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# Zinc concentration affects the functional groups of microbial communities in sugarcane-cultivated soil

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ABSTRACT

The addition of zinc (Zn) to the soil has significant gains in sugarcane productivity. The understanding of the consequences of zinc application requires insight into ecological aspects of changes in soil microbial communities. Here, we evaluated the effects of zinc application on richness, diversity, evenness and structure of microbial functional genes in sugarcane-cultivated soil, and response thresholds of abundance for groups of microbial functional genes on different zinc concentrations added to the soil (0, 5, 10 and 20 kg Zn $ha^{-1}$) in two sugarcane fields in the Northeastern São Paulo state, Brazil. Using a high-density functional gene array termed GeoChip 5.0, which contains 101,796 distinct probes belonging to gene categories involved in zinc transport, secondary metabolism, stress, virulence and nutrient cycling, differences were observed in the functional gene structure of microbial communities and abundance of specific gene families with a threshold between 5 and 10 kg Zn $ha^{-1}$. However, the richness, diversity and evenness of functional genes did not differ across the zinc gradients in soil. Cluster analysis based on microbial functional subcategories revealed variation in abundances across the gradient of zinc concentrations in soil mainly for virulence, stress, secondary metabolism, and carbon- and phosphorus-cycling-related gene families. A threshold in abundance was observed between 5 and 10 kg Zn $ha^{-1}$ with high abundance of microbial gene families associated with zinc transporter proteins, antioxidant enzymes, exoenzymes associated with infection, secretion proteins, virulence regulatory genes, carbon fixation, and phosphorus utilization in soils supplemented with up to 5 kg Zn $ha^{-1}$. In these same soils, reduced abundance was observed for gene families associated with methanogenesis, metabolism of halogen and carotenoid, and antiphagocytosis compared with soil supplements with 10 or 20 kg Zn $ha^{-1}$. The results suggest that changes in microbial functional gene structure and abundance could be used to evaluate the impact of soil management practices on soil microbial communities in sugarcane production fields.



## 1. Introduction

Zinc is an essential cofactor required by all living organisms. Zinc is the second most abundant transition metal in living organisms (after iron), has important functions in enzymatic activity, transcription factors (which regulate gene expression), and serves as a cofactor in more than 300 proteins (Palmgren et al., 2008). According to a global study of the micronutrient status of soils (Sillanpää, 1990), the zinc content in soils from Brazilian regions are highly variable, covering a wide range of values from high to low, potentially leading to zinc deficiency for important crops cultivated in this area (e.g., sugarcane, coffee, oranges, rice, maize, sorghum, cassava and rubber). In this sense, several studies have demonstrated significant gains in sugarcane productivity

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with zinc application (Cambria et al., 1989; Marinho and Albuquerque, 1981; Korndörfer et al., 1995; Wang et al., 2005; Mellis et al., 2016).

The number of zinc-binding proteins correlates linearly with the total number of proteins encoded by the genome of an organism, but the proportionality constant of Eukaryota (8.8%) is significantly increased compared with that observed in Bacteria and Archaea (from 5% to 6%) due to the larger portfolio of regulatory proteins in Eukaryota (Andreini et al., 2006). Zinc levels must be tightly regulated; too little zinc does not support cellular growth, whereas too much zinc is cytotoxic (Koh et al., 1996; Costello et al., 1997). The free zinc concentration in the cytoplasm is maintained well below the picomolar range (Outten and O'Halloran, 2001). It is generally accepted that accumulated trace metals reduce the soil microbial biomass (Brookes and McGrath, 1984) and (Chander et al., 1995) and the activity of various microbial enzymes (Kandeler et al., 1996).

Several microbial cellular processes, such as synthesis of secondary metabolites (Weinberg, 1990), and mechanisms associated with the infection and virulence of microorganisms (Campoy et al., 2002; Davis et al., 2009) seem to be sensitive to zinc stress (Kunito et al., 2001) and zinc homeostasis (Hantke, 2001) and thus might be useful for evaluation of the toxic effects of zinc on microorganisms. Soil microbial communities hold a central place in terrestrial ecosystems, performing essential ecological processes or functions, such as nutrient cycling, plant nutrition or disease suppression (Bardgett et al., 2008). Therefore, it is useful to understand the consequences of zinc application on diversity and structure of microbial functional genes in soil ecosystems.

Different methods and approaches have been used to study the functional diversity of soil microbial community. Biolog EcoPlate, a technique based on levels of activities on various substrates, was used in different studies to evaluate the metabolic profile of soil microbial communities (Pająk et al., 2016; Tosi et al., 2016; Kelly et al., 1999; Zak et al., 1994) and determine the relative zinc sensitivity on the metabolic activities of the isolated microorganisms (Lock and Janssen, 2005). A range of novel methods, most of which are based on rRNA and rDNA analyses, have been developed to assess the functional diversity and structure of microbial communities. Polymerase chain reaction (PCR)-based fingerprinting, such as terminal restriction fragment length polymorphism (T-RFLP), has been used to analyze changes in diversity and structure of soil microbial community based on specific functional genes (Bannert et al., 2011). Quantitative real-time PCR is useful to analyze differences in the abundance of microbial function-specific genes (Lammel et al., 2015). However, there are some problems and biases in the PCR amplification step; therefore, these methods cannot be used as definite indicators of richness of ecological communities. Shotgun metagenomic sequencing and gene expression array analysis constitute an alternative approach to taxonomic and functional characterization of soil microbial community that avoids these limitations. The GeoChip-based functional gene array method simultaneously monitors the expression levels of thousands of genes (He et al., 2007). Such information is very useful in functional diversity studies to track highly expressed genes and genes critical in biogeochemical pathways. It is also important to obtain information on the microbial community response to environmental changes and factors that regulate diversity (Cong et al., 2015; Li et al., 2014).

Considering the potential increases in sugarcane productivity provided by zinc application in natural low-fertility soils worldwide, it is necessary to obtain a better understanding of the effects of zinc application on specialized functioning of the soil performed by microbial groups. For this purpose, we applied a functional gene microarray (GeoChip v. 5.0) (*i*) to assess the effects of zinc application on richness, diversity, evenness and structure of microbial functional genes in sugarcane-cultivated soil and (*ii*) to identify the response thresholds in abundance for groups of microbial functional genes to the zinc concentration added to the sugarcane-cultivated soil.

## 2. Materials and methods

### 2.1. Field experiment

Regional-scale field experiments were performed with RB867515 variety of sugarcane at the two major producing regions of sugarcane in the State of São Paulo, Brazil: Serra Azul (latitude 21°18′41″ S and longitude 47°33′59″W) and Assis (latitude 22°39′40″ S and longitude 50°23′58″ W) municipalities. The climate in the region is humid subtropical. Oxisol and Ultisol are the soil types at the Serra Azul and Assis municipalities, respectively. The field experiments at both municipalities were characterized by sandy soil with low natural fertility and available zinc concentrations of less than 0.5 mg $dm^{-3}$ (Mellis et al., 2016). Four treatments with three replicate plots each were used in a completely randomized design in each location. Each experimental plot (75 $m^2$) consisted of five neighboring rows, each 10 m long and 1.4 m apart. Zinc was applied in the form of zinc sulfate ($ZnSO_4$) in the furrow at three different doses (5, 10 and 20 kg $ha^{-1}$) once at the beginning of the experiment on March 2011. The control treatment consisted of soil without application of zinc. In addition, fertilization was performed at sugarcane planting and consisted of 25 kg nitrogen (N) in the form of urea, 150 kg $P_2O_5$ (triple superphosphate), 100 kg $K_2O$ (potassium oxide) and 3 kg of sulfur (S) per hectare. Broadcast fertilization was performed six months after planting (September 2011) with 27 kg N and 40.5 kg $K_2O$ per hectare. In addition, 125 kg N and 100 kg $K_2O$ per hectare were applied in three subsequent cycles of sugarcane ratoon.

### 2.2. Soil sampling

Soil samples were collected from each of three treatment and control soils in both sugarcane field experiments (4 treatments × 3 soil samples per treatment × 2 experimental fields = 24 soil samples) after the third sugarcane ratoon. Soil sampling consisted of five points in the furrow for each experimental plot: one central sampling point and four other sampling points at least 2.8 m apart from the central point and directed towards the four cardinal points. The soil cores were taken from the 0 to 40 cm topsoil layer using a 5-cm diameter aseptic cylindrical core. Each soil sample consisted of a composite of five soil cores collected within each experimental plot. Soil samples for chemical determination were immediately processed. Aliquots of soil samples were transported to the laboratory on ice and stored at −20 °C until further processing within 72 h after sampling.

### 2.3. Soil chemical measurements

The chemical measurements of each of the 24 soil samples were determined according to EMBRAPA (2011). Soil pH was measured on a soil/0.01 M $CaCl_2$ (1:5) suspension. Aluminum (Al), calcium (Ca), and magnesium (Mg) were extracted with 1 M potassium chloride. Ca and Mg were determined by atomic absorption spectrometry, whereas Al was determined by acid-base titration. Available phosphorous (P) and potassium (K) were extracted by ion-exchange resin and determined by colorimetry and atomic emission spectroscopy, respectively. Combined results were used for calculation of exchangeable bases (SB) as the sum of Ca, Mg, and K; cation-exchange capacity (CEC) as the sum of Ca, Mg, K, Al, and H; base saturation (V) as the percent relation between SB and CEC;

aluminum saturation (m) as the percent relation between exchangeable Al and CEC; and potential acidity (H + Al) using an equation based on the pH determined in Shoemaker-McLean-Pratt (SMP) buffer solution. Soil zinc fractionation was performed according to the Standard U.S. Environmental Protection Agency (USEPA), 1998. Briefly, the fractionation involved a sequential extraction procedure with (a) $CaCl_2$ to extract water-soluble plus exchangeable zinc (Ex-Zn), (b) Na acetate (pH 5.0) to extract carbonate-bound zinc (CARB-Zn), (c) NaOCl (pH 8.5) to obtain organically bound zinc (OM-Zn), (d) $H_2C_2O_4$-$C_2H_8N_2O_4$-$C_6H_8O_6$ (pH 3.0) to extract oxide bound zinc (Ox-Zn), and (e) $HNO_3$-HCl to extract residual zinc (RES-Zn). The percent recovery of the sum of the extracted zinc in all fractions (Ex-Zn, CARB-Zn, OM-Zn, and Ox-Zn) plus the residual fraction (RES-Zn) was expressed as total zinc (TOT-Zn).

### 2.4. Soil DNA extraction, purification and labeling

DNA was extracted from 250 mg of soil using the PowerLyzer PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Three DNA extractions were performed per soil sample, combined and concentrated with the Genomic DNA Clean and Concentrator kit (Zymo Research Corporation, Irvine, CA, USA). Concentrated DNA was re-suspended in 20 μl of PCR grade water, and purity was assessed spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies, Inc., Wilmington, DE, USA) to determine absorbance at the following wavelengths: 230, 260, 280 and 320 nm. DNA concentration was measured with the Quant-iT PicoGreen kit (Molecular Probes/Invitrogen, Carlsbad, CA, USA). The DNA extraction from Assis and Serra Azul samples had an average of 26.90 ng $\mu l^{-1}$ ($\pm$ 1.34) and 23.72 ng $\mu l^{-1}$ ($\pm$1.61), respectively. All 24 of the DNA samples were stored at −20 °C until use.

Purified DNA was labeled with the fluorescent dye Cy-3 according to Wu et al. (2006). Briefly, DNA (600 ng) was mixed with random primers (300 ng $\mu l^{-1}$), denatured at 99.9 °C for 5 min and immediately chilled on ice. A solution containing 5 mM dAGCTP, 2.5 mM dTTP, Klenow fragment (40 U) and Cy-3 dUTP (25 nM) was added to the denatured DNA, and the reaction volume was adjusted to 50 μl with $H_2O$. The labeling solution was incubated at 37 °C for 6 h followed by 3 min at 95 °C. The labeled DNA was purified with the QIAquick Kit (Qiagen, Valencia, CA, USA), and dye incorporation was confirmed with a NanoDrop spectrophotometer. Labeled samples were vacuum-dried and stored at −20 °C until hybridization.

### 2.5. GeoChip 5.0 experiment, scanning and image processing

The GeoChip 5.0 was manufactured by Agilent (Agilent Technologies Inc., Santa Clara, CA, USA) in the 4 × 180 K format. The chip contains 167,044 distinct probes belonging to different key gene categories involved in carbon (e.g., degradation, fixation, methane), nitrogen (e.g., ammonification, nitrification, fixation), sulfur, phosphorus cycling, metal homeostasis (e.g., zinc transport), secondary metabolism (e.g., antibiotic metabolism, pigments), stress responses (e.g., oxidative stress), virulence (e.g., infection), and other microbial genes of known function. Here, gene sequence refers to each unique sequence targeted by GeoChip 5.0, and a gene family comprises all gene sequences assigned with the same name (e.g., named *Znu*C) and coding for the same class of proteins. The probes were designed to target Bacteria, Archaea, fungi, and viruses (bacteriophage) gene sequences.

Prior to hybridization, labeled DNA was resuspended in 27.5 μl of DNase/RNase-free distilled water and mixed completely with 99.4 μl of hybridization solution master mix containing 2 x Hi-RPM hybridization buffer (Agilent Technologies Inc., Santa Clara, CA, USA), 10 x aCGH blocking agent (Agilent Technologies Inc., Santa Clara, CA, USA), 10% formamide (J.T. Baker, Philipsburg, NJ, USA), 0.05 μg/μl Cot-1 DNA (Agilent, Technologies Inc., Santa Clara, CA, USA), and 10 pM Cy5-labelled universal standard DNA for normalization (Liang et al., 2010). The solution was then denatured at 95 °C for 3 min, incubated at 37 °C for 30 min, and hybridized with GeoChip 5.0 arrays. GeoChip hybridization proceeded at 67 °C in the presence of 10% formamide in a microarray hybridization oven (Agilent Technologies Inc., Santa Clara, CA, USA) for 24 h. After hybridization, the slides were washed using Agilent Wash Buffers 1 and 2 following the manufacturer's protocol. Then, the arrays were scanned at a laser power of 100% and a photomultiplier tube gain of 75% with a NimbleGen MS200 Microarray Scanner (Roche NimbleGen, Madison, WI, USA). The image data were extracted using the Agilent Feature Extraction program.

### 2.6. Raw data processing

Spot alignment and signal intensity determination were performed with the Feature Extraction 11.5 (Agilent Technologies Inc., Santa Clara, CA, USA). The raw data were submitted to the Microarray Data Manager at the Institute for Environmental Genomics website (http://www.ou.edu/content/ieg/tools/data-analysis-pipeline.html) using the following parameters: (i) spots with signal intensity lower than 1.3 times background or with a signal-to-noise ratio (SNR) less than 2.0 were removed as poor-quality spots (SNR = spot signal intensity – background signal average/SD background); (ii) unrepresentative data were removed using group cut 1 (by default) to remove singletons (probes that were positive in only one sample of an experimental group); (iii) normalization was performed by dividing the signal intensity of each spot by the average of the universal standard spots and then by the average of the signal intensities in each sample (i.e., mean-ratio approach).

### 2.7. Statistical analysis

One-way ANOSIM (analysis of similarity) was performed on Bray-Curtis dissimilarity matrices (incorporating 999 permutations for R statistics) after a process of standardization of all variables using the $L_1$ distance to determine the significance of differences across treatments regarding all soil chemical factors and only the zinc concentration in soil. All procedures were computed using PRIMER v6.1.9 (Primer-E Ltd, Plymouth, UK). One-way ANOVA (analysis of variance) with post hoc Tukey's HSD test was used to determine the significance of the differences among treatments for each soil factor within each of the two field experiments. The procedure was performed using the STATISTICA 10 package (StatSoft Inc., Tulsa, OK, USA). Spearman's rank correlation coefficients were calculated to explore the relationship between gene family abundance and zinc concentration in soil using the R package 'multtest' (version 2.6.0; R Core Team, 2015) and the false discovery rate controlling procedure (Benjamini and Hochberg, 1995). The abundance of each gene family was calculated based on the sum of all gene sequences assigned with the same name and coding for the same class of proteins. Ecological metrics, such as gene richness, diversity (Shannon and inverse Simpson indexes) and evenness (Sheldon index), were calculated using the 'diversity' function from the R package 'vegan' (R Core Team, 2015). A Tukey's test was used to determine the significance of the differences among treatments for each ecological metric within each of the two field experiments. The similarities in gene family composition across the samples were visualized by multidimensional scaling (MDS) ordination and tested by ANOSIM using Bray-Curtis index (Clarke, 1993) based on the abundance of gene families correlated to the zinc concentration in soil. To

**Table 1**
Total content of zinc (Zn) and its fractions in sugarcane-cultivated soils under different zinc concentration.

| Treatments (Zinc concentration) | Zinc fractions in soil | | | | | TOT-Zn (mg dm$^{-3}$) |
|---|---|---|---|---|---|---|
| | Ex-Zn (mg dm$^{-3}$) | CARB-Zn (mg dm$^{-3}$) | OM-Zn (mg dm$^{-3}$) | Ox-Zn (mg dm$^{-3}$) | RES-Zn (mg dm$^{-3}$) | |
| Assis (kg ha$^{-1}$) | | | | | | |
| 0 | 0.03[†]a[‡] ± 0.004[§] | 0.02a ± 0.004 | 0.05a ± 0.002 | 0.03a ± 0.008 | 0.11a ± 0.011 | 0.24a ± 0.006 |
| 5 | 1.03b ± 0.002 | 1.03b ± 0.006 | 1.08b ± 0.001 | 1.06b ± 0.002 | 1.35b ± 0.018 | 5.57b ± 0.013 |
| 10 | 2.04c ± 0.006 | 2.03c ± 0.004 | 2.08c ± 0.004 | 2.06c ± 0.008 | 2.65c ± 0.042 | 10.86c ± 0.032 |
| 20 | 3.04d ± 0.001 | 3.03d ± 0.004 | 3.08d ± 0.019 | 3.06d ± 0.001 | 3.48d ± 0.067 | 15.70d ± 0.058 |
| Serra Azul (kg ha$^{-1}$) | | | | | | |
| 0 | 0.04a ± 0.007 | 0.05a ± 0.014 | 0.09a ± 0.007 | 0.06a ± 0.025 | 0.14a ± 0.041 | 0.38a ± 0.093 |
| 5 | 1.03b ± 0.003 | 1.03b ± 0.010 | 1.09b ± 0.003 | 1.07b ± 0.005 | 1.33b ± 0.029 | 5.55b ± 0.021 |
| 10 | 2.04c ± 0.001 | 2.03c ± 0.002 | 2.08c ± 0.004 | 2.06c ± 0.007 | 2.55c ± 0.170 | 10.76c ± 0.165 |
| 20 | 3.05d ± 0.004 | 3.02d ± 0.012 | 3.08d ± 0.028 | 3.06d ± 0.002 | 3.45d ± 0.131 | 15.65d ± 0.100 |

Ex-Zn, exchangeable zinc; CARB-Zn, carbonate-bound Zn; OM-Zn, organically bound Zn; Ox-Zn, oxide bound Zn; RES-Zn, residual zinc; TOT-Zn, total zinc.
[†] Average for each of three replicates of soil.
[‡] Tukey's HSD test followed by Bonferroni correction for multiple comparisons was performed considering all treatments regarding each of two field experiments (Assis and Serra Azul) across replicates of soil for each treatment. Significance level: $P < 0.05$.
[§] Standard deviation of the average for each of three replicates of soil.

determine significant differences between two zinc rate categories defined by MDS, the Statistical Analysis of Metagenomic Profiles (STAMP) software package was used (Parks and Beiko, 2010). *P*-values were calculated using the two-sided Welch's *t*-test (Welch, 1947), whereas confidence intervals were calculated using Welch's inverted test. In addition, correction was performed using Benjamini-Hochberg false discovery rate. The total gene abundance in different functional subcategories was ordered in a heat map. Dissimilarity between samples and gene abundance within functional subcategories was estimated using the 'vegdist' function from the R package 'vegan' and with Bray-Curtis index (R Core Team, 2015). Clustering was performed using the average linkage algorithm (also called UPGMA for Unweighted Pair Group Method with Arithmetic Mean) (R Core Team, 2015).

## 3. Results

### 3.1. Content of zinc in the soil

The zinc concentration in soils revealed the intentional gradient defined by the treatments containing the different zinc concentration, the total content of zinc and zinc fractions that differed significantly among the treatments (Table 1) (ANOSIM, Assis: R = 0.989, $P < 0.001$; Serra Azul: R = 0.966, $P < 0.001$). Based on the soil chemical properties (Supplementary Table S1), the three zinc treatments did not form significantly distinct groups for either sugarcane field experiment (ANOSIM, Assis: R = 0.436, $P < 0.001$; Serra Azul: R = 0.448, $P < 0.001$).

### 3.2. Ecological metrics and structure similarity of microbial functional genes

A total of 40,489 out of 101,796 (40%) microbial functional genes targeted by GeoChip 5.0 were detected across all soil samples from Assis and Serra Azul experimental fields. These genes belonged to 525 different gene families and were associated with the following functional categories: zinc transport; carbon-, nitrogen-, phosphorus-, and sulfur-cycling; secondary metabolism; stress and virulence. Overall, 53 of the 525 detected gene families (10%) were significantly correlated with zinc concentration in soil (Table 2). Gene richness did not differ across the zinc gradient within each of the two experiments (Table 3). Functional gene diversity based on Shannon and inverse Simpson diversity indexes and evenness calculated using Sheldon index were also not significantly different across the zinc gradient within each of the two experiments (Table 3). Although the richness, diversity and evenness of gene and gene families did not differ between microbial communities across the zinc gradient in soil, we observed differences in the identity of genes correlated with zinc concentration (Fig. 1). ANOSIM revealed similar grouping with some differences for control treatment and soil amended with 5 kg Zn ha$^{-1}$ in both field experiments (Table 4, Fig. 1). Similar patterns were observed for soils amended with 10 and 20 kg Zn ha$^{-1}$ (Table 4, Fig. 1). However, clear difference were observed between both described clusters (R = 0.987).

**Table 2**
Quantity of gene families and gene sequences detected by GeoChip 5.0 and correlated with zinc (Zn) concentration belonging to gene categories in both field experiments.

| Gene categories | Gene families | | Gene sequences | |
|---|---|---|---|---|
| | Detected | Correlated with Zn[†] | Detected | Correlated with Zn[†] |
| Zinc transport | 14 | 5 | 2145 | 972 |
| Carbon cycling | 118 | 15 | 13145 | 785 |
| Nitrogen cycling | 29 | 2 | 1041 | 379 |
| Phosphorus cycling | 6 | 2 | 1668 | 23 |
| Sulfur cycling | 26 | 0 | 1730 | 0 |
| Secondary metabolism | 52 | 6 | 1356 | 127 |
| Stress | 85 | 8 | 9259 | 869 |
| Virulence | 195 | 15 | 10145 | 335 |
| **Total** | **525** | **53** | **40489** | **3490** |

[†] Values indicate the number of gene families and gene sequences significantly ($P < 0.05$) correlated with zinc concentration in soil (total content of Zn and its fractions).

**Table 3**
Ecological metrics based on functional gene sequences and gene families detected by GeoChip 5.0 and correlated with zinc concentration in sugarcane-cultivated soils.

| Assis (Zinc concentration) | | | | Serra Azul (Zinc concentration) | | | |
|---|---|---|---|---|---|---|---|
| 0 kg ha$^{-1}$ | 5 kg ha$^{-1}$ | 10 kg ha$^{-1}$ | 20 kg ha$^{-1}$ | 0 kg ha$^{-1}$ | 5 kg ha$^{-1}$ | 10 kg ha$^{-1}$ | 20 kg ha$^{-1}$ |
| *Richness estimates* | | | | | | | |
| Gene sequences detected | | | | | | | |
| 12628[†] ab[‡] ± 169[¶] | 12480 ab ± 100 | 12249 a ± 611 | 13366 b ± 721 | 13653 a ± 280 | 14024 a ± 157 | 13105 a ± 139 | 13393 a ± 275 |
| Gene sequences belonging to gene families correlated with zinc concentration | | | | | | | |
| 2632 a ± 40 | 2577 a ± 10 | 2547 a ± 131 | 2783 a ± 159 | 3007 ab ± 131 | 3266 b ± 105 | 2768 a ± 58 | 2874 ab ± 87 |
| Gene families detected | | | | | | | |
| 501 a ± 3 | 499 a ± 1 | 500 a ± 8 | 511 a ± 9 | 513 a ± 3 | 519 a ± 3 | 506 a ± 3 | 509 a ± 5 |
| Gene families correlated with zinc concentration | | | | | | | |
| 70 a ± 7 | 69 a ± 3 | 64 a ± 6 | 77 a ± 6 | 69 a ± 4 | 79 a ± 12 | 67 a ± 9 | 67 a ± 5 |
| *Shannon index* | | | | | | | |
| Gene families detected | | | | | | | |
| 4.81 a ± 0.005 | 4.80 a ± 0.003 | 4.80 a ± 0.01 | 4.81 a ± 0.01 | 4.82 a ± 0.002 | 4.83 a ± 0.002 | 4.81 a ± 0.003 | 4.82 a ± 0.005 |
| Gene families correlated with zinc concentration | | | | | | | |
| 2.97 a ± 0.005 | 2.98 a ± 0 | 2.97 a ± 0.01 | 2.99 a ± 0.01 | 2.99 a ± 0.01 | 2.99 a ± 0.005 | 2.98 a ± 0.01 | 2.98 a ± 0.005 |
| *Inverse Simpson index* | | | | | | | |
| Gene families detected | | | | | | | |
| 41.99 a ± 0.42 | 41.61 a ± 0.30 | 41.49 a ± 0.74 | 42.0 a ± 0.90 | 43.29 a ± 0.20 | 43.56 a ± 0.19 | 42.34 a ± 0.31 | 43.04 a ± 0.26 |
| Gene families correlated with zinc concentration | | | | | | | |
| 12.76 a ± 0.12 | 12.76 a ± 0.01 | 12.59 a ± 0.31 | 13.14 a ± 0.24 | 13.09 a ± 0.12 | 13.29 a ± 0.03 | 12.86 a ± 0.11 | 13.17 a ± 0.22 |
| Sheldon index | | | | | | | |
| Gene families detected | | | | | | | |
| 0.77 a ± 0.0002 | 0.77 a ± 0.0004 | 0.77 a ± 0.0005 | 0.77 a ± 0.0003 | 0.77 a ± 0.0004 | 0.77 a ± 0.0003 | 0.77 a ± 0.0003 | 0.77 a ± 0.0004 |
| Gene families correlated with zinc concentration | | | | | | | |
| 0.76 a ± 0.0017 | 0.76 a ± 0.0016 | 0.76 a ± 0.0046 | 0.76 a ± 0.0023 | 0.76 a ± 0.0018 | 0.76 a ± 0.0003 | 0.76 a ± 0.0021 | 0.76 a ± 0.0016 |

[†] Average for each of three replicates of soil.
[‡] Samples under different zinc addition were contrasted within each field experiment. Values with the same lower-case letters were not significantly different ($P < 0.05$) based on Tukey's HSD test followed by Bonferroni correction for multiple comparisons.

### 3.3. Variation in functional groups of microbial communities to zinc concentration in soil

Abundance-based cluster analysis of functional subcategories revealed four different bunches (I, II, III and IV) (Fig. 2). The highest variation in gene family abundances across the samples was noted for group III, encompassing virulence, carbon- and phosphorus-cycling and metabolic-related gene families. Virulence and stress-related gene families that clustered as group II also exhibited increased variation across all samples (Fig. 2). Zinc transporter gene families exhibited increased abundance in control soil and soil amended with 5 kg Zn ha$^{-1}$ compared with soils supplemented with higher zinc concentrations in both field experiments (Fig. 2).

Individual responses of gene families to increases in zinc concentration in soil are presented in Table 5. The majority of the gene families that were significantly correlated with total content of zinc and its fractions exhibited a decrease in abundance with increasing zinc concentration in soil. A similar pattern was observed based on the differences in abundance for gene families between two zinc rate categories (0 and 5 kg Zn ha$^{-1}$ and 10 and 20 kg Zn ha$^{-1}$), indicating a threshold of zinc effect on gene family abundance between 5 and 10 kg Zn ha$^{-1}$ (Fig. 3). Microbial functional gene families associated with zinc transport (e.g., cation-transporting membrane facilitators), secondary metabolism (e.g., indole-3-pyruvic acid metabolic routes, magnesium chelatase, and antioxidant enzymes peroxidase), oxidative stress (e.g., superoxide dismutase), oxygen, glucose, and phosphate limitation (e.g., transcriptional regulators), stress (e.g., regulator of the sigma factor, drought tolerance, envelope stress), infection (e.g., adherence, virulence, and dissolution of the extracellular

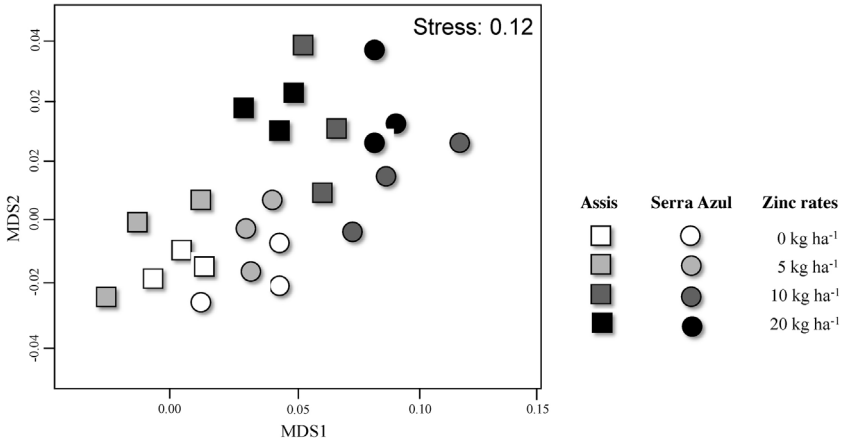


**Fig. 1.** Multidimensional scaling of functional gene families significantly correlated with zinc concentration in sugarcane-cultivated soils. Correlation was determined using Pearson coefficient, and we used a confidence level of $P < 0.05$.

**Table 4**
Analysis of similarity (ANOSIM). Pairwise comparison between functional gene family structures of microbial communities in sugarcane-cultivated soils under different zinc concentrations.

| | 0 kg Zn ha$^{-1}$ | 5 kg Zn ha$^{-1}$ | 10 kg Zn ha$^{-1}$ | 20 kg Zn ha$^{-1}$ |
|---|---|---|---|---|
| 0 kg Zn ha$^{-1}$ | – | 0.072 | 0.793 | 0.851 |
| 5 kg Zn ha$^{-1}$ | 0.005* | – | 0.754 | 0.810 |
| 10 kg Zn ha$^{-1}$ | 0.006* | 0.007* | – | 0.254 |
| 20 kg Zn ha$^{-1}$ | 0.005* | 0.005* | 0.006* | – |

Upper triangle indicates ANOSIM statistic based on Bray-Curtis dissimilarity between two different zinc concentrations applied to the soil. Lower triangle represents *P*-values of the test.
* $P < 0.05$.

matrix), secretion system (e.g., secretion protein, immune evasion), survival against host response (e.g., extracellular zinc metalloprotease, biofilm formation) and virulence (virulence protein, virulence regulatory gene, patulin biosynthesis) exhibited reduced abundance with increasing zinc concentrations in soil (Table 5). Additionally, microbial functional gene families associated with carbon degradation (e.g., glyoxylate cycle/isocitrate lyase, pectin, lignin, and phospholipids), carbon fixation (e.g., dicarboxylate/4-hydroxybutyrate cycle, reductive tricarboxylic acid cycle, 3-hydroxypropionate bicycle, Calvin cycle), denitrification, dissimilatory nitrogen reduction, and polyphosphate degradation also exhibited reduced abundance with increasing zinc concentrations in soil (Table 5). Increased abundance was demonstrated for gene families associated with methanogenesis, and secondary metabolism (e.g., halogenation and carotenoid) with increasing zinc concentrations in soil (Table 5). The microbial gene families '*sia*C' and 'lip', which are associated with anti-phagocytosis, exhibited contrasting responses to increasing zinc concentrations in soil, with increases in abundance for the '*sia*C' gene family. Gene probe origins for the gene families significantly correlated with zinc status in soil are presented in Supplementary Table S2.

## 4. Discussion

In agriculture, zinc is used as a nutritional supplement to promote plant growth. However, the incorporation of zinc into the soil may have complex effects on soil microorganisms, and the functional consequences of zinc supplementation are still not fully understood. In the present study, we used microarray-based high throughput genomic technologies to assess functional response at the community-level to zinc supplementation in sugarcane experimental fields. Our findings revealed significant differences in the functional gene structure of microbial communities and abundance of specific gene families across a gradient of zinc concentrations in these soils. However, the richness, diversity and evenness of functional genes did not differ across the zinc gradients in soil. This finding suggests that although the total number of functional genes within the microbial communities does not change, the identity of the present genes and the abundance of specific gene families are altered along the zinc

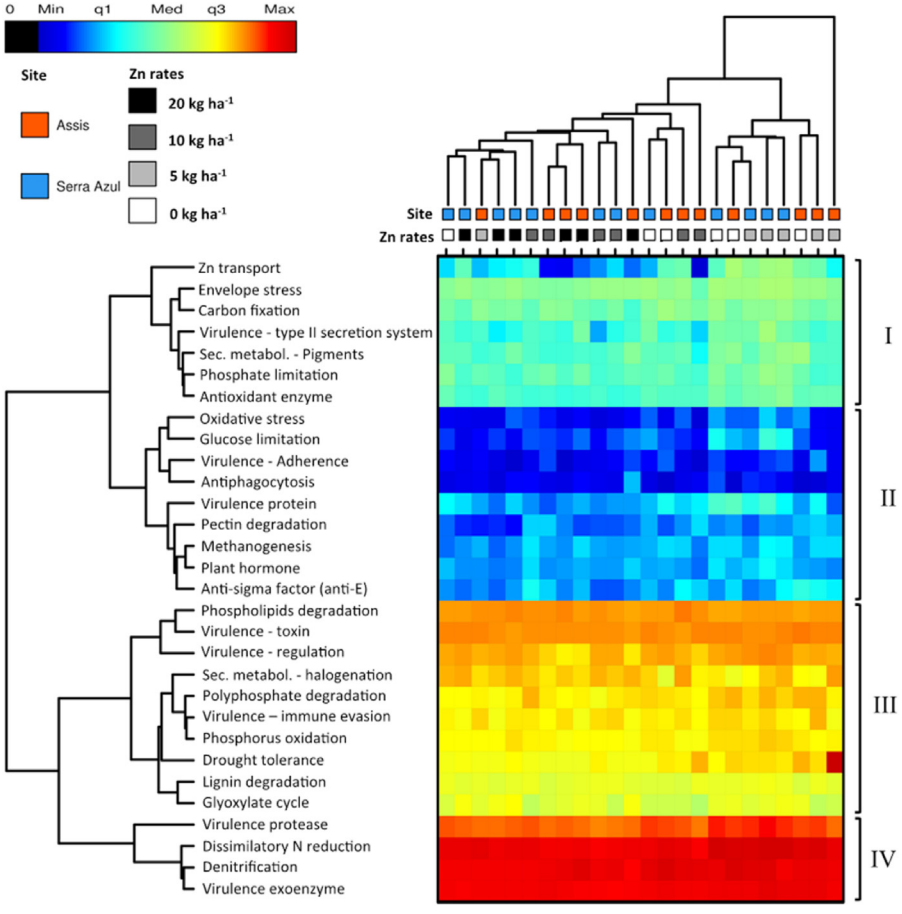


**Fig. 2.** Heatmap and cluster analysis of samples based on the dissimilarity in gene family abundance correlated with zinc concentration. Gene families (rows) and samples (columns) were clustered based on microarray abundance data of gene families significantly correlated with zinc concentration in soil. The heatmap colors correspond to the normalized signal intensity of the gene families. The color gradient represents the signal values across all genes/functional subcategories and samples, varying from cold colors (lower signal) to warm colors (higher signal). On the x-axis, samples were reordered and clustered according to their color intensity profile. On the y-axis, genes were grouped by functional subcategory, and their abundance was summed.

**Table 5**
Gene families significantly correlated with zinc concentration in soil (total content of zinc and its fractions) in both field experiments.

| Gene families | Estimate | Gene families | Estimate |
|---|---|---|---|
| **Zinc transport** | | **Survival against host response** | |
| MSC2 | −0.4287 | *sia*C | 0.4626 |
| YiiP (FieF) | −0.5333 | lip | −0.4083 |
| *Znt*A | −0.6826 | *zmp*A | −0.4698 |
| *Znu*C | −0.4544 | *ams*G | −0.4410 |
| *Zrt*2 | −0.4758 | **Virulence (others)** | |
| **Secondary metabolism** | | Vip | −0.4165 |
| per arc | −0.5249 | ler | −0.4629 |
| per fun | −0.4274 | patulin (6MSAS) | −0.4087 |
| V BPO | 0.4286 | **Carbon degradation** | |
| mg chelatase | −0.5011 | *Ace*A | −0.4178 |
| methoxyneurosporene desaturase | 0.4494 | Endopolygalacturonase | −0.4555 |
| IPyA | −0.4198 | Exopolygalacturonase | −0.5530 |
| **Oxidative stress** | | GLX | −0.4301 |
| *sod*A | −0.4940 | phospholipase C | −0.4310 |
| **Oxygen limitation** | | **Carbon fixation** | |
| Arca | −0.4698 | C CoA hydratase DiC4HB | −0.4566 |
| **Glucose limitation** | | FBPase | −0.4211 |
| *ccp*A | −0.4121 | *frd*A (rTCA) | −0.4796 |
| **Phosphate limitation** | | mdh | −0.4344 |
| *pho*B | −0.4110 | MMCE | −0.4616 |
| **Stress (others)** | | Rubisco | −0.5825 |
| *Rse*A | −0.4166 | *sdh*A | −0.4496 |
| tre fun | −0.6035 | *suc*A (DiC4HB) | −0.4583 |
| CpxR | −0.4929 | **Methanogenesis** | |
| *Psp*B | −0.4079 | *mrt*H | 0.4240 |
| **Infection** | | MtaB | 0.4528 |
| pilin | −0.4746 | **Denitrification** | |
| pap | −0.4730 | *nir*S | −0.4356 |
| *bfp*B | −0.4670 | **Dissimilatory N reduction** | |
| srt | −0.4404 | *Napa* | −0.4311 |
| *hys*A | −0.5013 | **Phosphorus utilization** | |
| **Secretion system** | | 5f1 (*ptx*D) | −0.4100 |
| *hrp*Q | −0.5414 | 5f1 (ppn) | −0.5730 |
| YopM | −0.5026 | | |
| *lcr*D | −0.4525 | | |

Gene families displayed in the table with Spearman's rank coefficient at the $P < 0.05$ level. Gene categories are indicated in bold.

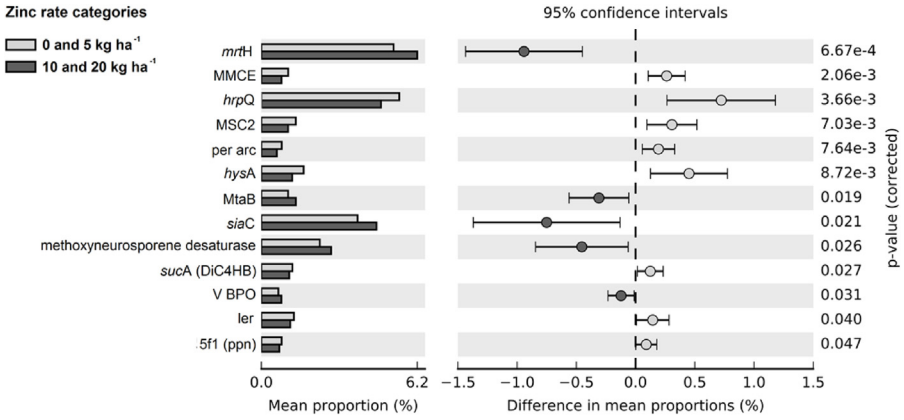


**Fig. 3.** Differences in abundance for gene families between two zinc rate categories revealed by using Statistical Analysis of Metagenomic Profiles (STAMP) software based on abundance of gene families correlated with zinc concentration in soil from both field experiments (Assis and Serra Azul).

gradient, with a clearer threshold in gene family abundance between 5 and 10 kg Zn $ha^{-1}$.

### *4.1. Impact of zinc inputs to the soil on richness, diversity, evenness and structure similarity of microbial functional genes*

Classical observations on toxic effects of heavy metals on field organisms, such as population size or ecological aspects at the community level (richness, diversity and evenness), are often difficult to interpret due to unexplained variation (or absence of variation) and unclear causality. It is often hypothesized that due to functional redundancy, the diversity of species in a community is more sensitive to toxicant stress than community functioning itself (Bååth, 1989; Moffett et al., 2003). However, Lock and Janssen (2005) showed that functional diversity of microbial communities decreased with increasing zinc concentrations in 11 soils contaminated with zinc runoff from galvanized electricity pylons. In the present study, analysis of similarity between the samples

suggested that there is a threshold in gene family abundance between 5 and 10 kg Zn $ha^{-1}$, which did not caused a change in richness, diversity and evenness of microbial functional genes in soil. This finding could, however, be expected as functional richness, diversity and evenness are not only influenced by the zinc concentration but also by numerous other soil factors.

### 4.2. Impact of zinc inputs to the soil on microbial functional groups

#### 4.2.1. Zinc transport

Cells require zinc transport mechanisms to allow efficient accumulation and distribution of zinc ions within the cell (Eide, 2006). In many bacteria, two well-characterized regulatory systems are used to ensure zinc homeostasis, and these genetic pathways act by cooperatively controlling the expression of membrane-bound transport systems that either import or export zinc cations (Hantke, 2001). The zinc uptake system, *Znu*, is employed by numerous Gram-negative bacteria to import zinc cations, and it is composed of an ABC-type transporter, where *Znu*C is the associated ATPase protein (Chandra et al., 2007). Additionally, the expression of the *Znu*ABC gene families is often controlled by a zinc-responsive transcriptional regulator of the *Fur* system called *Zur* (Hantke, 2005). The export of zinc from bacterial cells is often accomplished using the *Znt*A protein, which is an ATP-dependent transporter employed when cellular zinc concentrations are at toxic levels (Hantke, 2001). Previous studies have suggested that the efficient uptake of zinc could also play a critical role in virulence and during infection (Hantke, 2005; Ammendola et al., 2007; Sheehan et al., 2015). Our results are consistent with these previous observations given that zinc transport related gene families were adversely affected by increases in zinc concentrations, notably the cation-transporting membrane facilitators. The zinc effect on both microbial infection and virulence are discussed below.

### 4.3. Secondary metabolism

The secondary metabolism pathways potentially affected by zinc inputs were associated with the synthesis of auxin indole-3-acetic acid (IAA), chlorophyll and heme and the control of oxygen-reactive species. The associated gene families exhibited decreased abundance under high zinc conditions. In microorganisms, the indole-3-pyruvic acid (IPyA) pathway is the main route for IAA synthesis (Spaepen et al., 2007). Interactions between IAA-producing bacteria and plants lead to diverse outcomes on the plant side, varying from pathogenesis to phytostimulation (Spaepen et al., 2007). In photosynthetic organisms, the Mg-chelatase catalyzes the insertion of $Mg^{2+}$ into protoporphyrin IX, which is the first step unique to the synthesis of (bacterio) chlorophyll. Magnesium-protoporphyrin IX chelatase (Mg-chelatase) is located at the branch point of tetrapyrrole biosynthesis, at which point protoporphyrin IX is distributed for the synthesis of chlorophyll and heme. Regarding the control of oxygen-reactive species, microorganisms have developed efficient enzymatic and non-enzymatic mechanisms to eliminate reactive oxygen intermediates, e.g., superoxide radicals, hydrogen peroxide and hydroxyl radicals. Superoxide is eliminated by dismutation to $H_2O_2$ as catalyzed by superoxide dismutase, and the accumulation of $H_2O_2$ is prevented by the action of catalases and peroxidases (Fridovich, 1978; Zima et al., 1996).

In contrast, halogen metabolism (vanadium-dependent bromoperoxidases 'V-BPO') and carotenoid biosynthesis ('Methoxyneurosporene desaturase')-related gene families exhibited an increase in abundance with zinc input. The 'V BPO' gene family is related to antioxidative capacities (Ye et al., 2015). Carotenoids play important biological roles as accessory light-harvesting components of photosynthetic systems, photoprotecting antioxidants, and regulators of membrane fluidity.

### 4.4. Stress (oxidative stress, and oxygen, glucose, and phosphate limitation)

Decreases in abundance for superoxide dismutase '*sod*A' with increases in zinc concentration in our soils may be understood considering the key role played by SOD in the protection against harmful oxidative reaction resulting from metal stress (Monk et al., 1989). The '*sod*A' is a gene family related to oxidative stress. In regards to oxygen- and nutrient-responsive gene families ('*arcA*', '*ccpA*' and '*phoB*'), decreases in the expression of these proteins can alter transcriptional regulation, i.e., the means by which cells regulate the conversion of DNA to RNA, thereby influencing gene activity. Additionally, the stress-related gene family ('*RseA*') also exhibited reduced abundance with zinc inputs to our soils. '*RseA*' codes for a regulator of a sigma factor, a bacterial transcription initiation factor that enables specific binding of RNA polymerase to gene promoters. Taken together, these findings suggest adverse effects of zinc on the transcriptional process in microbial cells, which may be related to the fact that zinc is a component or activator of DNA-dependent RNA polymerase (Coleman, 1974), RNA-dependent DNA polymerase (Auld et al., 1974) and participates in the maintenance of ribosome integrity (Prask and Plocke, 1971) and the double helix of DNA (Chvapil, 1973). Finally, our results suggest a decrease in abundance for 'CpxR', a gene family related with envelope stress. The *Cpx* system responds to conditions associated with envelope stress, such as alkaline pH and overproduction of secreted proteins (Langen et al., 2001; Snyder et al., 1995), and to attachment of cells to surfaces (Otto and Silhavy, 2002).

### 4.5. Infection

Adherence is the main microbial function related to infection that was adversely affected by zinc inputs to our soils. The zinc effect on microbial adherence appears to be inconsistent in the literature and could be organism dependent. Based on assays with Gram-negative and Gram-positive bacteria, Sugarman et al. (1982) suggested that zinc can bind to bacterial pili and increase bacterial adherence. Crane et al. (2007) showed that zinc reduced the abundance of the RNAs encoded by bundle-forming pilus (*bfp*), a protein virulence factor in *Escherichia coli*. A potential adverse effect of zinc input to the soil was also highlighted in the exoenzyme hyaluronidase (also called hyaluronate lyase) encoded by the 'hysA' gene. Hyaluronidases are important for virulence in a number of Gram-negative and Gram-positive bacteria (Hart et al., 2009). These findings add evidence to the critical importance of tight regulation of zinc to soil microbial infection.

### 4.6. Secretion system

Microorganisms have evolved a wide variety of highly specialized macromolecular nanomachines that secrete a wide range of substrates, including small molecules, proteins and DNA, which may be necessary for zinc homeostasis (Serafini et al., 2014). These substrates have key roles in the response of bacteria to its environment and in several physiological processes (Costa et al., 2015). In our soils, increases in zinc concentrations adversely affected secretion systems related to microbial functions encoded by '*hrpQ*', '*YopM*' and '*lcrD*' genes. In phytopathogenic bacteria, the '*hrp*' system (for hypersensitive reaction and pathogenicity) encodes components of type III secretion system (TTSS) (Lindgren et al., 1986). TTSS translocates effector proteins into target cells to disrupt or modulate host cell signaling pathways and establish

replicative niches. *YopM*, a leucine-rich repeat TTSS protein, is a critical virulence factor in infection (McCoy et al., 2010). The *Yop* secretion system also involves *lcrD*, a gene of the *virA* locus, which encodes a 77-kDA inner membrane protein. Homologues of *lcr*D are involved in flagellar biogenesis in bacteria (Ramakrishnan et al., 1991). Taken together, these effects can potentially result in differences in physiological processes in microorganisms inhabiting our soils, such as adhesion, pathogenicity, adaptation and survival.

### 4.7. Survival against host response

Both specific and nonspecific host responses are critical for host survival. Without them, microorganisms would multiply and invade vital tissues and organs, resulting in severe damage to the host. Some nonspecific responses are biochemical, whereas others are cellular. Our findings revealed a negative effect of zinc on gene families related to cellular microbial responses associated with antiphagocytosis ('*lip*') and biofilm formation ('*amsG*') along with biochemical microbial responses such as extracellular zinc metalloprotease ('*zmpA*'). Zinc metalloproteases have been described in *Burkholderia cepacia*, a soil saprophyte Gram-negative bacteria (McKevitt et al., 1989; Corbett et al., 2003). The capsular gene families '*siaC*' and '*lip*' exhibited positive and negative responses to zinc inputs to our soils, respectively. Capsular gene expression is regulated both by phase variation via slipped-strand mispairing or reversible insertion of mobile elements (Hammerschmidt et al., 1996a,1996b) and at the transcriptional level. Indeed, evidence suggests that capsule biosynthesis and assembly are down-regulated during the early stages of the infectious cycle to facilitate adhesion to and invasion of the host cells (Deghmane et al., 2002).

### 4.8. Carbon degradation and fixation

The average composition of structural carbohydrates of sugarcane straw is 45–48% cellulose, 26–31% hemicellulose and 7–20% lignin (Singh et al., 2008). Endopolygalacturonase and exopolygalacturonase are enzymes that act on pectin, hydrolyzing its internal and external glycosidic bonds and producing shorter pectin molecular structures (Souza et al., 2003). Glyoxal oxidase (GLX) is presumed to generate radical species that participate in further reactions to degrade the lignin polymer (Kirk and Farrell, 1987). These enzymes involved in pectin and lignin degradation encoded by microbial genes were adversely affected by zinc inputs to our soils. Whereas microorganisms have metabolic requirements for trace metals, the same metals are often toxic at concentrations only a few times greater than those required (Hughes and Poole, 1991). For example, Davies and Sleep (1979) demonstrated inhibition of carbon fixation as a function of zinc uptake in natural phytoplankton assemblages. C-fixation is a highly important source of C in soils. In our soils, gene families associated with C-fixation pathways were also adversely affected by zinc inputs to the soil.

### 4.9. Methanogenesis

Although the inhibition of zinc on methanogens has been widely studied (Hayes and Theis, 1978; DeWalle et al., 1979; Bhattacharya et al., 1996), our results revealed an increase in the gene families involved with both hydrogenotrophic ('*mtrH*') and acetoclastic methanogenesis ('*MtaB*'). Some studies have shown that, up to a certain dosage, metals can work as stimulating agents in anaerobic digestion processes with a consequent increase in methane production (Demirel and Scherer, 2011; Mudhoo and Kumar, 2013).

### 4.10. Denitrification and dissimilatory nitrogen reduction

Microbial denitrification is a major pathway responsible for nitrate removal and a dominant source of nitrous oxide ($N_2O$) emissions from soil. Zheng et al. (2014) demonstrated that zinc substantially inhibited the gene expression and catalytic activity of key denitrifying enzymes in the same manner as noted in our findings. These negative effects of zinc on microbial denitrification may ultimately cause reduced nitrate removal and increased $N_2O$ emissions, which is likely to exacerbate global warming. An inhibitory effect of zinc on nitrogen reduction has also been reported by Stone et al. (2006).

### 4.11. Phosphorus utilization

Utilization of organic P by microorganisms and plants requires mineralization (hydrolysis) of substrates by phosphatase enzymes that may be of either microbial or plant origin, with some evidence that microbial enzymes exhibit increased efficiency for P release (Tarafdar et al., 2001; Richardson and Simpson, 2011). Zinc inputs to our soils reduced the abundance of gene families related to phosphorus oxidation and polyphosphate degradation. Herbert (1961) demonstrated that the distribution of P forms in microbial cells changes with environmental conditions, in particular with carbon and nutrient availability. However, zinc effects on microbial phosphorus utilization were not reported previously in the literature.

## 5. Conclusion

Taken together, our findings demonstrate that increases in zinc concentration in sugarcane-cultivated soil can alter the functional gene structure of microbial communities and abundance of specific gene families, with a threshold in gene family abundance between 5 and 10 kg Zn $ha^{-1}$ across the analyzed zinc concentrations. Responses of functional gene family abundance in microbial communities to zinc status in our soils were at least in part associated with zinc transport, antioxidative capacity, virulence, carbon-fixation, methanogenesis, phosphorus utilization, metabolism of halogen and carotenoid, and antiphagocytosis, which may reveal implications on soil microbial processes not only essential to ecosystem function but also for the sustainable management of agricultural ecosystems. Finally, these findings could assist in the identification of gene families with potential use as management-indicators of agricultural practices based on specialized functioning of the soil conducted by microbial groups in sugarcane production fields.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2016.12.009.

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