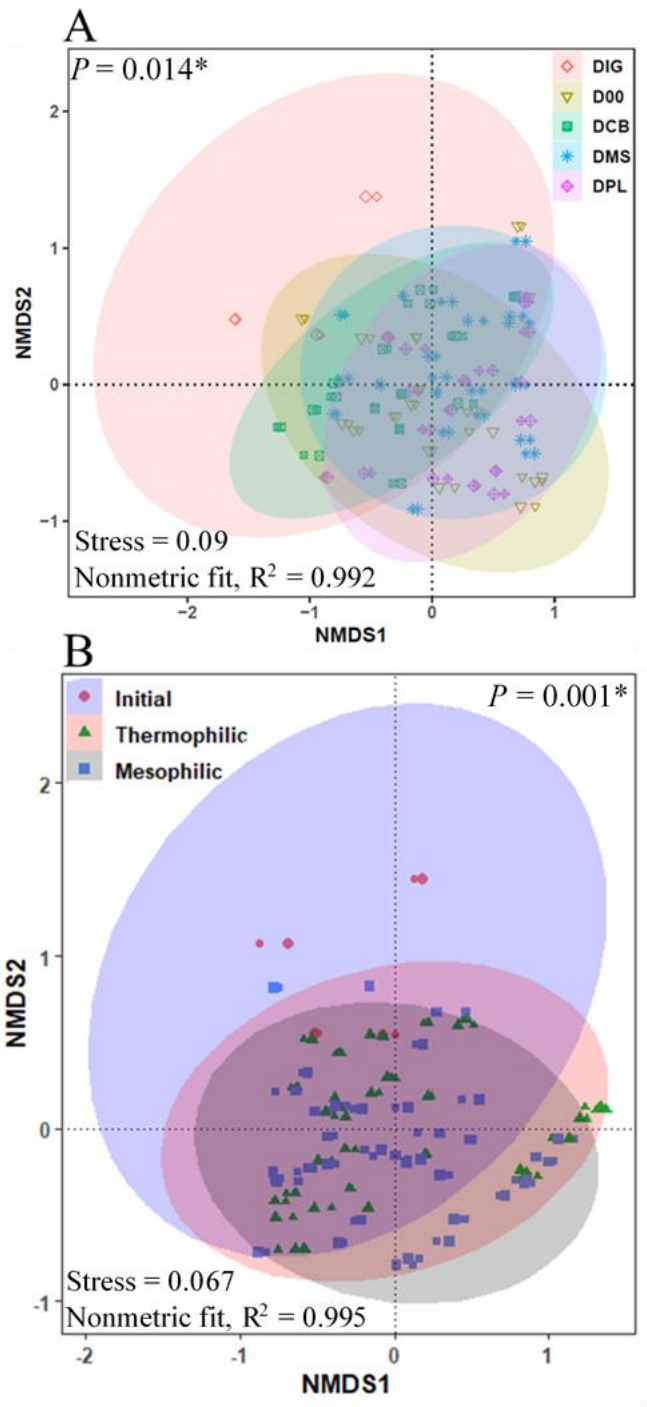

A
P = 0.014*
DIG
D00
DCB
DMS
DPL
NMDS2
NMDS1
Stress = 0.09
Nonmetric fit, R2 = 0.992
B
Initial
Thermophilic
Mesophilic
P = 0.001*
NMDS2
NMDS1
Stress = 0.067
Nonmetric fit, R2 = 0.995

Fig. 4

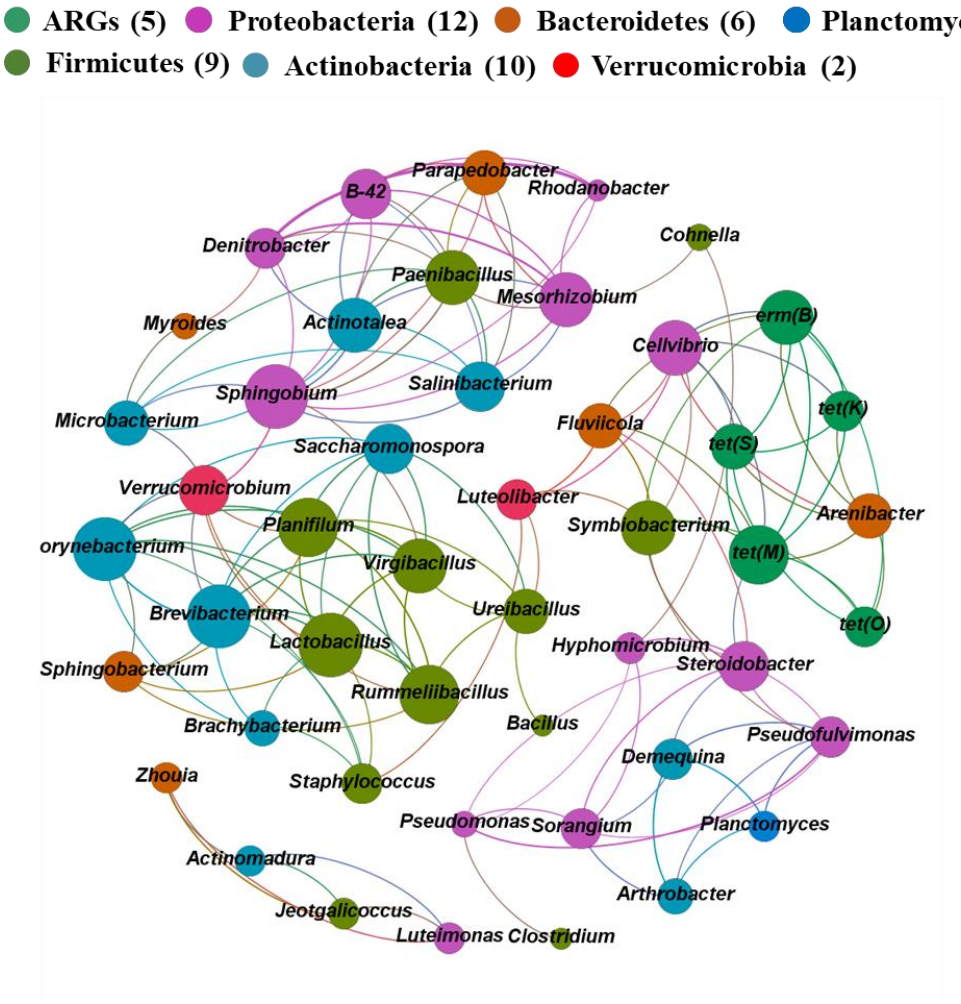

ARGs (5)
Proteobacteria (12)
Bacteroidetes (6)
Planctomycetes (1)
Firmicutes (9)
Actinobacteria (10)
Verrucomicrobia (2)
Parapedobacter
B-42
Rhodanobacter
Cohnella
Denitrobacter
Paenibacillus
Mesorhizobium
Myroides
Actinotalea
erm(B)
Cellvibrio
Sphingobium
Salinibacterium
tet(K)
Microbacterium
Fluviicola
tet(S)
Saccharomonospora
Verrucomicrobium
Luteolibacter
Arenibacter
Planifilum
Symbiobacterium
orynebacterium
Virgibacillus
tet(M)
Brevibacterium
Ureibacillus
tet(O)
Lactobacillus
Hyphomicrobium
Sphingobacterium
Steroidobacter
Rummeliibacillus
Brachybacterium
Bacillus
Pseudofulvimonas
Demequina
Zhouia
Staphylococcus
Pseudomonas
Sorangium
Planctomyces
Actinomadura
Arthrobacter
Jeotgalicoccus
Luteimonas
Clostridium

Fig. 5.

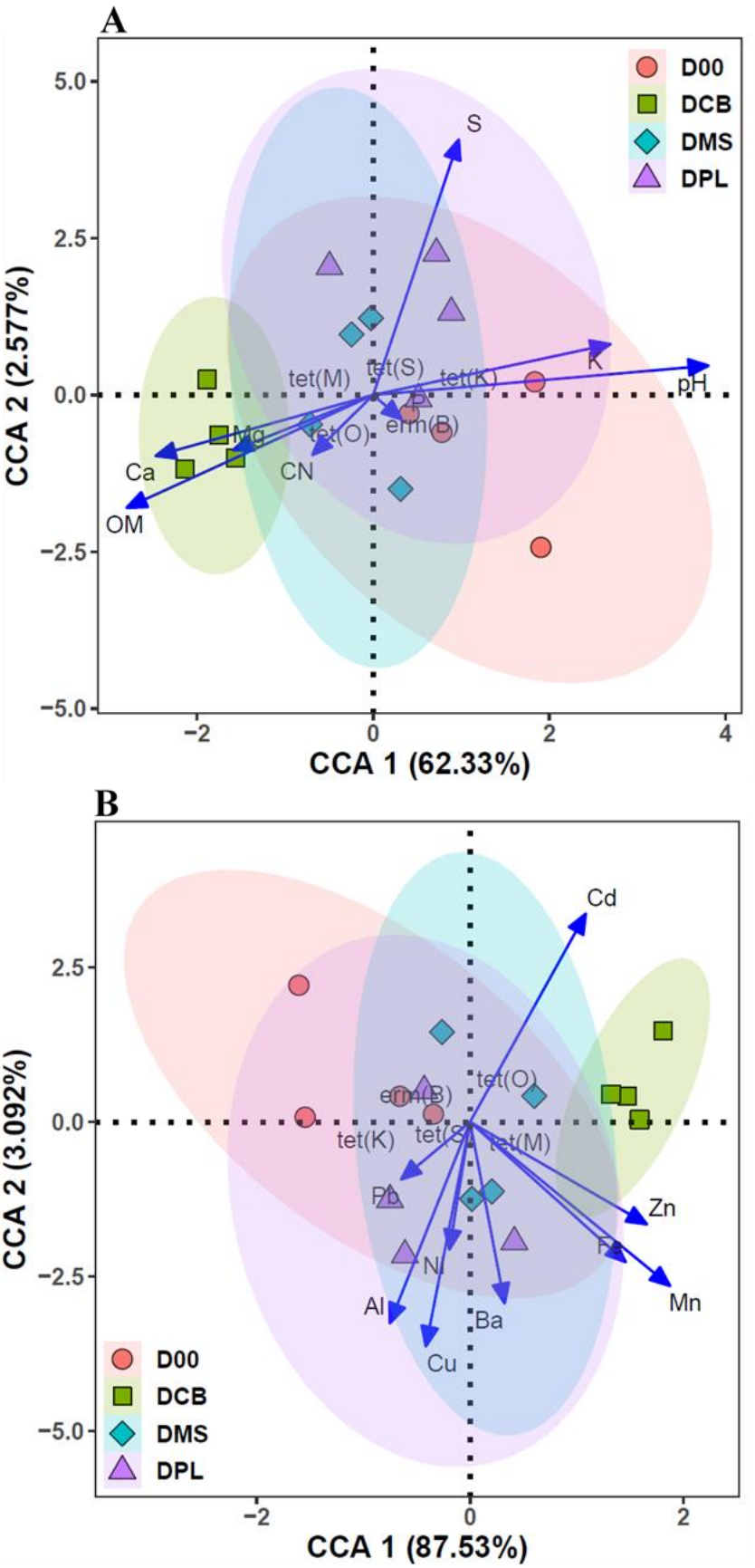

A
D00
DCB
DMS
DPL
S
K
pH
tet(M)
tet(S)
tet(K)
P
erm(B)
tet(O)
Mg
Ca
CN
OM
5.0
2.5
0.0
−2.5
−5.0
−2
0
2
4
CCA 2 (2.577%)
CCA 1 (62.33%)
B
Cd
tet(O)
erm(B)
tet(K)
tet(S)
tet(M)
Pb
Zn
Fe
Ni
Al
Ba
Mn
Cu
D00
DCB
DMS
DPL
2.5
0.0
−2.5
−5.0
−2
0
2
CCA 2 (3.092%)
CCA 1 (87.53%)

Fig. 6.