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Disentangling the influence of earthworms on microbial communities in sugarcane rhizosphere

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This thesis is dedicated
to my Parents, my Brothers and my Girl

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"Uma ciência saudável combina humildade com esperança: humildade para aceitar a extensão de nossa ignorância; e esperança de que novas descobertas irão expandir a Ilha do conhecimento. Porém, quando nos encontrarmos nas margens da Ilha e não pudermos contar com dados experimentais, a única estratégia à nossa disposição é a especulação bem fundamentada. Sem ela, sem o uso da imaginação, a ciência não pode avançar."

Marcelo Gleiser, A Ilha do Conhecimento; p153.

We are living the era of big data and it is our duty to transform our analytical capacity and use our minds to try to be more conclusive.

L.P.P.Braga (2016)

ABSTRACT

BRAGA, L. P. P. **Disentangling the influence of earthworms on microbial communities in sugarcane rhizosphere**. 2016. 96 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2016.

For the last 150 years many studies have shown the importance of earthworms for plant growth, but the exact mechanisms involved in the process are still poorly understood. Many important functions required for plant growth can be performed by soil microbes in the rhizosphere. To investigate earthworm influence on the rhizosphere microbial community, it was performed a macrocosm experiment with and without *Pontoscolex corethrurus* (EW+ and EW-, respectively) and followed various soil and rhizosphere processes for 217 days with sugarcane. In the second chapter of this thesis it was demonstrate that in EW+ treatments, N_2O concentrations belowground (15 cm depth) and relative abundances of nitrous oxide genes (*nos*Z) were higher in bulk soil and rhizosphere, suggesting that soil microbes were able to consume earthworm-induced N_2O. Shotgun sequencing (total DNA) revealed that around 70 microbial functions in bulk soil and rhizosphere differed between EW+ and EW- treatments. Overall, genes indicative of biosynthetic pathways and cell proliferation processes were enriched in EW+ treatments, suggesting a positive influence of worms. In EW+ rhizosphere, functions associated with plant-microbe symbiosis were enriched relative to EW- rhizosphere. Ecological networks inferred from the datasets revealed decreased niche diversification and increased keystone functions as an earthworm-derived effect. Plant biomass was improved in EW+ and worm population proliferated. Considering that earthworms contributed to with extra resources, it was evaluated in chapter three response of the soil resistome of sugarcane macrocosms under the influence of earthworms. Mechanisms of resistance against antimicrobial compounds appear to be an obligatory feature for the ecology and evolution of prokaryotic forms of life. However, most studies on resistance dynamics have been conducted in artificial conditions of anthropogenic inputs of antibiotics into very specific communities such as animal microbiomes. To resolve why and how resistance evolves, it is important to track antibiotics resistance genes (ARGs) (i.e., the resistome) in their natural hosts and understand their ecophysiological role in the environment. The results demonstrated that earthworms influenced changes of ARGs in bulk soil and rhizosphere. Negative correlations between ARGs and taxonomical changes were increased in EW+. Differential betweenness centrality ($DBC=nBC^{EW+} - nBC^{EW-}$) values comparing the network models with and without earthworms showed earthworm presence changed the composition and the importance of the keystone members from the models. Redundancy analysis suggested that ARGs may be associated with microbial fitness, as the variance of relative abundance of members of the group Rhizobiales could be significantly explained by the variance of a specific gene responsible for one mechanism of tetracycline detoxification.

Keywords: Microbial ecology. Soil metagenomics. N_2O. Soil resistome.

RESUMO

BRAGA, L. P. P. **Desvendando a influência de minhocas na comunidade microbiana de rizosfera de cana-de-açúcar.** 2016. 96 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2016.

Ao longo dos últimos 150 anos muitos estudos têm demonstrado a importância das minhocas para o crescimento de plantas. Porém o exato mecanismo envolvido neste processo ainda é muito pouco compreendido. Muitas funções importantes necessárias para o crescimento de plantas podem ser realizadas pela comunidade microbiana da rizosfera. Para investigar a influência das minhocas na comunidade microbiana da rizosfera, foi desenvolvido um experimento de macrocosmo com cana-de-açúcar com e sem *Pontoscolex corethrurus* (EW+ e EW-, respectivamente) seguindo diversos procedimentos por 217 dias. No Segundo capítulo da tese é demonstrado que no tratamento EW+, as concentrações de N_2O dentro do solo (15 cm profundidade) e a abundância relativa dos genes óxido nitroso redutase (*nos*Z) foram elevadas no solo e na rizosfera, sugerindo que microrganismos do solo foram capazes de consumir a emissão de N_2O induzida pelas minhocas. O sequenciamento do DNA total revelou que aproximadamente 70 funções microbianas no solo e na rizosfera apresentaram diferenças entre os tratamentos EW+ e EW-. No geral, genes associados a biossíntese e proliferação de células foram enriquecidos em EW+, sugerindo uma influencia positiva por parte das minhocas. Na rizosfera EW+, funções associadas a simbiose entre planta e microrganismos foram relativamente enriquecidas comparado com rizosfera EW-. Modelos de rede de interação ecológica revelam menor número de diversificação de nichos e aumento de funções importantes como um efeito derivado da influência das minhocas. A biomassa das plantas foi aumentada no tratamento EW+ e a população de minhocas proliferou. Considerando que as minhocas contribuíram com o aumento de nutrientes, foi avaliado no capítulo três a resposta do resistoma presente nas comunidades microbianas dos solos do experimento. Mecanismos de resistência contra compostos antimicrobianos parecem ser características obrigatórias para a ecologia e evolução de procariotos. Entretanto, a maior parte dos estudos sobre genes de resistência tem sido conduzida em condições artificiais utilizando fontes antropogênicas de antibióticos em comunidades microbianas muito específicas como por exemplo o microbioma animal. Para resolver por que e como a resistência evolui, é importante estudar genes de resistência a antibióticos (GRA) (i.e., resistoma) no seu ambiente natural e entender seu papel ecofisiologico no ambiente. Os resultados demonstraram que minhocas influenciaram a mudança na composição de GRA no solo e na rizosfera. Tratamentos EW+ apresentaram maior número de correlações negativas entre ARG e grupos taxonômicos. A medida de centralidade diferencial (DBC=nBC^{EW+} – nBC^{EW-}) comparando os modelos de rede de interações obtidos mostrou que a composição e o nível de importância dos indivíduos mais influentes é alterado nos tratamentos EW+ comparado com EW-. Além disso, por meio de uma análise de redundância (RDA) foi demonstrado que as alterações na abundancia relativa de GRA podem ser explicadas pelas alterações verificadas em grupos taxonômicos.

Palavras-chave: Ecologia microbiana. Metagenoma de solo. N_2O. Resistoma do solo.

SUMMARY

1. PRELUDE AND THESIS STRUCTURE

> "On the mountains of North Wales and on the Alps, worms, as I have been informed, are in most places rare (p.12). Worms are omnivorous. They swallow an enormous quantity of earth (p.35). […] also greatly facilitate the downward passage of roots of moderate size, and these will be nourished by the humus with which the burrows are lined (p.147). […] Many seeds owe their germination to having been covered by castings, and others buried to a considerable depth beneath accumulated casting lie dormant, until at some future time they are accidentally uncovered and germinate (p.147)".
>
> (Charles Darwin, The formation of vegetable mould through the action of worms with observations on their habits; 1881)

1.1. Introduction

The general interest on beneficial functions provided by earthworms started to grow manly after Darwin's book (1881), up to then, worms were considered to be garden pests that had to be removed from the soil (FELLER et al., 2003). Although Darwin's first observations addressed mainly the role of earthworms on sediments disposal and soil formation (MEYSMAN et al., 2006), he outlined some of the beneficial influence of worms on plant health. This phenomenon has been largely studied thenceforth (DARWIN, 1881; BROWN et al., 1999; VAN GROENIGEN et al., 2015). Nowadays it is of common sense that earthworms improve plant growth, but, so far, the mechanisms behind it are still poorly understood.

With the advent of industrial revolution, technological advances employed by agricultural systems transformed land use promoting essential benefits for modern societies. However, the large use of chemicals to control plagues and improve soil fertility has intensified soil degradation and lost of diversity (JAMES, 1997; ALTIERI; ROSSET, 1995; BUTTEL; GERTLER, 1982; CONWAY; PRETTY, 1991). Plant development is a process coordinated with microbial communities found in soil particles under the influence of roots. Soil microbes can produce vital compounds to plants (BRUTO et al., 2013).

In the gut soil bacteria ingested by earthworms can perform different metabolic processes (i.e., fermentation and denitrification). Recently it has been demonstrated that Brazilian earthworms can emit CH_4 e N_2O as a consequence of microbial metabolism activated by the anoxic and nutrient-rich conditions found in the alimentary channel

(DEPKAT-JAKOB et al., 2012; 2013). The influence of earthworms on microbial metabolic processes may be not strict to microbes inside the gut (DALLINGER; HORN, 2014). The beneficial influence of earthworms on plants and soil fertility is in great part attributed to the product of its digestion (i.e., casts) (MIKLÓS, 1996; LAVELLE, 1997). In some regions, studies demonstrate that a considerable proportion of soil (10-30 cm) has been in some moment ingested by worms. Worms can consume soil in a proportion of 5 to 30 times of their body weight per day (LAVELLE, 1988). However, so far, no study has addressed the effect of earthworms on rhizosphere microbial community.

The study of microbial communities in its natural environments is now possible by the advances in sequencing technology. One approach largely employed currently is the characterization of all genes present in environmental DNA. Such method is defined as metagenomic analysis and aims to provide a holist comprehension of the community (PROSSER, 2015). Therefore, the general hypothesis explored in the present thesis is that earthworms influence changes in microbial communities living in soil particles attached to plant roots. Metagenomic sequencing of soil microbial communities was employed to study the response of microbial communities to earthworms in sugarcane macrocosm experiment. The findings obtained from the experiment are presented next in the form of chapters. In the following two chapters this thesis aims to provide a comprehensive analysis of the influence of earthworms on microbial communities associated to plant roots. Each chapter addressees a different question and therefore each one has a specific hypothesis, specific objectives and different methodological approaches that where used to answer different questions as detailed further.

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2. DISENTANGLING THE INFLUENCE OF EARTHWORMS ON SUGARCANE RHIZOSPHERE

Abstract

For the last 150 years many studies have shown the importance of earthworms for plant growth, but the exact mechanisms involved in the process are still poorly understood. Many important functions required for plant growth can be performed by soil microbes in the rhizosphere. To investigate earthworm influence on the rhizosphere microbial community, It was performed a macrocosm experiment with and without *Pontoscolex corethrurus* (EW+ and EW-, respectively) and followed various soil and rhizosphere processes for 217 days with sugarcane. In EW+ treatments, N_2O concentrations belowground (15 cm depth) and relative abundances of nitrous oxide genes (*nos*Z) were higher in bulk soil and rhizosphere, suggesting that soil microbes were able to consume earthworm-induced N_2O. Shotgun sequencing (total DNA) revealed that around 70 microbial functions in bulk soil and rhizosphere differed between EW+ and EW- treatments. Overall, genes indicative of biosynthetic pathways and cell proliferation processes were enriched in EW+ treatments, suggesting a positive influence of worms. In EW+ rhizosphere, functions associated with plant-microbe symbiosis were enriched relative to EW- rhizosphere. Ecological networks inferred from the datasets revealed decreased niche diversification and increased keystone functions as an earthworm-derived effect. Plant biomass was improved in EW+ and worm population proliferated.

2.1. Introduction

Earthworms have a great capacity of modifying their habitats. These animals are recognized as ecosystem engineers due to their ability to convert soils into specialized functional domains, such as the drilosphere (BOUCHÉ et al., 1975) consisting of casts, burrows and the worms themselves) (that can regulate soil nutrient fluxes well beyond the life-span of an individual earthworm (LAVELLE, 2002). Therefore, earthworms can improve plant growth by enhancing organic matter mineralization and improving soil porosity and water content (JAMES, 1991; LAVELLE et al., 1992; SUBLER et al., 1997; BLANCHART et al., 1999; BROWN et al., 2000; SHIPITALE et al., 2004). However, the determination of the particular mechanisms connecting the promotion of beneficial soil functions and plant growth is more complex due to multiple interactions among the factors involved. For

example, nitrogen is an essential nutrient for plant growth and its availability is limited in most terrestrial ecosystems (KUZYAKOV; XU et al., 2013). A meta-analysis recently suggested that the benefits of earthworms would arise mainly from its capacity to improve the release of nitrogen trapped in organic matter (VAN GROENIGEN et al., 2014). Nevertheless, enhanced nitrogen release does not always explain plant growth in the presence of earthworms (BLOUIN et al., 2006). Blouin et al. (2006) tested the effect of earthworms on plant biomass over N-gradient conditions, and demonstrated that the beneficial effect on biomass improvement was independent of the variations in N concentrations. In this study, the hypothesis that the main effect of earthworms on plant production is due to increased N mineralization was rejected. Therefore suggesting a more complex mechanism in which not only mineralization of nutrients but also plant growth regulators would be involved in the process by which earthworms improve plant biomass (BLOUIN et al., 2006; PUGA-FREITAS et al., 2015). Such compounds have already been demonstrated to be present in earthworm dejections (MUSCOLO et al., 1998; NARDI et al., 2000; CANELLAS et al., 2002).

Soil influenced by roots, namely the rhizosphere, is considered an environment of complex biological interactions, where many different species soil microorganisms can grow using the large amount of organic compounds released by roots (MENDES et al., 2013). Rhizosphere microorganisms play important roles in plant physiology. They can facilitate the uptake of many important nutrients such as nitrogen, phosphorus and iron (MENDES et al., 2013; BRUTO et al., 2014) and also synthetize complex compounds known to participate in plant growth regulation process[17]. Most of the microbes growing in the rhizosphere are organothrophs (MENDES et al., 2013). Therefore rhizosphere microbes are likely to be positively influenced by the organic compounds that are egested by earthworms.

Compared to the pre-ingested soil, gut contents can contain more concentrated levels of ammonium, amino acids and fatty acids. Further, compounds such as glucose, maltose, formate, acetate, lactate and succinate, which normally cannot be detected in soils, are found in the alimentary canal in large amounts DRAKE; HORN, 2007). Additionally, the *in situ* conditions of earthworm gut are likely to favor denitrification. N_2O and N_2 emissions from the earthworms and denitrification genes were reported to be enriched in the alimentary canal of earthworms (DEPKAT-JAKOB et al., 2013). Likewise, the products of metabolic processes happening in the gut can be released in the soil (KARSTEN et al., 1995; FURLONG et al., 2002; IHSSEN et al., 2003; HORN et al., 2005) and promote benefits to microbial communities living even in a range beyond the drilosphere

(DALLINGER; HORN 2014). However, little is known of the effects of earthworms on microbial functions in rhizosphere.

Sugarcane is one of the most efficient plants in converting sun energy into sugars. Besides that, this plant has also a remarkable necessity of accumulating silicon (Si), absorbing it more than any other mineral nutrient (SAVANT et al., 2008). Si is proposed as an essential element for sugarcane, being necessary to support cell growth and protect against water loss, pathogens and heavy metal toxixity (SAVANT et al., 2008). The production of sugarcane is of great importance for developing countries, especially Brazil, occupying more than 10 million hectares. Sugarcane cropland receives huge amounts of fertilizers and pesticides annually (FISOLO et al., 2015). Elucidating soil processes and the mechanisms by which earthworms can improve biomass production and plant health is of great concern in order to develop more sustainable use of natural resources in agroecoystems.

2.2. Hypothesis

It is hypothesized that microbial functions in sugarcane rhizosphere are altered by earthworms and that functional changes are associated with plant beneficial functions.

2.3. Objectives

To investigate the soil microbial functions in response to the presence of *Pontoscolex corethrurus*, a peregrine earthworm species commonly found in sugarcane fields (SPAIN et al., 1990) and throughout the tropics and sub-tropics (BROWN et al., 2006), in pots growing sugarcane seedlings. As earthworms are known to emit N_2O as a consequence of denitrification happening in their gut, and as some soil microbial communities have the potential to be a sink for N_2O (JONES et al., 2014) by reducing it to N_2 through the nitrous oxide reductase enzyme, it was also monitored N_2O concentrations belowground along the experiment and determined the abundance of the nitrous oxide reductase gene (*nos*Z) in bulk soil and rhizospheric community at the end of the experiment. CO_2 concentrations belowground were also reported as an indicative parameter of respiration. Advanced methods of molecular biology for metagenomic whole community shotgun sequencing were performed for revealing the functional profile of soil microbial community.

2.4. Methods

2.4.1. Experimental design

A greenhouse experiment was conducted for 217 days using 100-L plastic pots filled (41cm height; 71 cm diameter at the top; 54 cm diameter at the bottom) with 70 kg of sieved and homogenized soil (podzolic dark red oxisol; 30% sand, 8% silt and 62% clay), collected from the University of São Paulo - Experimental Station (Piracicaba, Sao Paulo, Brazil) above a 3 cm layer of washed stones. The pots were subjected to natural lighting cycle and natural variation of temperature inside the greenhouse. Piracicaba has a tropical climate, the average of the maximum temperatures along the experiment were around 28.25ºC (±1.38). The soil sieved and homogenized was left resting in pots for 2 weeks until the beginning of the experiment, which was when sugarcane was planted and worms were inoculated. The resting period before beginning the experiment was to stabilize the production of gases resulting from the soil sampling. Before the beginning of the experiment an airstone (aquarium bubbler, 4 cm height and 1.5 cm diameter) was placed inside the soil, buried in the center of the pot, at 15 cm depth. The airstone was connected with the atmosphere through a silicon tube with a plastic cap that was closed prior to gas sampling. This approach was designed to collect gas samples inside the soil in each one of the pots in order to obtain the concentrations of N_2O and CO_2 belowground.

A total of six pots including three replicates with earthworms (EW+) and three without earthworms (EW-) were used to test the influence of earthworms on soil microbiome with growing sugarcane. Soil moisture was monitored with specific sensors (Extech MO750, Nashua, NH, USA) and the humidity was determined at 15 cm depth and maintained at the 40% by watering the pots with distilled water when necessary. Plants were obtained from the Sugarcane Center of Technology (CTC). Six seedlings produced by tissue culture, from the same variety (CTC22) and at the same development stage, were planted in each pot. After 90 days, 3 plants from each pot were culled randomly in order to reduce nutrient competition between the remaining plants of the macrocosms. Earthworms (*Pontoscolex corethrurus*) were purchased from Minhobox (Juiz de Fora, MG). Worms were acclimated for 24 hours in extra pots containing the same soil used in the experiment. After this period they were transferred to a plastic container with wet tissue paper and kept for 4 hours for gut "clearance". Twenty individuals per pot were inoculated in three of the six experimental units just after planting sugarcane seedling.

Destructive soil and plant sampling was performed at the end of the experiment (217 days). Under field conditions sugarcane is harvested from 12-18 moths after planting. For the specific case of this experiment, the decision was based on the concentrations of gases belowground and the size of the plants. Significant differences in N_2O emissions were observed only during the first 60 days and after 200 days some of the plants were over 2 meters tall, stretching the limits of the greenhouse. Bulk soil soil was collected (0-10 cm depth) from three equidistant points, considering a 10 cm distance between samples and the position of silicon tube from the airstone as the centroid. Soil from the different points was homogenized and stored at -80ºC prior to the molecular analysis. The soil samples were subsampled for soil chemical analysis at the Soil Analysis Laboratory of University of São Paulo (Department of Soil Science). The three plants in each pot were removed and rhizosphere samples collected by scratching root-attached soil, homogenized and stored at -80ºC prior to molecular analysis. Plant parts (roots and shoots) were oven (60º C) dried and weighed. Finally, the pots containing earthworms were hand-sieved and all the animals removed and counted.

2.4.2. N_2O and CO_2 determination

Twenty-two soil atmosphere (belowground) samples were collected per pot from the aeration stones, using syringes periodically along the experiment. The samplings were taken all in the morning around 10:00 h, and the time in between the samplings were as follows: the first 16 samplings were taken using an interval of ~7 days, after that, 3 samplings used an interval of ~10 days, and the following 2 samplings used an interval of ~15 days with the last one taken using an interval of ~30 days. N_2O and CO_2 were determined using gas chromatography (SRI 8610C Model, Torrance, CA, USA) configured with the same analytical conditions as described elsewhere (NAVARRETE et al., 2015) (HayeSep-D and N-packed columns at 81ºC). Average of concentrations was calculated as follows: the values of concentrations measured along the experiment were summed and divided by the number of samplings. A timeline plot of the average concentrations for each gas is presented.

2.4.3. Molecular analysis

Total DNA from soil was extracted using the Power Lyzer Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to instructions provided by the manufacturer. After extraction, DNA quality was determined in a microliter spectrophotometer (NanoDrop). The quantifications of 16S rRNA genes from Bacteria, Archaea and *nos*Z (encoding for nitrous oxide reductases) were performed using the StepOnePlus^{tm} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The standard curves were obtained from dilutions (10^3-10^8 copies of gene per µL) of a known amount of the gene amplified by PCR previously. The reaction mixture included 5 µL of SYBR green 2x reaction mix (Fermentas, Thermo Scientific, Wilmington , DE, USA), 1 µL of each primer (5 µL), 2 µL of ultrapure water and 1 µL of template DNA. The conditions for amplification of the genes 16S rRNA from Bacteria, 16S rRNA form Archaea, and *nos*Z were performed as described by Heuer et al. (1997), Yu et al. (2008) and Henry et al. (2006), respectively. Analysis of melting curve of amplicons was performed to confirm the specificity of amplification. After quantification the results were analyzed using the StepOnePlus^{tm} Real Time software v.2.2 (Applied Biosystems, Foster, CA, USA).

Shotgun sequencing of total DNA libraries was performed with Nextera kit according to the manufacturer instructions for the MiSeq reagent kit v2 (500 cycles; Illumina, San Diego, CA, USA). The quality and quantity of DNA used in the kit reactions were determined using spectrophotometer (NanoDrop ND-2000; Thermo Scientific, Wilmington, DE, USA) and fluorometric measurement with the Qubit dsDNA BR assay kit (Moleculas Orobes Life Technologies, Foster, CA, USA). The quantification of DNA in the libraries prior to the last dilution before sequencing, as determined by the manufacturer, was performed using KAPA SYBRFAST qPCR. Libraries were sequenced using an in-house MiSeq Personal Sequencing System (Illumina, San Diego, CA, USA). The metagenomic datasets raw reads are available via MG-RAST under the project name “Metagenomics of sugarcane soils”, via the link “http://metagenomics.anl.gov/linkin.cgi?project=19145” (files 1-3 refer to the EW- samples, files 4-6 refer to the EW+ samples, letters “b” and “r” indicates whether the reads are from bulk soil or rhizosphere, respectively).

2.4.4. Statistical analysis

A multivariate analysis was performed for the variables measured using metagenomic approach, for all the other a univariate analysis was performed. In both cases homogeneity of variance and normality were tested in order to define the most appropriate statistical test to be used in order to detect the significant differences between EW+ and EW-. The significance level (alpha) considered for all the tests was 0.05. For the univariate analysis, to test the null hypothesis of homogeneity and normal distribution the tests Levene and Shapiro-Willk were applied using R statistical computing (R DEVELOPMENT CORE TEAM, 2007). For alpha < 0.05 in any of the tests, Kruskal-Wallis test was implemented, otherwise t-test was implemented using PAST (HAMMER et al., 2001). For the multivariate analysis, the homogeneity of variances was tested using Marti Anderson's (PERMDISP2) procedure, a multivariate procedure analogue of the Levene's test in the R package VEGAN (OKSANEN, et al., 2001). Respectively, in bulk soil and rhizosphere, a total of 2,243 and 2,043 variables were assigned as functions encountered in the metagenomic datasets. Hence, the hypothesis of normal distribution was tested based on skewness (Mardia's test) univariatedly. Only 12 % and 15 % of the variables, respectively from bulk soil and rhizosphere datasets, were found to be nearly asymmetric as their skew values were found to be two times greater than the standard error of the skewness (TABACHNICK; FIDEL, 1968; HAE-YOUNG, 2013). However, none of the skew values of the variables were above the critical threshold (KLINE, 2011), therefore the datasets were considered to fall within the hypothesis of normal distribution. The analysis of the metagenomic datasets was performed according to the best practices as determined by the Statistical Analysis of Metagenomic Profiles (STAMP) methods, using the effect size and the confidence intervals for assessing biological importance (PARKS et al., 2014). The t-test (two-sided) was selected using t-test inverted as the method to calculate the confidence intervals of the effect sizes. The effect size is the difference in proportion (DP) of sequences assigned to a given feature in two samples, and it was calculated as follows: DP= p_1 - p_2. Where p_1 and p_2 are the number of sequences in the two samples assigned to the features of interest (x_1 and x_2) divided by the total number of sequences in the profile (C_1 and C_2) (i.e., $p_1 = x_1/C_1$; $p_2=x_2/C_2$). Error bar plots indicating the p-value with the effect size and associated confidence interval for each function detected to be of significant biological relevance (t-test, p-value<0.05) were generated (Supplementary Figure S2).

2.4.5. Computational analysis

Using PEAR (ZHANG et al., 2014), metagenomic datasets were merged (R1 and R2) and the leftover (not merged) reads from R1 included within the output. Sequences below 50 nucleotides length and Q20 were removed. The screening of the datasets was performed using MEGAN6 (HUSON et al., 2016) by providing the alignments resulting from DIAMOND (BUCHFINK et al., 2015) against an NCBI-NR database (Feb/2016). The read counts were normalized to the smallest number of reads (HUSON et al., 2016). Functional profiling was investigated using the INTERPRO2GO database (MITCHELL et al., 2015), resulting matrixes were exported using STAMP format for the statistical analysis as described above.

The correlations between the most abundant microbial functions (i.e., all those with abundance grater than the average abundance) were built according to the technique for inferring the sparse correlations for compositional data (SparCC) (FRIEDMAN et al., 2012). This method uses a permutation-based (n=100) approach to calculate p-values for the interactions, so that only significant (p-values<0.05) and strong (-0.9> r >0.9) correlations were maintained in the network graph. The graph was visualized with the interactive platform GEPHI (BASTIAN et al., 2009) using the Fruchterman Reingold algorithm. The degree of importance of the nodes was determined by the value of betweenness centrality and the clusters were determined by the modularity of the network, both measures were extracted from GEPHI.

2.4.6. Microcosm experiment

A validation experiment was performed in order to understand and compare plant contribution on N_2O emissions belowground. Plastic pots (5L) were filled with sieved and homogenized soil collected from the same location as was for the macrocosm experiment (University of Sao Paulo –Experimental Station, Piracicaba) and incubated in the greenhouse for 30 days with and without sugarcane seedlings and with and without earthworms (n=4). The earthworms from the same specie (*P. corethrurus*) were obtained from a specialized producer (Minhobox) and followed the same procedure of pre-incubation as described for the macrocosm experiment. Two sugarcane seedlings at the same developmental stage were planted per pot and 20 individuals of young earthworms were inoculated per pot.

For sampling the gas belowground it was used the same method described for the macrocosm experiment with airstones buried (15 cm depth) in the center of pots.

After 30 days gas samples belowground were collected and a destructive sampling was performed, and rhizospheric soil and earthworms were incubated for gas measurements. 1 gram (fresh weight) of rhizospheric soil (n=3) from the pots with earthworms (EW+) and without it (EW-), or 1 individual of earthworm (n=3) from the pots with sugarcane (SC+) and without it (SC-) was incubated for 5 minutes inside 10 ml syringes in the dark at room temperature. Prior to the incubation, worms were washed in sterilized water, dried with a paper towel and transferred to the 10 ml syringe. After transferring the rhizospheric soil or the earthworm, the volume of the syringe was set to 10 ml and the plastic cap (replacing the needle) was closed, so that there was no variation in the pressure and the atmosphere inside was not exchanged with the outside. After incubation time the syringe was connected to the chromatograph, the plastic cap of the syringe was opened and a sample of air was injected. After injection of the syringe air into the chromatograph, worms were removed from the syringes and weighted (0.29 g ±0.07). Therefore the gas emission from earthworms was normalized per gram of the individual by dividing the concentrations obtained by the fresh weight of the worm incubated. The variables measured were tested statistically for homogeneity and normal distribution prior to the identification of appropriate statistical tests for detecting differences between the means.

2.5. Results

2.5.1. Effect of earthworms on plant biomass and soil chemical parameters

Data collected at the end of the experiment revealed that mean plant dry mass was significantly higher in the pots with earthworms (EW+) (t-test, p-value =0.018) (Figure 2.1a) and the level of Si in soils was considerably lower than in the pots without earthworms (EW-) (t-test, p-value=0.11) (Figure 2.1b). No significant differences were observed for the levels of total nitrogen (N) (t-test, p-value=0.29) (Figure 2.1c) and organic carbon (OC) in soils (t-test, p-value=0.63) (Figure 2.1d). The detailed results of other chemical parameters of soil are included in the appendices (Table S2.1)

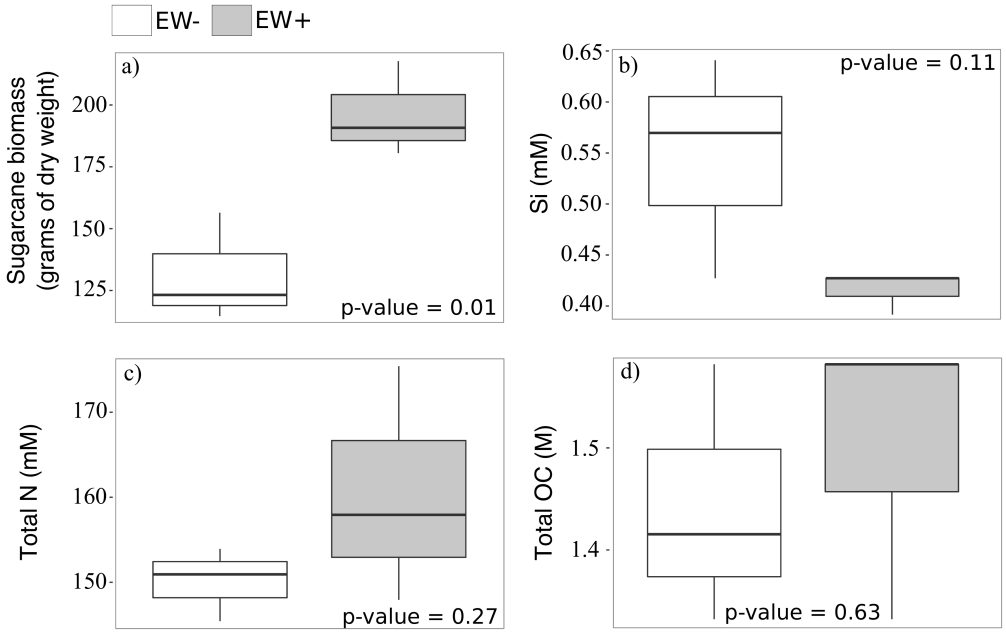


Figure 2.1 - Plant and soil parameters determined after 217 days of greenhouse experiment. Panel a) indicates plant total biomass (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.01). Panel b) indicates levels of silicon (Si) determined in bulk soil soil samples at the end of the experiment (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.11). Panel c) indicates levels of total nitrogen (N) determined at the end of the experiment (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.28). Panel c) indicates the levels of total organic carbon (OC) determined at the end of the experiment (levene's test, F>0.05; Shapiro-Wilk's test, p<0.05; Kruskal-Wallis, p-value=0.63). Empty boxes represents the values obtained in the pots without earthworms (EW-) and striped boxes represents the values obtained in the pots with earthworms (EW+).

2.5.2. The earthworm biomass

At the end of the experiment a mean of 92 individuals (±28.71) of *P. corethrurus* were counted per pot and several eggs were observed in the three pots. The increase in the number of individuals per pot from the beginning to the end of the experiment was 72 ± 28.71. The mean of the earthworm total biomass (sum of individuals weight) at the end of the experiment was 9.43 (±5.14) grams (g) of fresh weight per pot, almost the same as inoculated (9 g ±0.57). However, the average weight of the individuals (grams per worm) was considerably lower compared to the initial. The average weight of the individual inoculated at the pots was 0.45 g (±0.18) and the average weight of the individuals recovered from the pots was 0.10 g (±0.13). This result indicates that the experimental conditions favored worm development and reproduction.

2.5.3. N_2O and CO_2 production belowground

The accumulated mean of N_2O concentration belowground (i.e., the sum of all the measurements of concentration obtained from an experimental unit divided by the number of samplings) was significantly higher in EW+ than EW- pots (Kruskal-Wallis test, p-value=0.049) (Figure 2.2a). However averages of N_2O concentration in a timeline series (Figure 2.2b) were significantly higher in EW+ than EW- (Kruskal-Wallis test, p-values<0.05) only at the beginning of the experiment. After the 60th day (starting from date 30/04), the concentration averages were decreased until nearly the same levels found in EW- pots and apart from the samplings collect at date 22/05 and 18/07, in which N_2O was significantly higher in EW+ than EW- (Kruskal-Wallis test, p-values<0.05), all the others showed no significant differences (Kruskal-Wallis test, p-values>0.05).

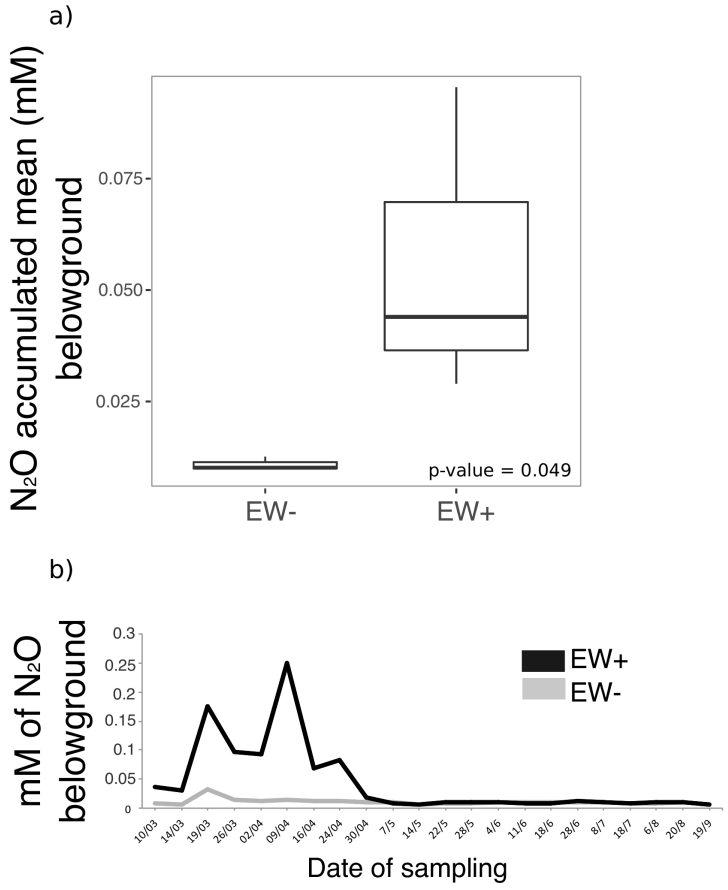


Figure 2.2 - N_2O concentration belowground (15 cm depth) monitored along the experiment. Panel a) indicates the accumulated mean of N2O concentrations in pots with earthworm (EW+) and without earthworms (EW-) (levene's test, F>0.05; Shapiro-Wilk's test, p<0.05; Kruskal-Wallis, p-value=0.04). Panel b) indicates 22 values (x-axis) of N2O mean collected along the experiment (217 days) according the date of sampling. The black line represents the values obtained in the pots with earthworms (EW+), and the gray line represents the values obtained in the pots without earthworms (EW-).

The accumulated mean of CO_2 concentration belowground was not different in EW+ compared to EW- (t-test, p-value=0.25) (Figure 2.3a). The averages of CO_2 concentrations in a timeline series (Figure 2.3b) were higher in both EW+ and EW- only at the beginning, and started to decrease around day 60th. However, CO2 started to decrease a little earlier in EW-, so that CO_2 concentrations were significantly higher in EW+ for at least 7 days, from date 30/04 until 07/05. Worth noting that the decline period coincide with decline of N_2O in EW+.

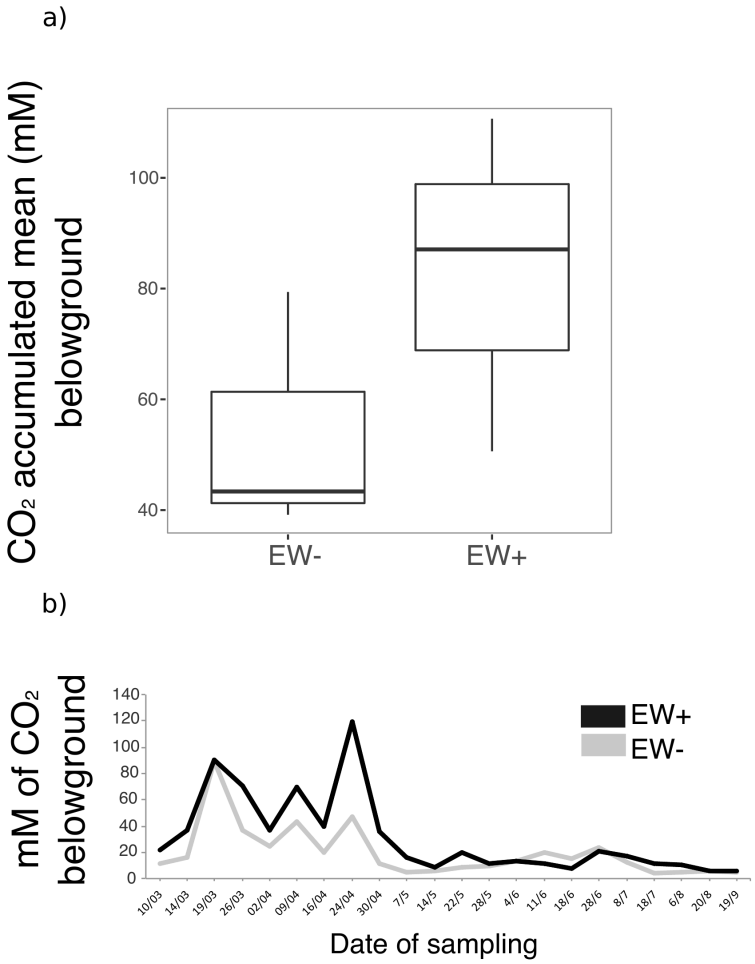


Figure 3 - CO_2 concentration belowground (15 cm depth) monitored along the experiment. Panel a) indicates the accumulated mean of CO_2 concentrations in pots with earthworm (EW+) and without earthworms (EW-) (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.25). Panel b) indicates 22 values (x-axis) of CO_2 mean collected along the experiment (217 days) according the date of sampling. The black line represents the values obtained in the pots with earthworms (EW+), and the gray line represents the values obtained in the pots without earthworms (EW-).

2.5.4. Quantification of 16S rRNA (Bacteria and Archaea) and nitrous oxide reductase gene (nosZ)

Bacteria 16S rRNA gene abundance was enriched significantly in the bulk soil (t-test, p-value=0.01) from EW+ relative to EW- pots (Table 2.1). No significant difference was observed for Archaea. The abundance of *nos*Z gene was increased considerably in the bulk soil (t-test, p-value=0.07) (Figure 2.4a) and significantly in the rhizosphere from EW+ compared to EW- (t-test, p-value=$7.4x10^{-4}$) (Figure 2.4b). The proportion of *nos*Z for the prokaryotic community, expressed as the ratio of the abundance of *nos*Z and the 16S rRNA gene abundances, showed the same tendency (t-test, p-value=0.05) in rhizosphere of EW+ relative to EW- (Figure 2.4c-d).

Table 2.1 - Means and standard error for the values of *Bacteria* and *Archaea* abundances comparing the treatments with earthworms (EW+) and without earthworms (EW-).

	EW+		EW-	
Bacteria ($x10^5$)				
Bulk soil	**4.3**	±0.6	2.6	±0.2
Rhizosphere	3.8	±0.4	3.3	±0.1
Archaea ($x10^2$)				
Bulk soil	7.3	±4.6	5	±2.2
Rhizosphere	6.2	±0.7	7.6	±1.6

Values from qPCR were normalized according to the DNA concentration (ng/µl) measured in each sample after extracted from soil. Significant differences between treatments (EW+ and EW-) are represented in bold (t-test, p-value<0.05).

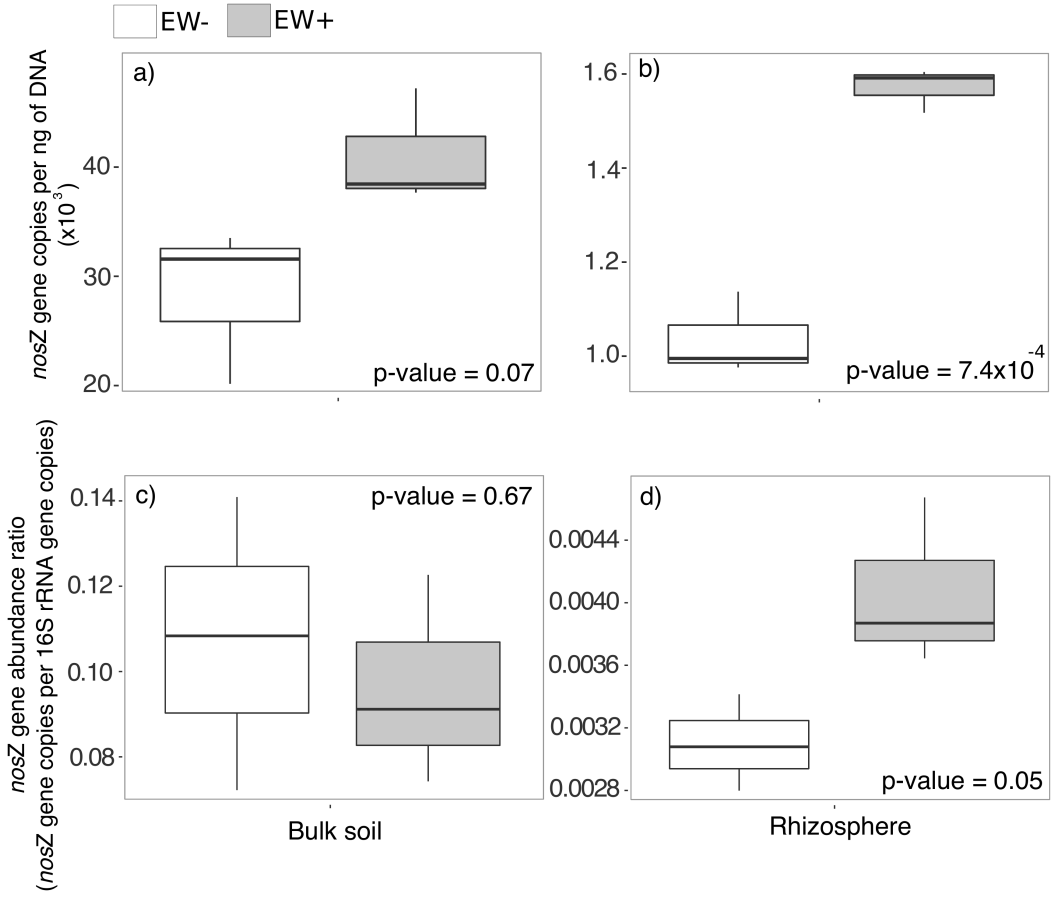


Figure 2.4 - Abundance of nitrous oxide reductase gene (nosZ) determined at the end of the experiment. Panel a) and b) indicates the total number of *nosZ* gene copies quantified in bulk soil (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.07) and rhizosphere (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=$7.4x10^{-4}$), respectively. Panel c) and d) indicates the ratio of *nosZ* gene within the prokaryotic community obtained in the bulk soil (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.67) and rhizosphere (levene's test, F>0.05; Shapiro-Wilk's test, p>0.05; t-test, p-value=0.05), respectively. The ratio values were obtained by dividing the total abundance of *nosZ* gene copies by the sum of the total abundance of 16S rRNA genes from archaea and bacteria. Empty boxes represents the values obtained in the pots without earthworms (EW-) and striped boxes represents the values obtained in the pots with earthworms (EW+).

2.5.5. Metagenomic profiling of microbial functions

12 metagenomic datasets were obtained (samples from the bulk soil and rhizosphere of the 6 macrocosms). In average, a total of 268,468±149,394 reads passed the quality and length filter per dataset. Analysis of the rarefaction curves revealed good coverage of the diversity of microbial functions (Figure 2.5). The profiling of metagenomic datasets (total DNA) revealed that earthworm presence significantly changed around 70 microbial functions in both bulk soil and rhizosphere (t-test, p-value<0.05). For both environments the functions

were assigned to major categories based on their descriptions available on the reference database (INTERPRO2GO) or based on current literature when necessary. The entire list of the functions found to be significantly different (t-test; p-value<0.05) comparing EW+ with EW- can be found in Figure 2.6. Figure 2.7 summarizes the variance of the major categories, in a low-dimensional space using the method of principal component analysis.

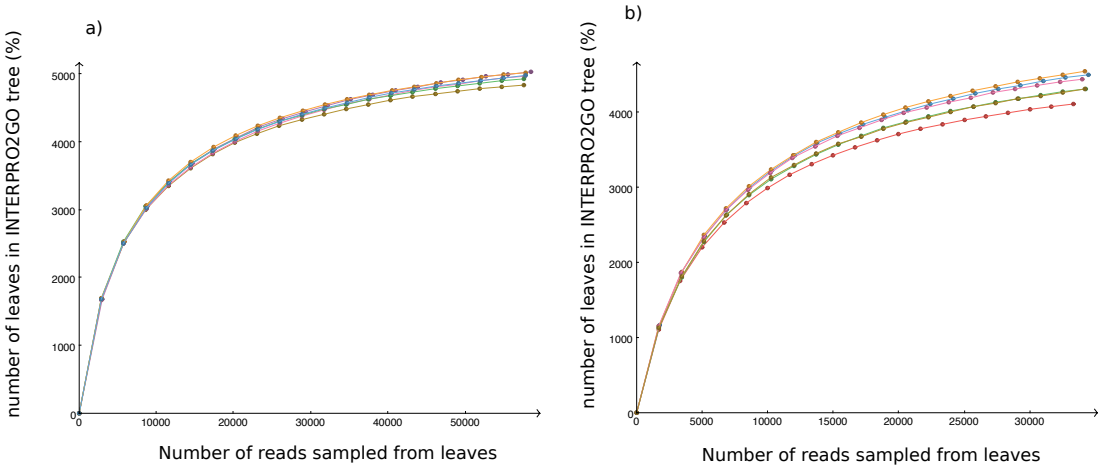


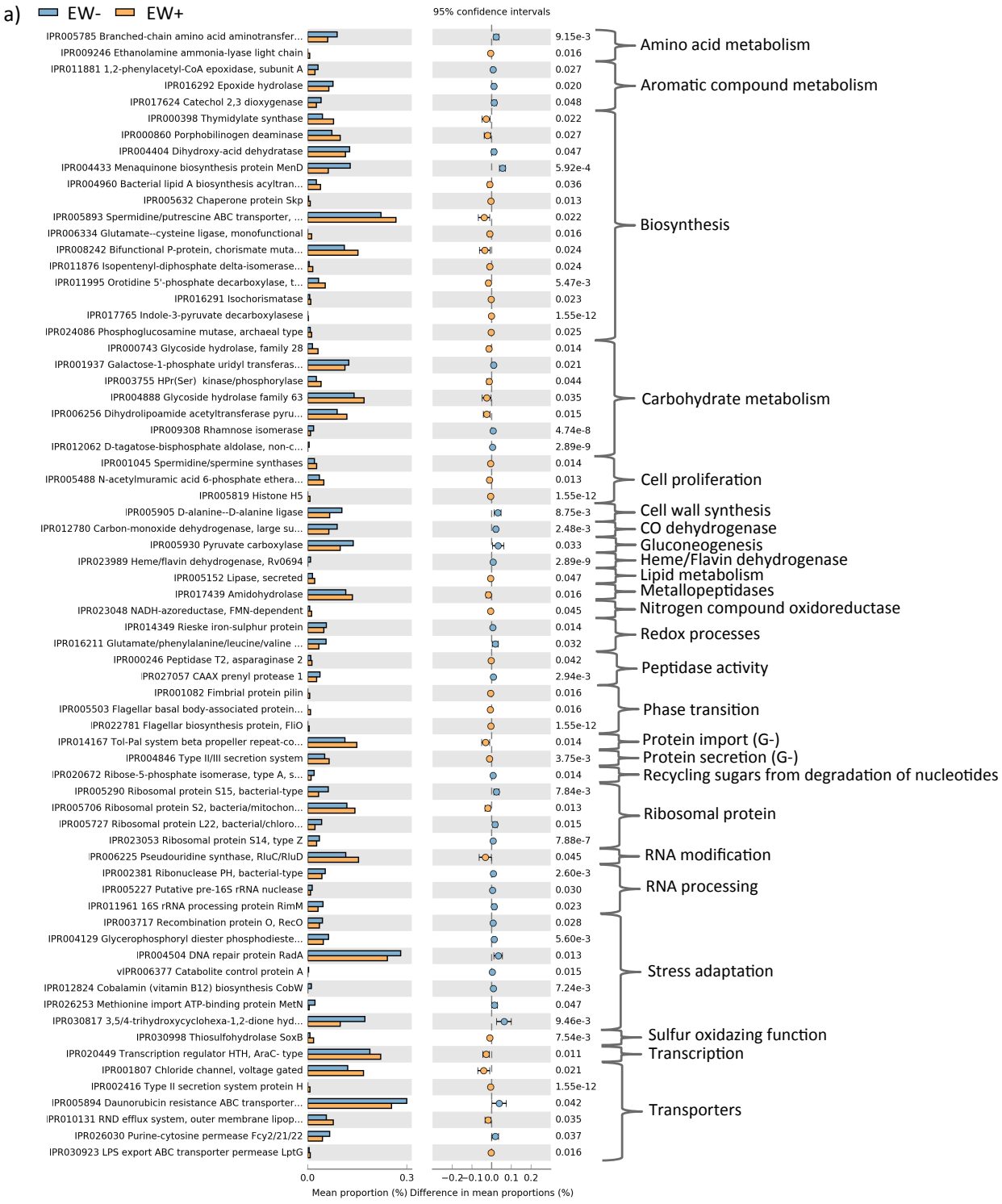
Figure 2.5 - Rarefaction curves obtained from the functional profiling of the metagenomic datasets in bulk (a) and rhizosphere (b). The curves indicate the coverage of microbial functions assigned to the INTERPRO2GO database for each dataset. The read counts were normalized to the smallest number of reads (MEGAN6). Each leave represents a different function assigned within the tree of functions obtained from MEGAN6. The lines in the plots represent each one of the 12 metagenomic samples obtained at the 217th day after the beginning of the experiment.

Some of the major categories assigned reveal a major pattern. In the EW+ bulk soil, functional genes associated with phase transition, carbohydrate and lipid metabolisms, biosynthesis, translation, protein import/export by Gram-negative (G-) bacteria, redox processes involving sulfur and nitrogen compounds and cell proliferation were enriched relative to EW- bulk soil (Figure 2.7a). More specifically, the phase transition major category refers to functions involved in cell motility such as the flagellum (IPR022781, IPR005503) and cell adhesion, referring to a cellular component (pilus) responsible for adhesion (IPR001082). The latter contains phylogenetic signs from G- bacteria. Within the carbohydrate and lipid metabolisms major categories, some functions associated with rapidly metabolisable carbon source (i.e., glucose and fructose) (IPR006256, IPR003755) and catabolism of lipids (e.g., the secretion of lipases) (IPR005152) can be highlighted,

respectively. Within the major category of biosynthesis, some of the functions assigned indicate synthesis of complex compounds such as the chaperone protein Skp function (IPR005632), which is involved in the biogenesis of outer membrane proteins (JARCHOW et al., 2008). Moreover, although in a very little proportion, genes associated with the production of plant growth regulators were identified (IPR017765).

In the bulk soil from EW-, among others, functions altered were assigned within the major categories of stress adaptation, peptidase activity, and amino acid and aromatic compound metabolisms (Figure 2.7a). Additionally, carbon-monoxide dehydrogenase, a function related with a diverse group of facultative chemolitoautotroph bacteria (IPR012780) was enriched in EW- compared to EW+.

In the rhizosphere, worth noting that microbial functions associated with plant-microbe symbiosis, transcription, biosynthesis, transporter and cell proliferation were significantly higher in EW+ compared to EW- (Figure 2.7b). More specifically, the functions included within the major category of plant-microbe symbiosis were part of metabolic processes referring to cell host colonization, by microbes known to perform nitrogen fixation (IPR003766) (SANTI et al., 2013), and to plant growth regulators (IPR005955) (ESTABROOK; SENGUPTA-GOPALAN, 1991; GONG et al., 2005), and to processes mediating cellular interactions within symbiotic interactions (IPR004453) (MARCHETTI et al., 2013), and to processes participating in secretion systems of protein effectors (IPR007688) (NELSON; SADOWSKY 2015). Likewise in bulk soil, metabolic processes involving G- bacteria were also reported in rhizosphere of EW+ (IPR004463). While in EW- rhizosphere, among others, the major categories of stress adaptation and peptidase activity were again enriched. Interestingly, EW- conditions presented higher level of genes associated with functions referring to gas vesicle (IPR009430).


a)
EW-
EW+
95% confidence intervals
IPR005785 Branched-chain amino acid aminotransfer... 9.15e-3
IPR009246 Ethanolamine ammonia-lyase light chain 0.016
IPR011881 1,2-phenylacetyl-CoA epoxidase, subunit A 0.027
IPR016292 Epoxide hydrolase 0.020
IPR017624 Catechol 2,3 dioxygenase 0.048
IPR000398 Thymidylate synthase 0.022
IPR000860 Porphobilinogen deaminase 0.027
IPR004404 Dihydroxy-acid dehydratase 0.047
IPR004433 Menaquinone biosynthesis protein MenD 5.92e-4
IPR004960 Bacterial lipid A biosynthesis acyltran... 0.036
IPR005632 Chaperone protein Skp 0.013
IPR005893 Spermidine/putrescine ABC transporter, ... 0.022
IPR006334 Glutamate--cysteine ligase, monofunctional 0.016
IPR008242 Bifunctional P-protein, chorismate muta... 0.024
IPR011876 Isopentenyl-diphosphate delta-isomerase... 0.024
IPR011995 Orotidine 5'-phosphate decarboxylase, t... 5.47e-3
IPR016291 Isochorismatase 0.023
IPR017765 Indole-3-pyruvate decarboxylasese 1.55e-12
IPR024086 Phosphoglucosamine mutase, archaeal type 0.025
IPR000743 Glycoside hydrolase, family 28 0.014
IPR001937 Galactose-1-phosphate uridyl transferas... 0.021
IPR003755 HPr(Ser) kinase/phosphorylase 0.044
IPR004888 Glycoside hydrolase family 63 0.035
IPR006256 Dihydrolipoamide acetyltransferase pyru... 0.015
IPR009308 Rhamnose isomerase 4.74e-8
IPR012062 D-tagatose-bisphosphate aldolase, non-c... 2.89e-9
IPR001045 Spermidine/spermine synthases 0.014
IPR005488 N-acetylmuramic acid 6-phosphate ethera... 0.013
IPR005819 Histone H5 1.55e-12
IPR005905 D-alanine--D-alanine ligase 8.75e-3
IPR012780 Carbon-monoxide dehydrogenase, large su... 2.48e-3
IPR005930 Pyruvate carboxylase 0.033
IPR023989 Heme/flavin dehydrogenase, Rv0694 2.89e-9
IPR005152 Lipase, secreted 0.047
IPR017439 Amidohydrolase 0.016
IPR023048 NADH-azoreductase, FMN-dependent 0.045
IPR014349 Rieske iron-sulphur protein 0.014
IPR016211 Glutamate/phenylalanine/leucine/valine ... 0.032
IPR000246 Peptidase T2, asparaginase 2 0.042
IPR027057 CAAX prenyl protease 1 2.94e-3
IPR001082 Fimbrial protein pilin 0.016
IPR005503 Flagellar basal body-associated protein... 0.016
IPR022781 Flagellar biosynthesis protein, FliO 1.55e-12
IPR014167 Tol-Pal system beta propeller repeat-co... 0.014
IPR004846 Type II/III secretion system 3.75e-3
IPR020672 Ribose-5-phosphate isomerase, type A, s... 0.014
IPR005290 Ribosomal protein S15, bacterial-type 7.84e-3
IPR005706 Ribosomal protein S2, bacteria/mitochon... 0.013
IPR005727 Ribosomal protein L22, bacterial/chloro... 0.015
IPR023053 Ribosomal protein S14, type Z 7.88e-7
IPR006225 Pseudouridine synthase, RluC/RluD 0.045
IPR002381 Ribonuclease PH, bacterial-type 2.60e-3
IPR005227 Putative pre-16S rRNA nuclease 0.030
IPR011961 16S rRNA processing protein RimM 0.023
IPR003717 Recombination protein O, RecO 0.028
IPR004129 Glycerophosphoryl diester phosphodieste... 5.60e-3
IPR004504 DNA repair protein RadA 0.013
vIPR006377 Catabolite control protein A 0.015
IPR012824 Cobalamin (vitamin B12) biosynthesis CobW 7.24e-3
IPR026253 Methionine import ATP-binding protein MetN 0.047
IPR030817 3,5/4-trihydroxycyclohexa-1,2-dione hyd... 9.46e-3
IPR030998 Thiosulfohydrolase SoxB 7.54e-3
IPR020449 Transcription regulator HTH, AraC- type 0.011
IPR001807 Chloride channel, voltage gated 0.021
IPR002416 Type II secretion system protein H 1.55e-12
IPR005894 Daunorubicin resistance ABC transporter... 0.042
IPR010131 RND efflux system, outer membrane lipop... 0.035
IPR026030 Purine-cytosine permease Fcy2/21/22 0.037
IPR030923 LPS export ABC transporter permease LptG 0.016
Amino acid metabolism
Aromatic compound metabolism
Biosynthesis
Carbohydrate metabolism
Cell proliferation
Cell wall synthesis
CO dehydrogenase
Gluconeogenesis
Heme/Flavin dehydrogenase
Lipid metabolism
Metallopeptidases
Nitrogen compound oxidoreductase
Redox processes
Peptidase activity
Phase transition
Protein import (G-)
Protein secretion (G-)
Recycling sugars from degradation of nucleotides
Ribosomal protein
RNA modification
RNA processing
Stress adaptation
Sulfur oxidazing function
Transcription
Transporters
0.0
0.3
−0.2 −0.1 0.0 0.1 0.2
Mean proportion (%)
Difference in mean proportions (%)

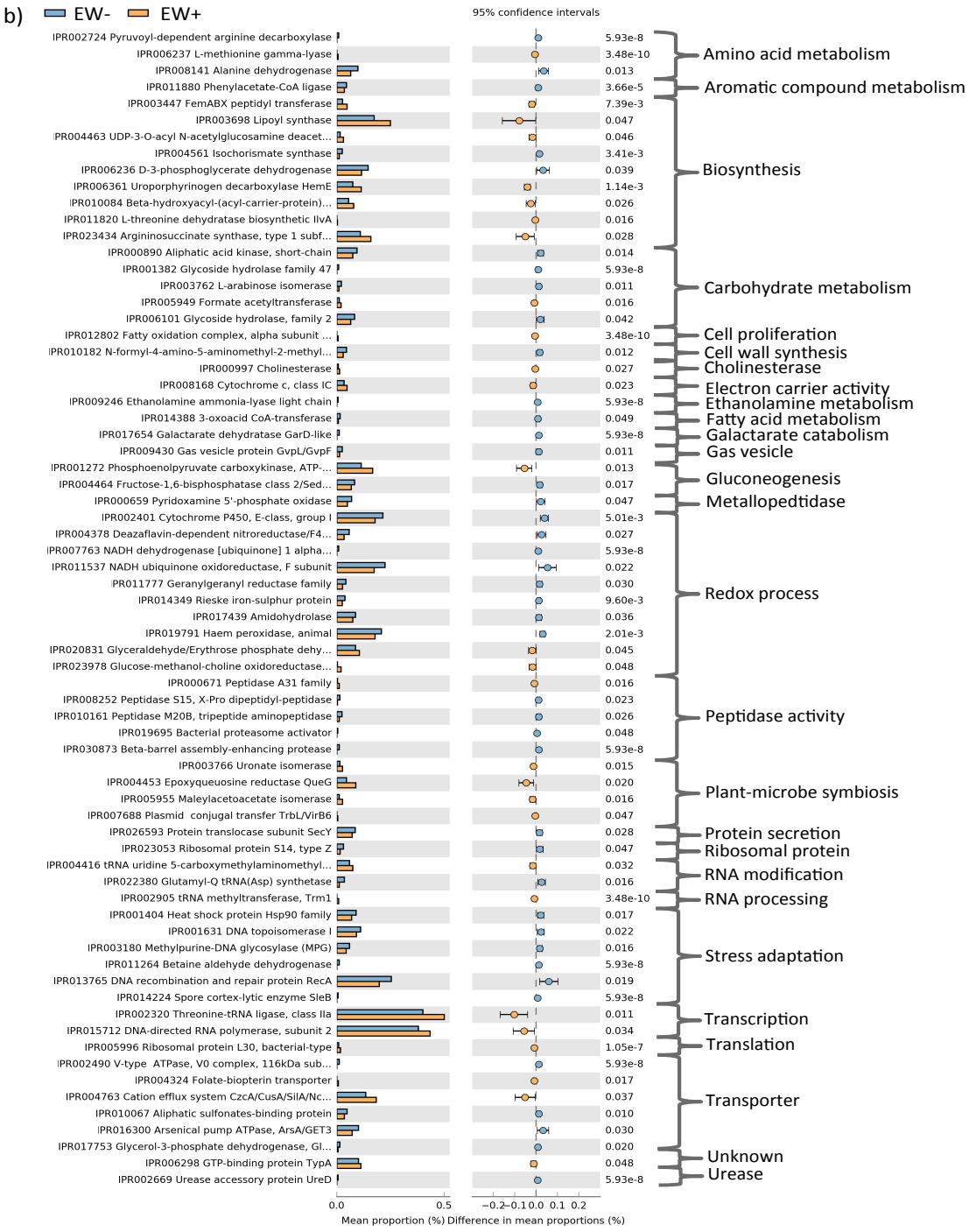


Figure 2.6- Microbial functions altered in bulk (a) and in rhizosphere (b). The error bar plots indicates the p-value of the functions with the effect size and associated confidence interval for each function detected to be of significant biological relevance (t-test, p-value<0.05). The color code indicated in the confidence intervals shows if the enrichment was higher in EW- (blue) or in EW+ (orange).

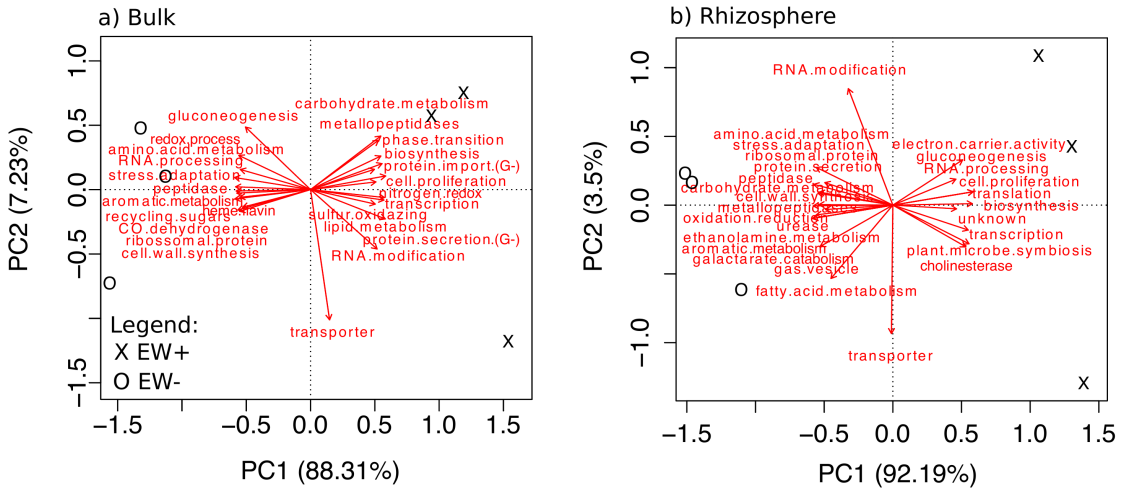


Figure 2.7 - Principal component analysis summarizing the variance of major categories of microbial functions as determined in the metagenomic profiles from bulk soil (a) and rhizosphere (b) at the end of the experiment. The major categories of functions are composed by more specialized pathways. The complete list of specific pathways of biological importance can be found in Figure 2.6.

2.5.6. Ecological network interactions of microbial functions

The presence of earthworms in the macrocosms altered ecological interactions among microbial functions, as revealed by the network models (Figure 2.8). A decline in the number of clusters (i.e., communities) and an increase in the level of importance (i.e., keystone) of the functions (i.e., nodes) most influencing the models, as indicated by the increase in the values of betweenness centrality of the nodes (Table 2.2), was detected as a major effect of earthworms on microbial communities. Bulk soil of EW- presented 16 clusters while the model built for EW+ bulk soil presented 10 clusters, and 13.97 % of the keystone functions in bulk EW+ presented a degree of importance greater than the keystone functions in bulk EW- (Table 2.2). In the EW- rhizosphere, microbial functions were grouped into 20 clusters, and 3.79 % of the keystone functions presented greater importance than the keystone function in EW- bulk (Table 2.2). In EW+ rhizosphere, microbial functions were grouped into 15 clusters, and 7.26 % of the keystone functions presented greater importance than the keystone function in EW- bulk (Table 2.2).

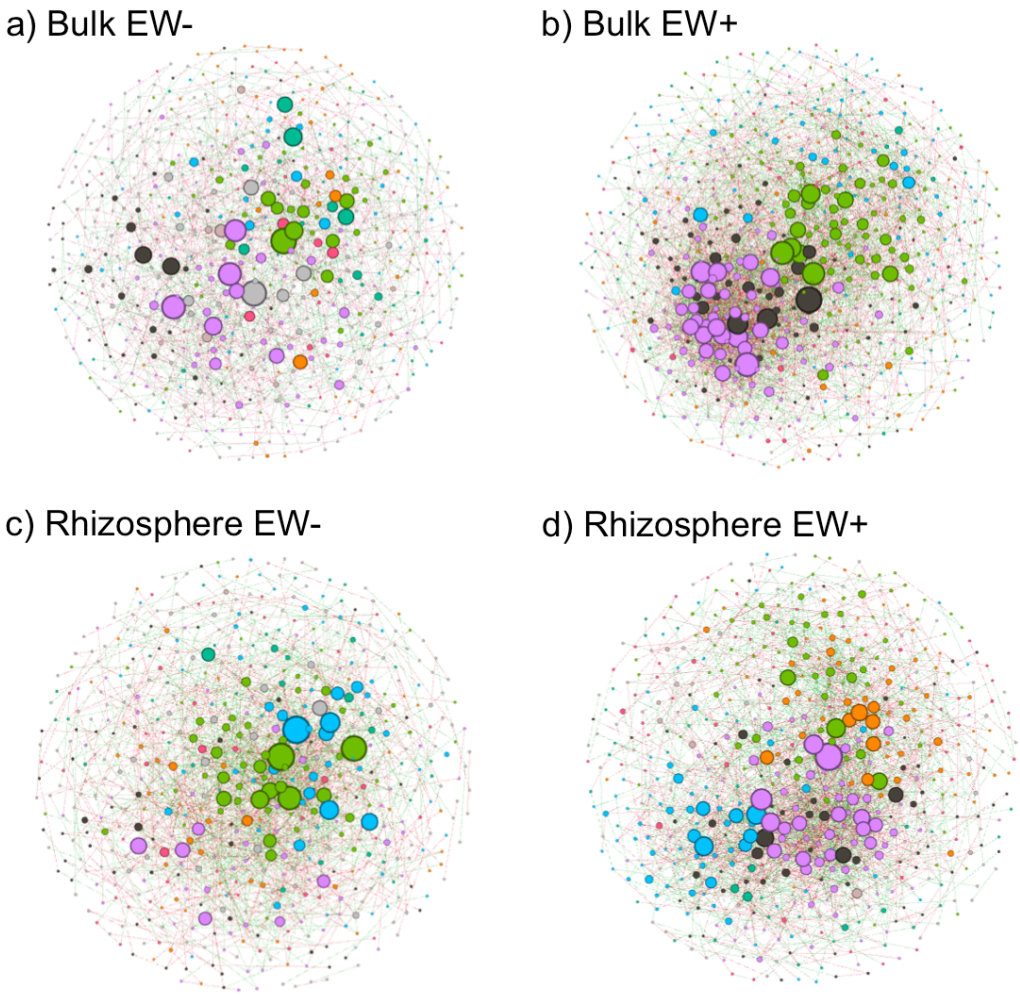


Figure 2.8 - Ecological interactions of microbial functions. Significant (p-value>0.05) and strong (-0.9> r >0.9) correlations among the most abundant microbial functions. Nodes represent functions and edges represent the correlation between them. Network a) represents interactions built for bulk EW-, with 642 nodes and 1418 edges (53.88 % positive correlations). Network b) represents interactions built for bulk EW+, with 651 nodes and 3201 edges (52.17 % positive correlations). Network c) represents interactions built for rhizosphere EW-, with 579 nodes and 1737 edges (50.83 % positive correlations). Network d) represents interactions built for rhizosphere EW+, with 564 nodes and 2360 edges (51.91 % positive correlations). Different colors indicate different clusters (i.e., modularity), and the nodes were sized according to their importance for the model (i.e., betweenness centrality).

Table 2.2 -Values of betweenness centrality* obtained for the first 20 keystone functions found in each network model with (EW+) and without earthworms (EW-)

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
IPR010067	488.51	IPR003346	2424.90	IPR016407	1293.17	IPR025703	1668.95
IPR001406	482.58	IPR000269	2217.13	IPR005215	1285.72	IPR005372	1255.95
IPR032465	448.96	IPR005771	2157.45	IPR022694	1191.86	IPR000101	1142.79
IPR005746	434.33	IPR004808	2083.24	IPR010228	1089.42	IPR004713	1119.53
IPR004387	412.82	IPR001241	1963.69	IPR004589	898.97	IPR003514	1112.62
IPR005748	336.82	IPR000821	1895.82	IPR004196	855.25	GO000451	1111.28
IPR000748	336.16	IPR002301	1881.19	IPR001062	792.38	IPR026259	1093.10
IPR006200	326.78	IPR002292	1829.40	IPR001238	743.54	IPR006468	1019.87
IPR004809	311.19	IPR011275	1787.26	IPR026019	714.30	IPR002295	955.54
IPR002028	301.71	IPR010226	1727.91	IPR000269	712.73	IPR003568	942.36
IPR011217	284.82	IPR000206	1661.76	IPR017121	650.79	IPR002292	940.66
IPR004506	275.75	IPR004576	1641.33	IPR001701	648.96	IPR011833	876.80
IPR003995	269.25	IPR004670	1632.89	IPR001406	635.34	IPR004791	866.77
IPR005064	267.36	IPR002524	1571.73	IPR002023	630.31	IPR002151	855.63
IPR006443	266.53	IPR015712	1552.21	IPR001719	623.83	IPR002303	847.44
IPR006314	266.17	IPR012394	1516.03	IPR016202	557.10	IPR004807	819.57
IPR003997	260.17	IPR023042	1421.11	IPR008248	545.89	IPR005750	818.25
IPR000522	256.95	IPR023051	1413.71	IPR001088	541.72	IPR000043	816.09
IPR000787	246.50	IPR002320	1411.89	IPR005704	539.29	IPR002139	815.65
IPR019927	233.39	IPR001088	1408.84	IPR004373	534.73	IPR005746	813.39

* Measured according to the number of shortest paths between any two nodes that pass through one particular node. High values indicate high influence of the node on the model.

2.5.7. Microcosm experiment: gas emissions from the incubations

The N_2O emissions (Figure 2.9a) detected belowground were different (Kruskal-Wallis, p-value=0.01) among the treatments. N_2O emissions from the pots with only sugarcane (EW-SC+) were not different from the emissions of the pots with only soil (EW-SC-) (Dunn's test. p-value=0.60). However, the N_2O emissions from the pots with earthworms were significantly higher compared to the emissions of the pots without worms (i.e., EW+SC- compared to EW-SC-, Dunn's test, p-value=0.009346; and EW+SC- compared to EW-SC+, Dunn's test, p-value=0.03759; and EW+SC+ compared to EW-SC-, Dunn's test, p-value=0.01157; and EW+SC+ compared to EW-SC+, Dunn's test, p-value=0.04496). Although, the N_2O emissions between the pots with worms were not different (i.e., EW+SC- compared to EW+SC+, Dunn's test, p-value=0.94).

Differences were also detected for the CO_2 emissions (Figure 2.9d) from belowground (Kruskal-Wallis test, p-value=0.008). CO_2 was higher in all the pots with earthworms or with sugarcane (i.e., EW-SC+ compared to EW-SC-, Dunn's test, p-value=0.08; EW+SC- compared to EW-SC-, Dunn's test, p-value=0.04; EW+SC+ compared to EW-SC-, Dunn's test, p-value=0.0006), and no significant difference were detected between them (Dunn's test, p-value>0.05). N_2O and CO_2 emissions of rhizospheres, (Figure 2.9b and Figure 2.9e), were not different (t-test, p-value>0.05). *In vivo* emissions of worms (Figure 2.9c and Figure 2.9f) were not different for N_2O (t-test, p-value>0.05), but CO_2 emissions of worms from pots with sugarcane were significantly higher compared to the pots without plants (t-test, p-value=0.00929). Further, *in vivo* emissions of N_2O of *P. corethrurus* were significantly higher than the N_2O emissions of rhizospheric soil from the pots with earthworms (t-test, p-value=$2.5x10^{-6}$).

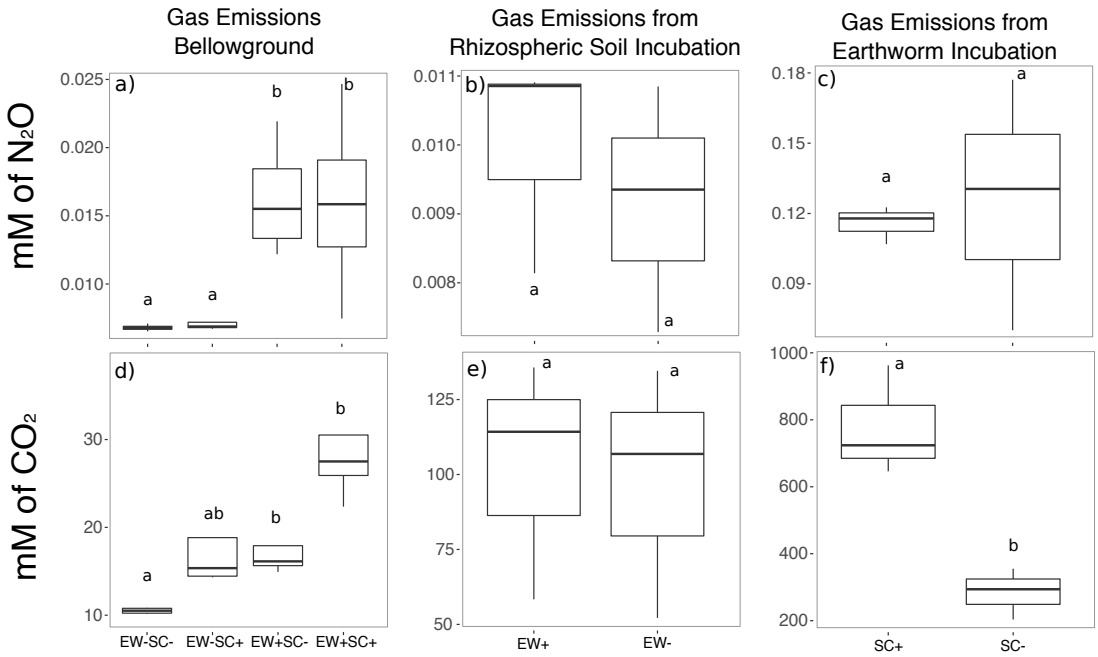


Figure 2.9 - Verification study: N_2O and CO_2 emissions belowground, from rhizospheric soils, and earthworms. Panels a), b) and c) show N_2O concentrations (mM). Panels d), e) and f) show CO_2 concentrations (mM). Panels a) and d) show the gas concentrations belowground at the end of the experiment (day 30^{th}) for the treatments (n=4): without earthworms and sugarcane (EW-SC-), with sugarcane (EW-SC+), with earthworms (EW+SC-), and with both (EW+SC+). Panels b) and e) show the mean concentration of gas emitted of rhizospheric soils (1 gram of fresh weight) from the pots with earthworms (EW+) and without it (EW-). The panels c) and f) show the *in vivo* gas emissions per gram (fresh weight) of earthworm from the pots with sugarcane (SC+) and without it (SC-). Different letters above the boxes in the panels indicate significant differences ($p<0.05$).

2.6. Discussion

Sugarcane biomass was significantly improved in EW+ macrocosms (Figure 2.1a). Although not significant, the considerable decrease in Si (Figure 2.9b) can be a result of biomass improvement and earthworm-induced microbial activity. Sugarcane is a strong accumulator of Si, and Si fertilization is associated with yield improvements (SAVANT et al., 2008). Further, it has been recently demonstrated that earthworms can improve the Si uptake by plants (BITYUSKII et al., 2016). Bityuskii et al. (2016) proposed that ingested microbes that can produce exoenzymes in the earthworm gut would be responsible to enhance the release of Si derived from the degradation of complex organic matter. These findings thus extend previous studies to the earthworm-sugarcane system.

The earthworm-induced N_2O emissions are the consequence of their feeding habits. Experiments have repeatedly demonstrated that N_2O emissions are associated with microbial processes happening in the gut (DEPKAT-JAKOB, 2013) and worm-worked soils (LUBBERS et al., 2013), where the populations of denitrifiers and dissimilatory nitrate reducers can be more abundant than in bulk soil (DRAKE; HORN, 2007). Furthermore, the physical process of ingesting microbial cells might kill some of them, releasing N trapped in microbial biomass.

Hence, assuming that earthworms have the constant capacity to increase N_2O emissions inside the soil, why did they decrease in the present experiment around 60 days after the experiment began? The nitrous oxide reductase, encoded by the gene *nos*Z, is the enzyme that converts N_2O to N_2, representing the last step in denitrification (HENRY et al., 2006). Denitrification is an anaerobic respiratory process in which microbes produce and/or consume N_2O, representing a biotic source or sink for N_2O (JONES et al., 2014). Therefore, the decrease in N_2O belowground was a consequence of the increase in *nos*Z gene activity. Indeed, part of the question remains: why after the 60^{th} day? The CO_2 timeline indicates that soil respiration declined around 60 days after the beginning of the experiment in both EW+ and EW- conditions (Figure 2.3b). Further, except for the dates 30/4 and 07/05 (around 60^{th} and 67^{th} days), no significant differences were detected between the CO_2 means, which indicates that decrease in CO_2 was an event independent of the influence of earthworms. Assembling the pots with sieved soil caused extra aeration between the soil particles. Oxygen plays an important role on enhancing CO_2 emissions from soils by aerobic metabolism belowground (THIET et al., 2006; MANZONI et al., 2012). Thus, these three findings, namely i) the increase in *nos*Z, ii) the decrease in N_2O, and iii) decrease in CO_2, suggest that

the accumulated earthworm-induced N_2O was respired by N_2O reducers mainly after day 60^{th} because the conditions before that could be favoring aerobic respiration due to soil aeration during macrocosm assembling.

The dataset also suggests that G- bacteria were favored in bulk soil and in rhizosphere of EW+. This agrees with previous findings, suggesting that G- bacteria may have a better ability to survive gut passage than gram-positive (G+) bacteria (PEDERSEN; HENDRIKSEN, 1993; LIU et al., 2011; DALLINGER; HORN, 2014). Or, as an alternative mechanism, previous findings demonstrated that G- population predominates in rhizosphere while G+ predominates in bulk soil (SÖDERBERG et al., 2004). Rhizosphere is known to be dominated by r-strategists while bulk soil is dominated by k-strategists (BLAGODATSKAYA et al., 2014). Therefore, survival from the gut passage, if possible, may not be the only mechanism by which G- can be more positively affected by earthworms. As r-strategists, they could colonize first and grow faster than G+. Bacterial 16S rRNA gene was significantly increased in bulk soil from EW+ (Table 2.1). It was found evidences of functional pathways associated with cell motility from G- in bulk soil (IPR001082), which could be due to the need of moving towards the soil zone were earthworms have released nutrients. New experiments need to be performed in order to test this hypothesis.

Earthworm-worked soils can contain large amount of nutrients concentrated (i.e., ammonium, and sugars) or even nutrients generated exclusively by metabolic processes in their gut (i.e., fermentation) (SCHULZ et al., 2015) such as formate, acetate, succinate and lactate (DRAKE; HORN, 2007). Those easily available organic compounds released in the soil by earthworms may positively affect the metabolism of soil microbes. This may explain why biosynthetic processes were more enriched in EW+ (Figure 2.6ab and Figure 2.7ab) and cell proliferation functions were observed (Figure 2.6ab and Figure 2.7ab). Microbes in EW- were lacking this additional source of nutrient. Some of the functions assigned to the major category of stress adaptation response may indicate that microbes in EW- were thriving under relative poor conditions. Mechanisms associated to DNA repair (IPR003717, IPR004504, IPR003180, IPR001631, IPR013765), cytoprotection against diverse environmental stresses (IPR004129) (MICHELL, 2008), disturbance in organismal homeostasis (IPR001404), adaptation to nutrient limiting conditions (IPR026253) (GIANOULIS et al., 2008), activation of minimal catalytic activity under growth-limiting conditions (IPR006377) (KOWALCZYK; BARDOWSKI, 2007) were all higher in EW- from bulk or rhizosphere compared to EW+. Sugarcane croplands present a high demand for fertilizers in order to reach a satisfactory level of biomass development. For example, in Brazil, 60-100 kg of nitrogen is applied per hectare

annually (ROBINSON et al., 2011). In the present experiment, no additional source of nutrient (i.e., fertilizers) was applied to the soils. Therefore, considering the intense competition for nutrients between roots and microbes (KUZYAKOV; XU, 2013), it is acceptable that growth conditions were relative limited in EW-.

Rhizosphere microbes in EW+ were capable to invest in functions associated with plant symbiosis (Figure 2.6b and Figure 2.7b). For example, uronate isomerase (IPR003766) (CAMPBELL et al., 2003) gene shares homology with hormogonium-regulating genes. Hormogonia are gliding filaments specialized for dispersal which are associated with cell host colonization. In some organisms, such as cyanobacteria, this is the phase preceding the differentiation to heterocyst and the expression of nitrogenase (CAMPBELL et al., 2003). This mechanism has been demonstrated to be important for biological fixation of nitrogen in non-legume plants (SANTI et al., 2013). The uronate isomerase gene can be found in the genome of several rhizobacteria from the genera *Azorhizobium* (KEGG ID: AZC_3342), *Azospirillum* (KEGG ID: AZLd01370), *Mesorhizobium* (KEGG ID: mll4056), *Sinorhizobium* (KEGG ID: SM_b21354) and *Rhizobium* (KEGG ID:NGR_c32910), among others. Another case of plant-microbe symbiosis is the maleylacetoacetate isomerase (IPR005955), which belongs to a glutathione S-transferase family. These enzymes were demonstrated to be directly involved in regulation of plant growth (GONG et al., 2005) and their respective genes can be found in plant-growth promoting rhizobacteria from the genera *Pseudomonas* (ESTABROOK; SENGUPTA-GOPALAN, 1991) and others such as *Bradyrhizobium* (KEGG ID: bll0109), *Sinorhizobium* (KEGG ID: SMc03206), and *Rhizobium* (RHE_CH01748). Additionally, the datasets from EW+ rhizosphere also presented higher levels of functions involved in modulate cell-host interactions (IPR004453) (MARCHETI et al., 2013), and functions associated with secretion system (type IV) (IPR007688), responsible for transferring t-DNA and effector proteins to plant cells, which can also participate in beneficial interactions (NELSON; SADOWSKY, 2015). In comparison to EW-, EW+ rhizosphere had lower enrichment of gas vesicle function (IPR009430). This is a subcellular structure known to happen in several phyla of bacteria and archaea, which may facilitates buoying cells to the oxygenated layers, working strategically under situations of competition for O_2 (WALSBY, 1994). The source of O_2 in rhizosphere are the root cells, which may loose part of the O_2 which is delivered to them to the surrounding soil (ARMSTRONG, 1971; COLMER; PEDERSEN, 2008). The decrease in the need for gas vesicle could be an effect connected with the extra supply of N_2O by earthworm activity and the increase in *nos*Z gene abundance.

The analysis of network interactions (Figure 2.8) suggests that the specific changes observed by contrasting EW+ with EW- (Figure 2.6 and Figure 2.7) are supported by modifications that earthworm presence caused to the structure of ecological interactions among microbial functions. The low number of clusters in EW+, compared to EW-, demonstrates that EW+ presented lower need for functional diversification (FAUST; RAES, 2012). Perhaps, because they were supplied with extra source of nutrients which from the earthworm-worked soil. Further, the increase in the number of important functions (Table 2.2) in EW+ reflects that more functions were controlling the structure of ecological interactions (FAUST; RAES, 2012). Together, these patterns are in consistency with changes detected by the functional profiling (Figure 2.6 and Figure 2.7), supporting that worms may have contributed with extra resources to microbes.

Here it was only measured the abundance of *nos*Z gene clade I (*nos*Z I), however recently, a new clade of this gene (*nos*Z II) has been identified (SANFORD et al., 2012; JONES et al., 2013). There is a possible niche differentiation between these clades. Although both were reported to be present in microbes colonizing roots, *nos*Z I was shown to be significantly more abundant in the rhizosphere (GRAF et al., 2016). Here it was shown that *nos*Z I is also important for microbial communities in rhizosphere under the influence of the earthworm-induced N_2O emission. In the dataset, the proportion of *nos*Z I in EW+ bulk was not different from EW-. However, the bacterial population was significantly enriched in EW+ bulk compared to EW-. Therefore, the dataset can support only limited conclusions about the influence of earthworms on nitrous oxide reducers in the bulk soil. Based on recent research (GRAF et al., 2016), it would be expected for *nos*Z II in bulk soil to show the same response as detected here for *nos*Z I in the rhizosphere. However, further research should address this hypothesis.

The rhizosphere is considered a hotspot for denitrifiers (KLEMEDSTSSON et al., 1987; HENRY et al., 2008; GRAF et al., 2016), and abundant literature supports that plants can increase the N_2O emissions from soils (PRADE; TROLLDENIER, 1988; DING et al., 2007; SEY et al., 2010; NI et al., 2012). However, it was performed a microcosm experiment (Figure 2.9), using a similar approach (i.e., same plant, same worm and soil from the same origin). It was verified that N_2O production belowground from pots growing sugarcane was not different from the pots without the plant (Figure 2.9a). In the microcosm experiment, the same effect was observed as was detected in the macrocosm experiment presented here: pots with earthworms showed higher N_2O emissions belowground. Further, the incubation of rhizospheric soils from the pots with and without earthworms showed no significant

difference for N_2O emissions. While the *in vivo* emissions of N_2O from *P. corethrurus* were significantly higher than the N_2O emissions of rhizospheric soil from the pots with earthworms (t-test, p-value=$2.5x10^{-6}$). These results reinforces that N_2O production belowground is dominated by earthworm activity rather than root processes. Additionally, it also reinforces that earthworm-induced N_2O emission belowground might have little effect on rhizosphere N_2O respiration in a short-term scale (30 days), as observed in the macrocosm experiment (60 days).

2.7. Conclusion

Overall, the present study demonstrates that earthworms seem to be important players that positively influence rhizosphere microbes, providing extra resources that may favor them to invest in biosynthetic processes and plant-microbe symbiosis functions. The *nosZ* gene activity was significantly important for microbial community in rhizosphere soils from EW+. It is proposed, as a hypothetical mechanism, that the production of plant beneficial functions by microbes in the rhizosphere influenced by earthworms may result from the increase in availability of high quality electron donors (i.e., glucose, maltose, formate, acetate, lactate, and succinate) and the increase in N_2O as electron acceptor, both products which can escape from the earthworm gut (Figure 2.10). The proposed mechanism needs to be tested in further research, in which the influence of the bioturbation process should also be evaluated.

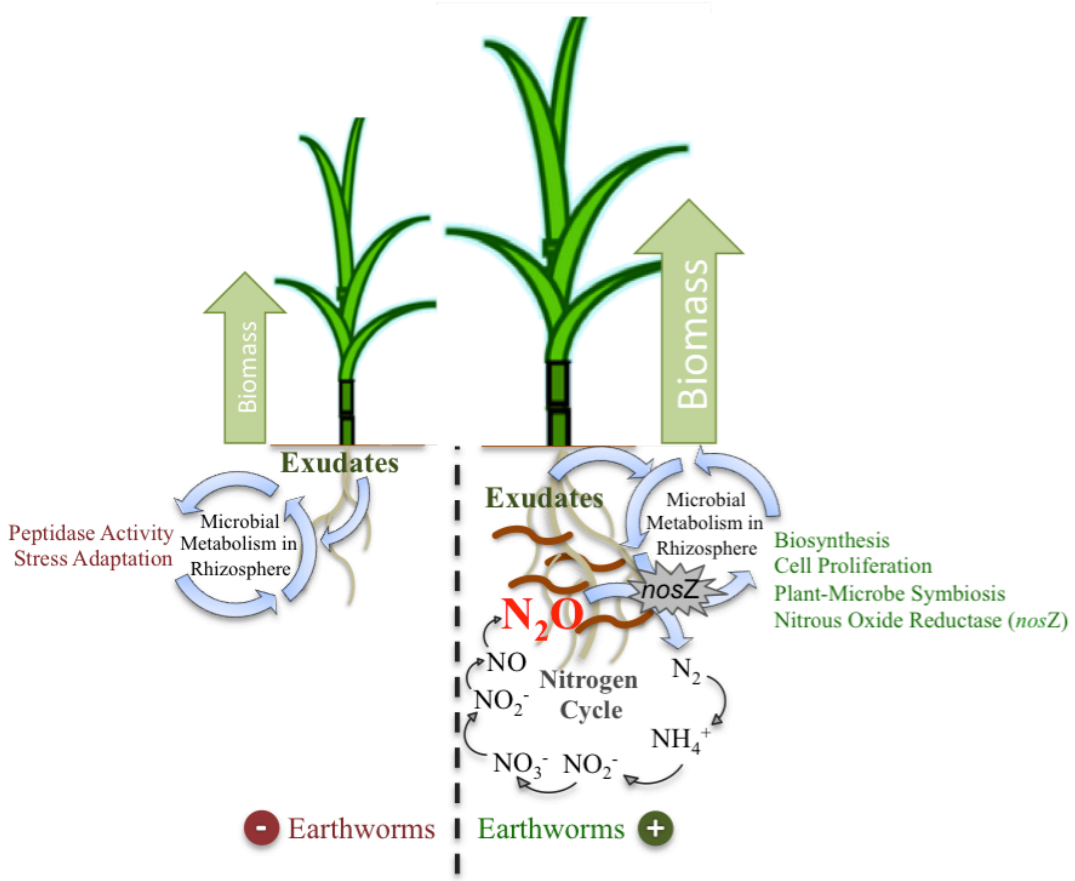


Figure 2.10 - Hypothetical model representing the mechanism by which earthworms may influence rhizosphere microbes in sugarcane. The collective findings in the present study demonstrate that earthworm activity alter microbial functions in the soil (bulk soil and rhizosphere). It is proposed that the cause for that is the increase in the availability of nutrients and the elevated abundance of N_2O, both are known to be originated during the process of soil digestion inside worm gut, and therefore they may scape from the alimentary channel and be available to the soil microbial communities. Although the complete mechanism might be more complex than the represented, the dataset suggests that these factors may play important role on enhancing microbial biosynthesis, cell proliferation and plant-microbe symbiosis in rhizosphere under the influence of earthworms.

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3. EARTHWORMS ALTER RESISTOME DYNAMICS AND TAXONOMICAL PROFILES OF SOIL MICROBIAL COMMUNITIES

Abstract

Mechanisms of resistance against antimicrobial compounds appear to be an obligatory feature for the ecology and evolution of prokaryotic forms of life. However, most studies on resistance dynamics have been conducted in artificial conditions of anthropogenic inputs of antibiotics into very specific communities such as animal microbiomes. To resolve why and how resistance evolves, it is important to track antibiotics resistance genes (ARGs) (i.e., the resistome) in their natural hosts and understand their ecophysiological role in the environment. The present study reports the response of the soil resistome from bulk and rhizsophere of sugarcane macrocosms under the influence of earthworms (*Pontoscolex corethrurus*). The resistome was detected to be highly prevalent, and diverse ARGs were enriched. The results demonstrated that earthworms influenced changes of ARGs in bulk soil and rhizosphere. Negative correlations between ARGs and taxonomical changes were increased in response to earthworm influence. Differential betweenness centrality (DBC=nBC^{EW+} – nBC^{EW-}) values comparing the network models with and without earthworms showed that earthworm presence changed the composition and the importance of the keystone members from the models. Redundancy analysis suggested that ARGs may be associated with microbial fitness, as the variance of relative abundance of members of the group Rhizobiales could be significantly explained by the variance of a specific gene responsible for one mechanism of tetracycline detoxification. Overall, the results presented in this Chapter demonstrate that the structure of the community of indigenous ARGs found in soil resistome can be (re)configured by natural processes occurring in soils, such as rhizosphere depositions and earthworm activities

3.1. Introduction

Since their discovery, over 80 years ago, antibiotics have been largely employed in human and animal health care in order to resolve pathogenic infections caused by bacteria (NESME; SIMONET, 2015). However pathogens can quickly evolve resistance to antibiotics, threatening pathogens inhibition and creating a vicious circle (LEWIS, 2013; BERENDONK et al., 2015). Most molecules capable of microbial antagonism are also produced by microorganisms (BÉRDY, 2005), so that for most antibiotics, if not all, a possible resistance mechanism might already exist, otherwise not even the producers would be able to avoid the effect of poisoning (CUNDLIFFE, 2010). Although resistance can be generated by selective pressure and be broadly spread by horizontal gene transfer processes (HEUER; SMALLA, 2012), the mechanisms of origin and spread of resistance in the environment are still poorly understood (BERENDONK et al., 2015).

Antibiotics resistance genes (ARGs), also called resistome, when referring to the global pool of ARGs, are common in environmental samples (NESME et al., 2014). This indicates that they might play an important role in microbial physiology from an ecosystem perspective (DAVIES; DAVIES, 2010). According to classical views, antibitiotics are molecules secreted to inhibit growth of neighbor cells under in situ conditions of competition (LENSKI; RILEY, 2002; KIRKUP; RILEY, 2004; HIBBING et al., 2010). However, an alternative hypothesis proposes that antibiotics are molecules that work as collective regulators of microbial homeostasis (DAVIES et al., 2006; LINARES et al., 2006). Nevertheless, experiments so far extensively confirm that antibiotics are related to an 'arms-shield' race (CHERIF; LOREAU, 2007; FOSTER; BELL, 2012; KOCH et al., 2014; ABRUDAN et al., 2015). This point of view has been stressed by Nesme and Simonet (2015), and explains the existence of ARGs as a mean of defense against antibiotics.

Most studies on dynamics of resistance mechanisms have been conducted in artificial conditions of anthropogenic inputs of antibiotics into very specific communities such as animal microbiomes. Neutralizing molecules with potential inhibitory effects is a strategy inherent to microbial existence, and is probably an obligatory condition. For example, ARGs were reported from ancient DNA samples of permafrost (>30,000 years) and also in caves isolated for over 4 million years (D'COSTA et al., 2011; BHULLAR et al., 2012). Most known antibiotics have been isolated from soil, so not surprisingly, recent reports describing resistome analysis of environmental samples indicate soil as the greatest reservoir for ARGs (NESME; SIMONET, 2015). To better comprehend resistance dynamics it is necessary to consider studies evaluating ARGs in their natural hosts. Soil is characterized by oligotrophic conditions (HU et al., 1999). Soils generally have low levels of organic matter and recalcitrance of organic carbon and these conditions determine the ecology and evolution of soil microbiomes (FIERER et al., 2008). However, in a long-term perspective, soils can be seen as open systems shaped by the interactions of biotic and abiotic factors, and pulses of nutrients may arise intermittently from these interactions.

Plant roots are a very potent source of pulse of nutrients to soil microbial communities. For instance, most of the carbon released in plant exudates can be consumed in a few hours by microbes associated to the roots (FISCHER et al., 2010). Root activity converts soils into functional domains defined as rhizosphere (root associated soil, i.e. < 5mm), thereby influencing microbial functional and taxonomical compositions (MENDES et al., 2013). Another important source of nutrients for soil microbes are the metabolic products from the on going fermentation and denitrification in earthworm gut that

may scape within the casts and be readily consumed by soil microorganisms (DRAKE; HORN, 2007; ZHANG et al., 2013). High quality nutrients can be found within the earthworm-worked soil (DRAKE; HORN, 2007) therefore soils influenced by earthworms are considered hotspots for microbial life (BROWN et al., 2000). Although, neither plants nor earthworms are permanent from the perspective of an established soil microbial community, thus, selective pressure forces microbes to be adapted to a (non even) balance between oligotroph and copiotroph conditions. These interactions have been in place probably since the transition of life from the oceans to the land over 500 million years ago (WISNIEWSKI-DYÉ et al., 2011; CHIN et al., 2013). Considering the relationship between nutrients, competition and ARGs, the concept of nutrient pulses into microbial communities affected by biological processes in soil ecosystems might help to shed light on the question of the origins and causes of resistance mechanisms and their spreading soils.

3.2. Hypothesis

In a previous analysis (see Chapter 2) it has been demonstrated that earthworm presence can influence positively rhizosphere microbes, favoring the enrichment of plant beneficial functions. Therefore, it was used the same dataset to investigate the response of the soil resistome in the rhizosphere with and without the influence of earthworms (EW+ and EW- respectively) to test the hypothesis that rhizosphere microbial community under the influence of earthworms presents a different composition of antibiotic resistance genes.

3.3. Objectives

To annotate ARGs from metagenomic datasets (DNA^{total}) obtained from a macrocosm experiment using the RESFAM database. To compare and correlate ARGs changes with changes in taxonomical profiles of microbial communities.

3.4. Methods

3.4.1. Experimental design

Macrocosms (100L) were filled with 70 kg of sieved soil (podzolic dark red oxisoil; 30% sand, 8% silt and 62% clay) and maintained in greenhouse conditions. The soil was collected from an experimental farm of the University of São Paulo, no crop had being grown

for at least 1 year until the sampling. Sugarcane seedlings were provided by the Sugarcane Center of Technology (CTC) and planted in a number of 6 per pot (3 of them were later randomly harvested). Earthworms were provided by the distributor Minhobox (Juiz de Fora, MG). Twenty individuals of *Pontoscolex corethrurus* were inoculated in the macrocosms after passing through a careful process of 24 hours of previous incubation for acclimatization in the soil used for the experiments and subsequent gut 'cleaning' for 4 hours in a plastic container with wet tissue paper. 3 experimental units with and without earthworms were set up (total of 6 macrocosms). After 217 days, destructive sampling was performed, and in each of the experimental units bulk soil samples were collected in 3 equidistant points (10 cm from each other and from the center of the pot) and homogenized. Rhizosphere soil from the plants was collected in each pot and homogenized. In pots with earthworms all animals were manually removed from the soil, resulting in 100% survival.

3.4.2. Molecular analysis

DNA extraction was performed using the Power Lyzer Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to instructions provided by the manufacturer. Samples were evaluated in NanoDrop to guarantee minimum quality. Libraries from Nextera kit were prepared according to the manufacturer instructions for the MiSeq reagent kit v2 (500 cycles; Illumina, San Diego, CA, USA) and sequenced using an in-house sequencing system.

3.4.3. Computational and Statistical Analysis

The reads (R1 and R2) were merged and the leftovers (not merged) reads from R1 were included in the final output file, sequences below 50 length and Q20 were removed. These steps were performed using PEAR (ZHANG et al., 2014). The gene calling was performed using PRODIGAL to identify open read frames (ORFs) in the reads (HYATT et al., 2010). The annotation of resistance genes was performed with HMMSCAN (EDDY, 1998) by using the hidden Markov models (HMM) profiles available in RESFAM database, a curated database of protein families confirmed for antibiotic resistance function and organized by ontology (GIBSON et al., 2015). All the ARGs related to this study are described in the text with respective RESFAM ID number (in brackets), corresponding exactly to the family of proteins associated to resistance genes available in the database (GIBSON et al., 2015).

The number of hits for all the families of ARGs annotated was normalized dividing it per total number of ORFs that were detected in the respective metagenomic dataset. Taxonomical profiling of the datasets was performed using MEGAN6 (HUSON et al., 2016) by using the output of DIAMOND (BUCHFINK et al., 2015) after aligning the sequences from the datasets against a NCBI Non Redundant (NR) database (Feb/2016). The ARGs rarefaction curves were built experimentally *in silico*, by using USEARCH (EDGAR, 2010) for extracting random subsets of the datasets and subsequently annotations of each one of the subsets using the same procedure as described before, with HMMSCAN and RESFAM database. The rarefaction curves for taxonomical profiling were performed using MEGAN6.

The significance level (alpha) considered for all the tests was 0.05. To test the null hypothesis of homogeneity and normal distribution the tests Levene, and Marti Anderson's (PERMDISP2), from VEGAN package (OKSANEN et al., 2016), for the case of multivariate, and Shapiro-Willk were applied using R statistical computing (R DEVELOPMENT CORE TEAM, 2007). The null hypothesis could not be rejected (alpha>0.05) and ANOVA followed by Tukey HSD was implemented to detect significant differences between the resistome abundance. The analysis of the metagenomic datasets was performed according to the best practices as determined by the Statistical Analysis of Metagenomic Profiles (STAMP) methods, using the effect size and the confidence intervals for assessing biological importance (PARKS et al., 2014). A pairwise comparison EW+/EW- was performed using the t-test (two-sided) and t-test inverted as the method to calculate the confidence intervals of the effect sizes. The effect size is the difference in proportion (DP) of sequences assigned to a given feature in two samples, and it was calculated as follows: DP= p_1 - p_2. Where p_1 and p_2 are the number of sequences in the two samples assigned to the features of interest (x_1 and x_2) divided by the total number of sequences in the profile (C_1 and C_2) (i.e., $p_1 = x_1/C_1$; $p_2=x_2/C_2$). Error bar plots indicating the p-value with the effect size and associated confidence interval for each function detected to be of significant biological relevance (t-test, p-value<0.05) were generated. To test if the variance of the relative abundance of taxonomic groups can be explained by the variance of the relative abundance of RGs, a redundancy analysis (RDA) was performed. A model of permutations was built to test the operational taxonomic unit OTU matrix against the ARGs matrix. Significance values were set at p<0.05.

3.4.4. Network models for predicting ecological interactions

The OTU table and the ARGs table, not normalized (i.e., read count), were both filtered and rare observations were removed (abundance <10%). The co-occurrence network tool (CoNet) (FAUST et al., 2012) implemented in Cytoscape platform, was used to detect strong and significant correlations between the objects observed in both tables. The Spearman correlation coefficient threshold used was $-0.8>\rho>0.8$ and p-value <0.05 corrected (Bonferroni). The intersection method was used as network merge strategy and the significant relationships between the OTU and ARGs tables were interpreted as positive (copresence) or negative (mutual exclusion) (FAUST et al., 2012). The models were visualized using the interactive platform GEPHI 9.1 (BASTIAN et al., 2009) applying the Fruchterman algorithm. The objects (ARGs or OTUs) predicted to have strong and significant correlations were evaluated according to their degree of importance for model. This information can be extracted from the model by evaluating the measure of betweenness centrality (BC) of the object. BC can be defined as the amount of control (i.e., influence) that an object (i.e., node) exerts over the interactions of the other nodes (YOON et al., 2006). This measure was calculated using NetworkAnalyzer, a cytoscape tool, implementing a fast algorithm for BC described by Brandes et al. (2001).

The change in importance of a node between the EW+ and EW- models is named in this study as Differential Betweenness Centrality (DBC). This measure is proposed in order to detect impact of a node to the community when earthworms were present in the system. DBC was calculated as the formula: $DBC = nBC^{EW+} - nBC^{EW-}$; where '*n*' refers to one specific node and BC means the value of Betweenness Centrality attributed 'n' in both networks: the network inferred according to the correlations in the model with earthworms (BC^{EW+}), and the network inferred according to the correlations in the model without earthworms (BC^{EW-}). The value of DBC was calculated for all the nodes detected in the network models and the full results are available in the Table S3.2.

3.5. Results

3.5.1. Microbial taxonomical composition

The major predominant groups over the soil samples were Terrabacteria group (55%) and Proteobacteria (26%) (Figure 3.1). At a higher taxonomical level a total of 380 OTUs were identified. The rarefaction curves inferred from the datasets support a considerable coverage of the microbial diversity (Figure 3.2).

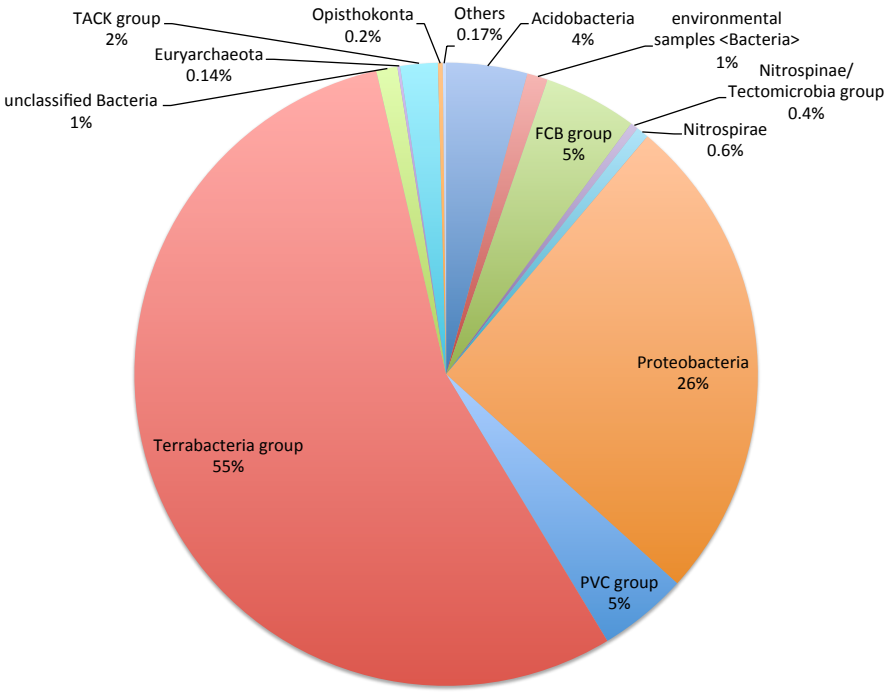


Figure 3.1 – Taxonomical composition found in the metagenomic datasets classified in a low taxonomical level. The sequences assigned as TACK group refer to Thaumarchaeota. Sequences assigned as "Others" refer to rare taxa (<0.1%). The sequences assigned to FCB group refer to Bacteroidetes and Gemmatimonadetes. The sequences assigned to PVC group refer to Planctomycetes and Verrucomicrobia. The sequences assigned to Terrabacteria group refer to Actinobacteria, Armatimonadetes, Chloroflexi, Cyanobacteria/Melainabacteria group, Deinococcu-Thermus and Firmicutes. The taxonomical levels were defined according to the NCBI-NR classification of taxonomical groups (MEGAN6).

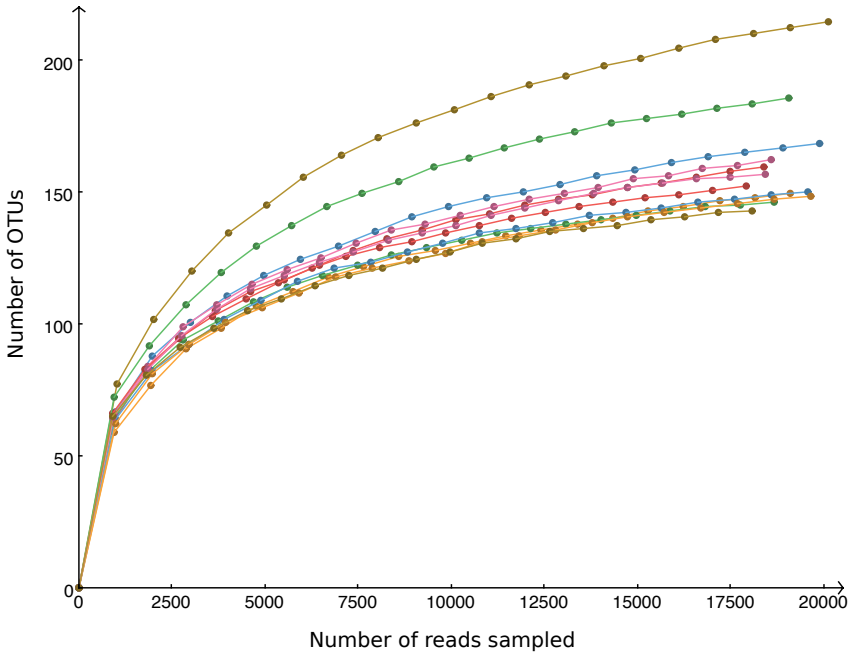


Figure 3.2 – Rarefaction curves reporting the number of operational taxonomical unities (OTUs) per number of reads sampled. Each line represents one of the 12 metagenomic dataset evaluated.

The abundance of several microbial groups was changed in EW+ compared to the taxonomical profiles obtained in EW- (Figure 3.3). The statistical analysis (t-test; p-value<0.05) revealed that *in situ* conditions in EW+ bulk soil favored the increase in abundance of 11 different microbial groups and the inhibition of 10 different microbial groups compared to EW- (Figure 3.3a). In the rhizosphere, EW+ conditions favored the increase in abundance of 13 different taxa while only 1 was inhibited compared to EW- (Figure 3.3b; t-test, p-value<0.05).

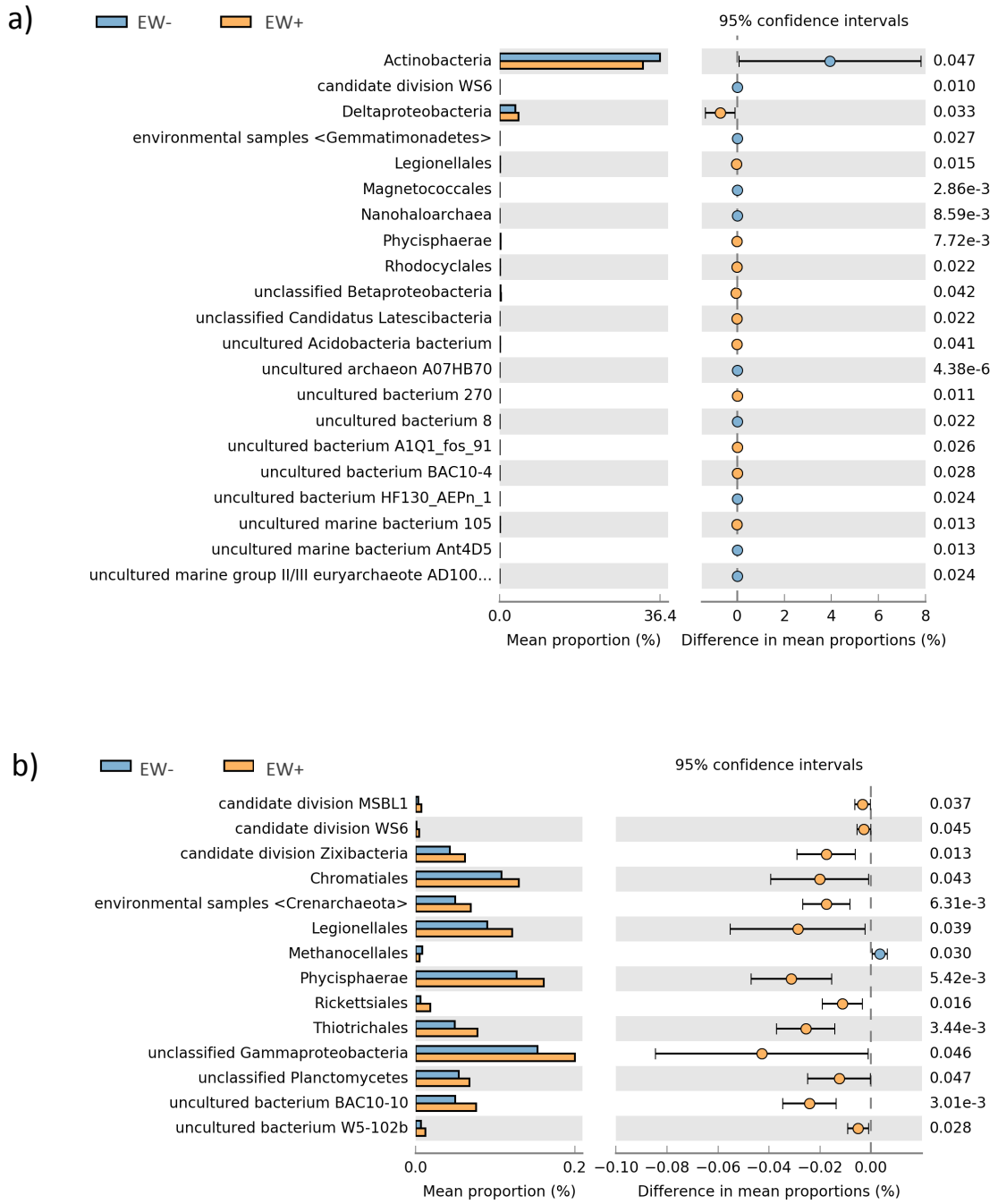


Figure 3.3 – Microbial taxonomical changes detected in bulk (a) and rhizosphere (b) with and without the influence of earthworms (EW+ and EW- respectively). Error bar plots with the effect size and associated confidence interval indicates the variation of microbial groups detected to be of significant biological relevance (t-test, p-value<0.05).

3.5.2. Soil resistome relative abundance, prevalence and composition

On average, 280,146 (±154,488) ORFs were found per metagenomic dataset. From this set of genes, a total of 170 different genes conferring microbial resistance to antibiotic molecules were annotated. The microbial communities from the soil samples analyzed in the present work were found to harbor a highly representative resistome, with around 5-7% of the ORFs assigned as ARG. This information is confirmed by the analysis of the rarefaction curves constructed for each one of the experimental units tested. The plateau in the rarefaction analysis was achieved after the screening of 40% of the gene pool in all datasets (Figure 3.4).

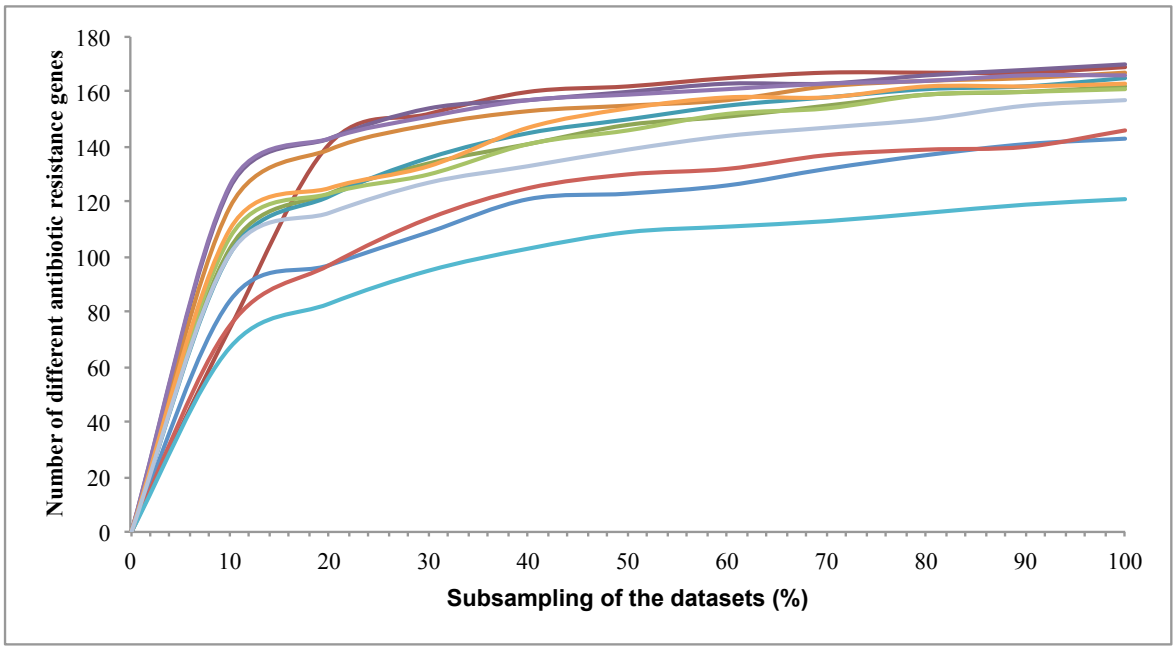


Figure 3.4 - Rarefaction curve of the metagenomic datasets. X-axis represent the exact number of different families associated with antibiotic resistance genes (ARGs) found in randomized subsamples of the datasets (y-axis). Each line represents the values from each one of the datasets obtained from the experimental units with and without earthworm (n=3).

No significant difference between the resistome abundance (sum of relative abundance of all ARGs) was detected comparing the EW+ and EW- conditions (Tukey HSD, p-value>0.05) (Table 3.1). Resistome composition was evaluated by grouping the ARGs according to the type of antimicrobial compounds that they antagonize (Figure 3.5). It was detected that half of the ARGs belongs to genes conferring resistance against diverse antibiotics (49%), followed by glicopeptides (19%), daunorubicin (8%), aminoglicosides (7%), beta-lactams (7%) and others (<5%)

Table 3.1 - Relative abundance of resistome (%)

-	*EW**-		*EW+*	
Bulk	6.83	±0.28	7.25	±0.46
Rhizosphere	5.81	±0.24	5.87	±0.14

*EW: earthworms

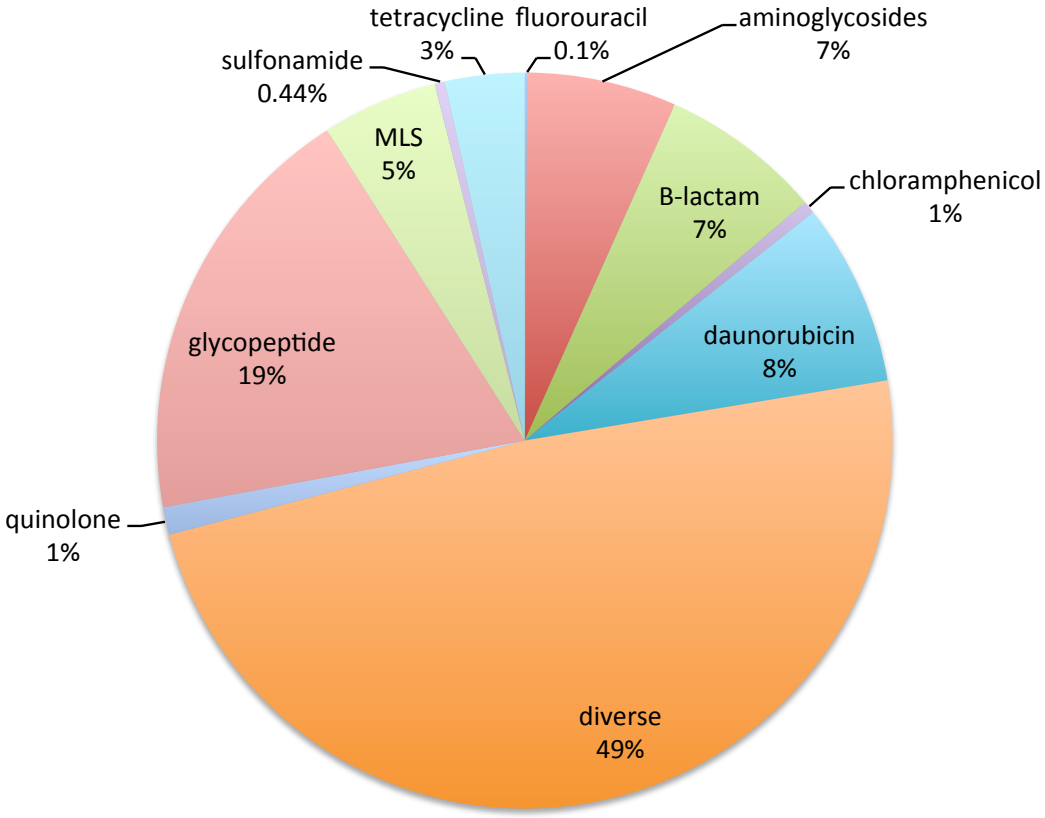


Figure 3.5 – Antibiotic resistance genes (ARGs) composition found in the metagenomic datasets classified according to the antimicrobial compound they antagonize.

3.5.3. Changes in ARGs profiles

Metagenomic profiling revealed that the *in situ* conditions found in EW+ favored significant changes in the proportions of several different ARGs compared to EW- (Figure 3.6; t-test, p-value<0.05) for distinct mechanism of resistance. A detailed list of the ARGs altered including the mode of action of each antimicrobial compound and the respective mechanisms of resistance can be found in APPENDICES section (Table 3.1). In EW+ bulk soil (Figure 3.6a), compared to EW-, it was detected higher proportions of ARGs responsible for the enzymatic degradation of (RF0155), tetracycline (RF0168), beta-lactams (RF0105 and RF0055), 5-fluorouracil (RF0146), and aminoglycosides (RF0030). One type of major facilitator superfamily (MFS) efflux pumps, associated with tetracycline detoxification (RF0130), and three different types of efflux pumps, conferring resistance to diverse antibiotics (RF0079, RF0021 and RF0164), all belonging to the family of resistance-nodulation-division (RND) were also found in higher proportions in EW+ bulk soil. In EW- bulk soil the ARGs found in higher proportions are responsible for the enzymatic degradation of beta-lactams (penicillin) (RF0148), aminoglycoside (RF0004), and the efflux of three types of MFS efflux pumps (RF0065, RF0104 and RF0109) and one type of ABC transporter, all efflux systems associated with multidrug detoxification.

In the rhizosphere EW+ (Figure 3.6b), only ARGs responsible for the enzymatic modification of the vancomycin biding-target (RF0154) and ARGs responsible for one type of MFS efflux pump, activated for tetracycline detoxification (RF0128), were found in higher proportions compared to EW-. In EW- rhizosphere, compared to EW+, it was found in higher proportions mechanisms associated with enzymatic degradation of aminoglycosides (RF0012, RF0173, RF0030) and beta-lactams (RF0105), and mechanisms associated with the MFS efflux pump activated for tetracycline detoxification (RF0131). Overall, earthworms presence influenced the change of several different types of mechanisms of resistance against diverse antimicrobial compounds. Compared to EW- conditions, more different ARGs were found in higher proportions in EW+ bulk and only 2 were changed in EW+ rhizosphere.

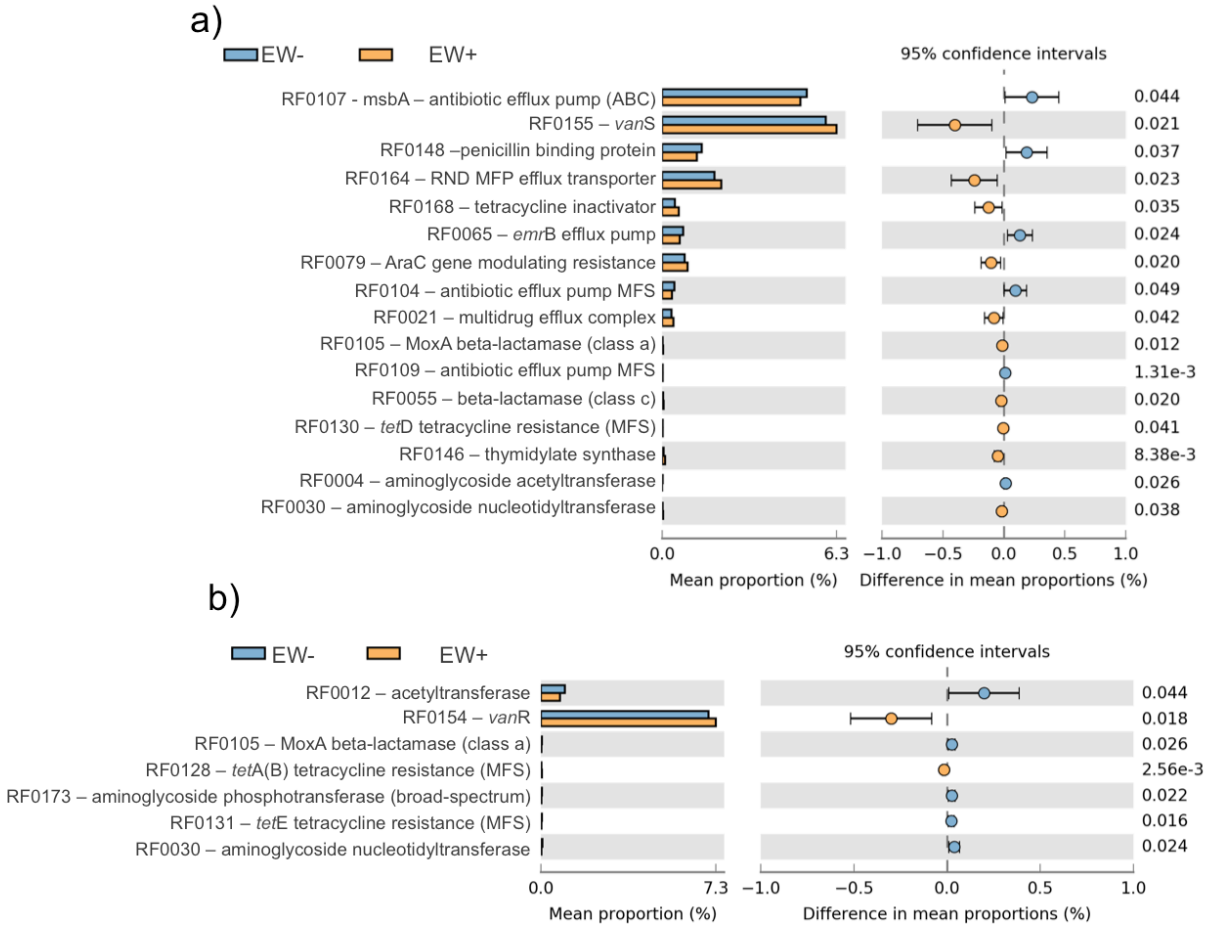
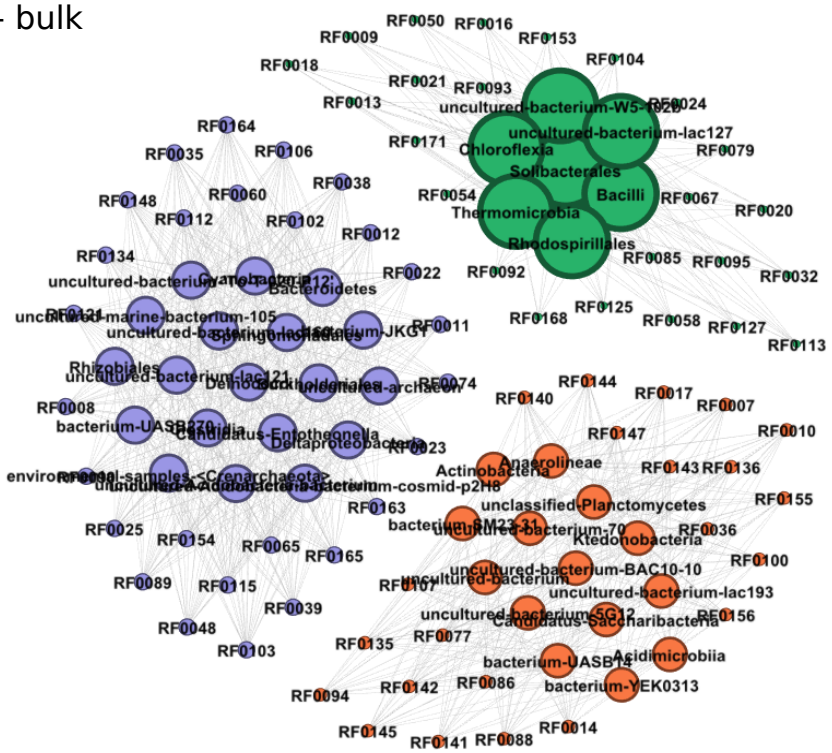


Figure 3.6 – Antibiotic resistance genes changes detected in bulk (a) and rhizosphere (b) with and without the influence of earthworms (EW+ and EW- respectively). Error bar plots with the effect size and associated confidence interval indicates the variation of microbial groups detected to be of significant biological relevance (t-test, p-value<0.05).

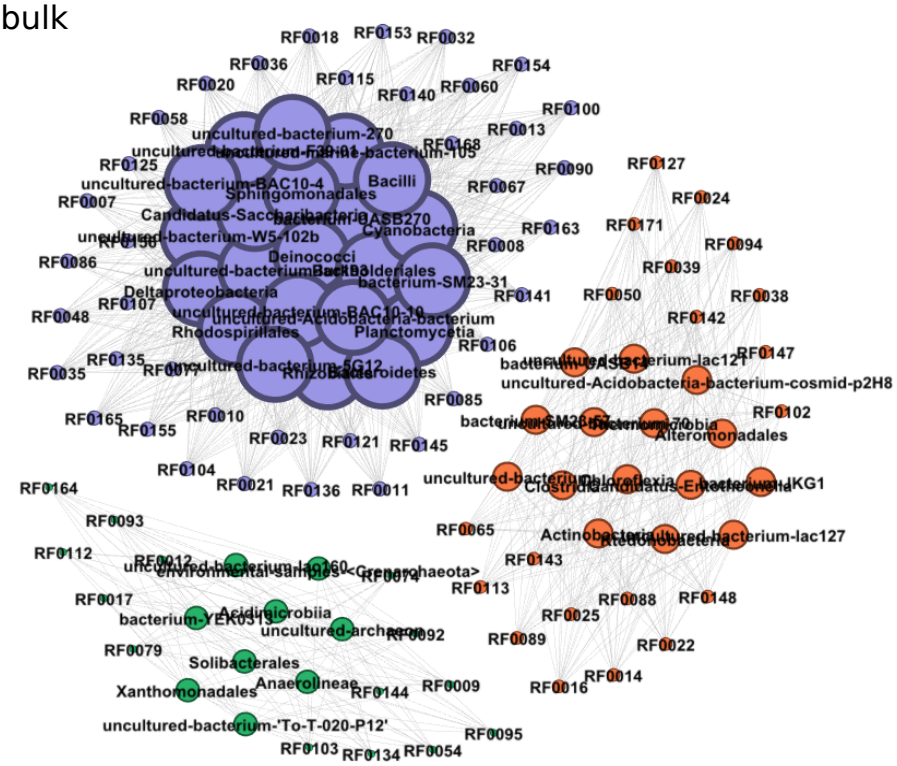
3.5.4. Ecological models of interactions between microbial groups and ARGs

Ecological models built on strong and significant correlations (-0.8>ϱ>0.8; p-value corrected < 0.05; Figure 3.7) detected more interactions between OTUs and ARGs in rhizosphere and less in bulk (Table 3.2). The proportion of negative relationship between ARGs and OTUs were increased in EW+ bulk and rhizosphere compared to EW- (Table 3.2). The measure of DBC indicates the proportion in which the degree of importance of a member of the community (ARGs and OTUs) was altered (Figure 3.8). This comparison demonstrates that members predicted to play less influence in the models in EW- were found to play higher influence in EW+ models (APPENDICES section Table 3.2), and vice-versa. This effect is verified for both ARGs and OTUs. Further, in general, the BC values were decreased in the models predicted for the interactions in EW+ (APPENDICES section Table 3.2), and the BC values attributed to ARGs were increased in the EW+ models compared to EW- models (APPENDICES section Table 3.3).

a) EW- bulk



a) EW+ bulk



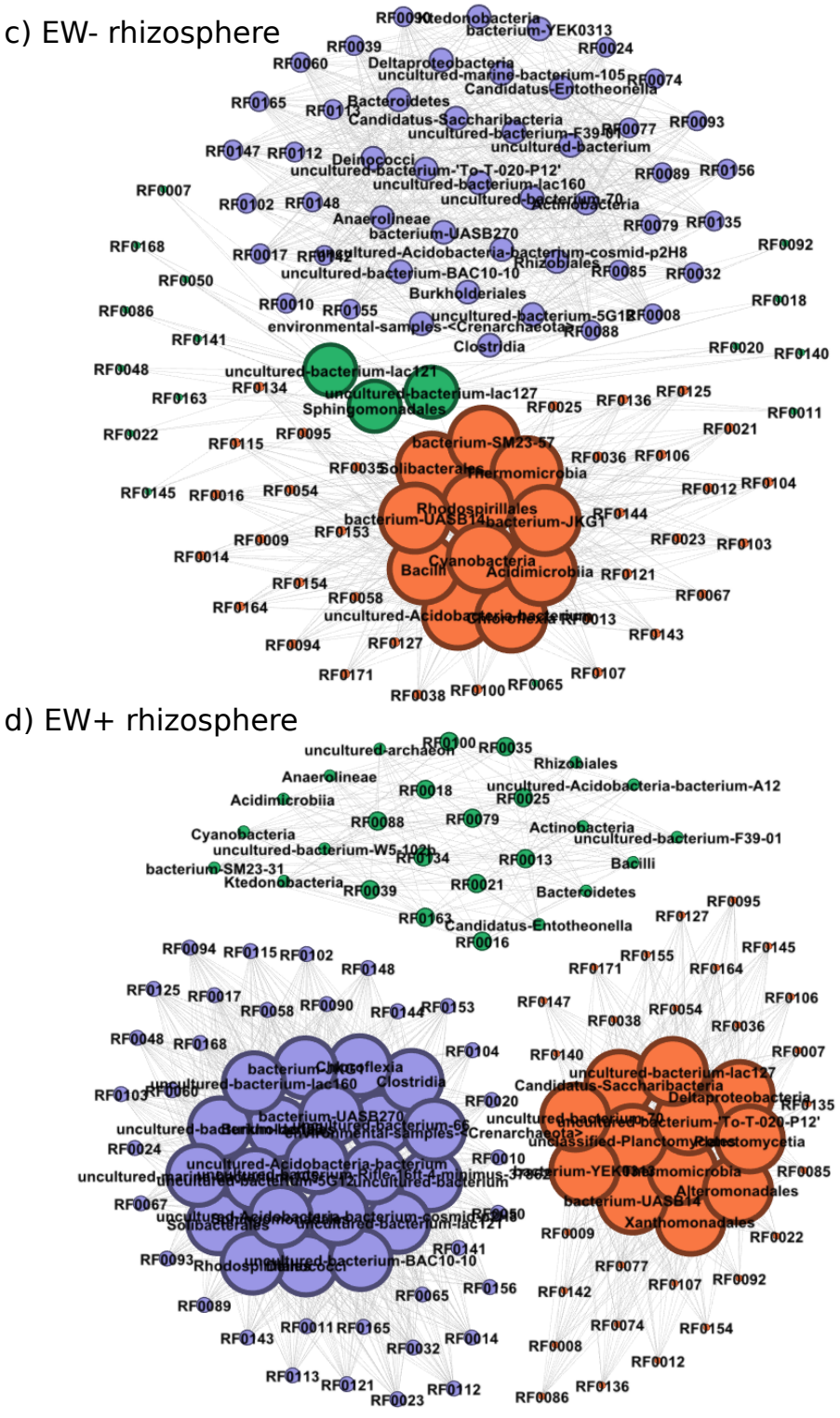


Figure 3.7 – Network models representing the ecological interactions predicted between microbial taxonomical groups and antibiotic resistance genes in (a) bulk soil without earthworms (EW-), (b) bulk soil with earthworms (EW+) and (c) rhizosphere EW- and (d) rhizosphere EW+.

Table 3.2 – Network parameters

Network model	nodes	interactions	mutual exclusion* (%)
Bulk EW-	113	989	34.78
Bulk EW+	119	1284	52.8
Rhizosphere EW-	110	983	37.84
Rhizosphere EW+	120	1206	51.32

*negative correlation

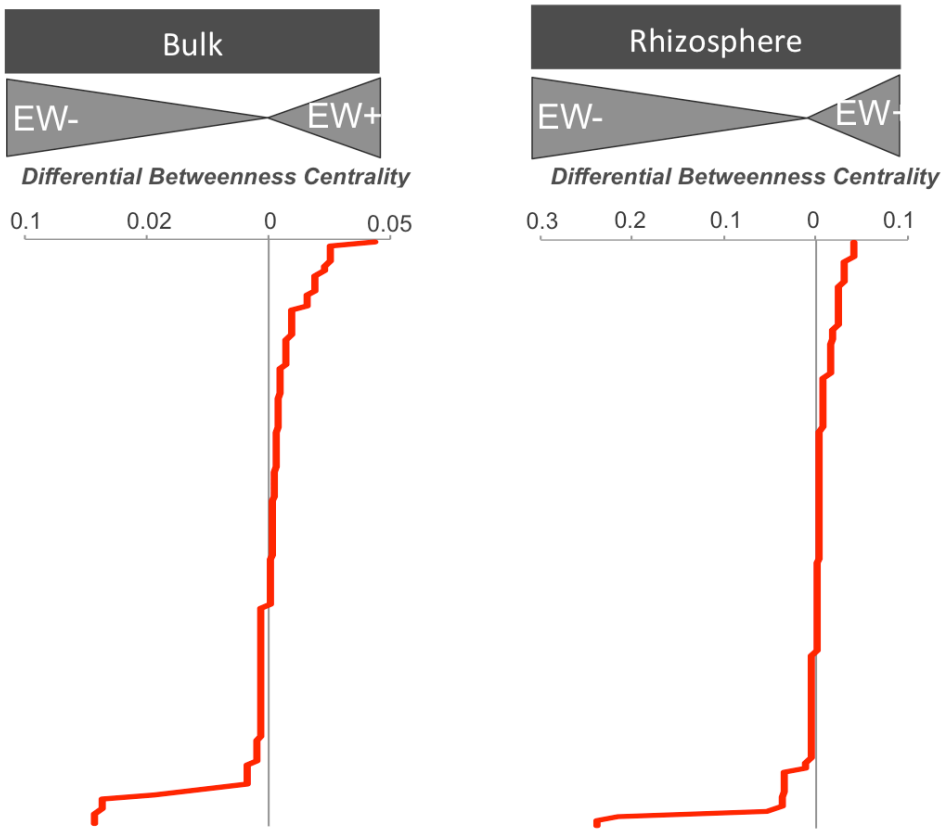


Figure 3.8 – Differential betweenness centrality (DBC) indicating the proportion of change in importance of a node comparing the model with and without earthworms (EW+ and EW- respectively). DBC = $n\text{BC}^{\text{EW+}} - n\text{BC}^{\text{EW-}}$; where '*n*' is the node of the model. Values on x-axis on the right indicate nodes that were found to less influence in the EW- models than in EW+ models. Values on x-axis on the left indicate nodes that were found to less influence in the EW+ models than in EW- models. The larger the values (DBC) the greater the change in the importance of the node for the model as illustrated by crescent triangles in gray above x-axis. Each value attributed in y-axis represent a unique node.

3.5.5. ARGs and taxonomic variance

The variance of the relative abundance of specific groups of microbes can be significantly explained by the variance of the relative abundance of specific ARGs (Figure 3.9). The variance of members from the groups Gemmatimonadales, Deltaproteobacteria and Planctomycetales in bulk soil of pots with earthworms can be explained by the variance found for the relative abundance of resistance mechanisms associated to vancomycin (RF0154) and to multidrug efflux pumps of types AcrBDF family (RF0017) and RND (RF0115). The variance of the relative abundance of members of the group Rhizobiales found in rhizosphere from the pots with earthworms can be explained by the variance of the relative abundance of mechanisms of resistance associated to tetracycline (RF0137). Rhizobiales members were enriched considerably in the rhizosphere under the influence of earthworms, while the other taxonomical groups (Gemmatimonadales, Deltaproteobacteria and Plactomycetales) demonstrated the tendency of being negatively correlated to the presence of earthworms (ANOVA, $F>0.05$) (Figure 3.10). Without earthworms, the variance of the relative abundance of taxonomical groups appears to be associated to the variances of a type of beta-lactamase (RF0076) and aminoglycosidase (ANT2) (RF0026).

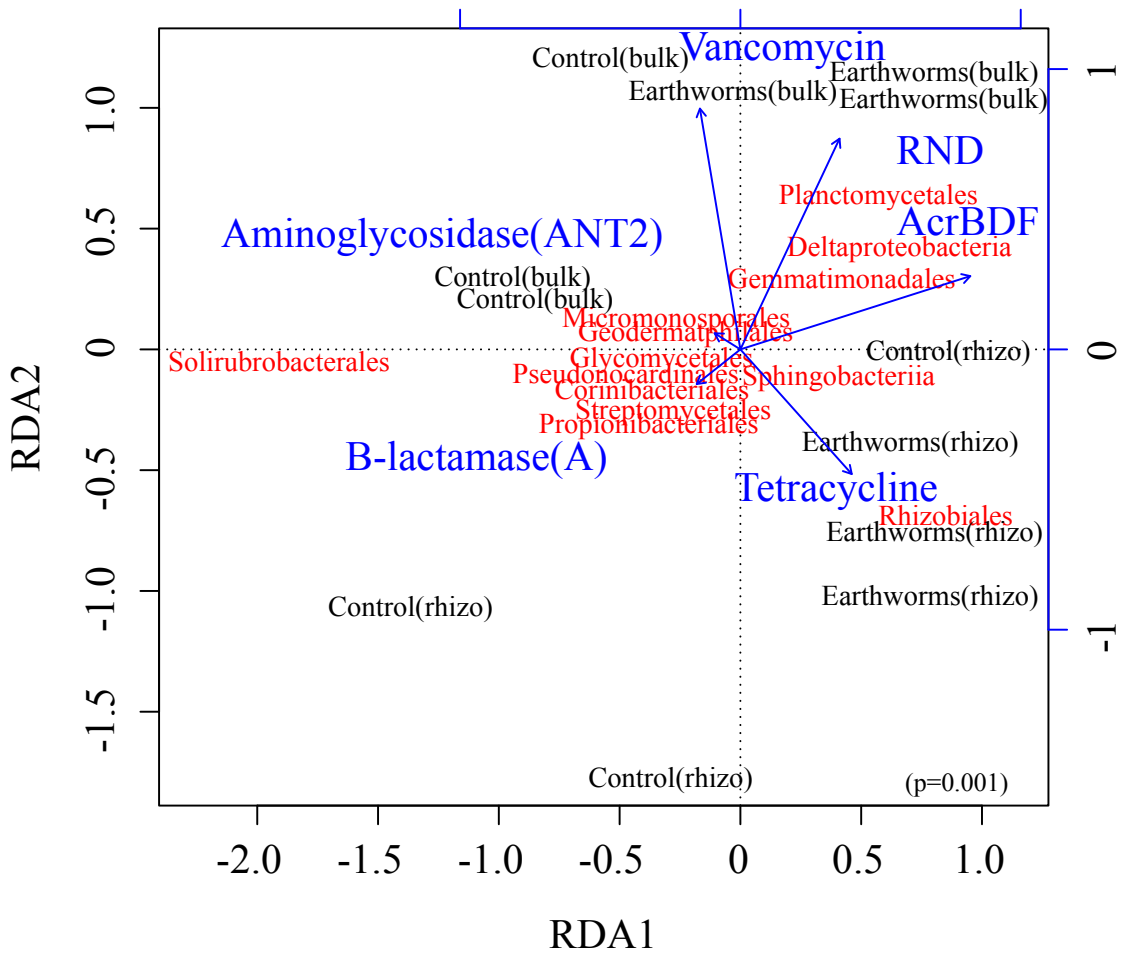


Figure 3.9 - Redundancy analysis (RDA) showing the family of proteins associated with antibiotic resistance gene (ARGs) whose variance in relative abundance significantly explains the changes in the variance of relative abundance of microbial taxonomical groups. P-values were selected according a permutation test ($p<0.05$) and the significance of the model fitting the significant variance was tested with permutation test for all variables ($p=0.001$).

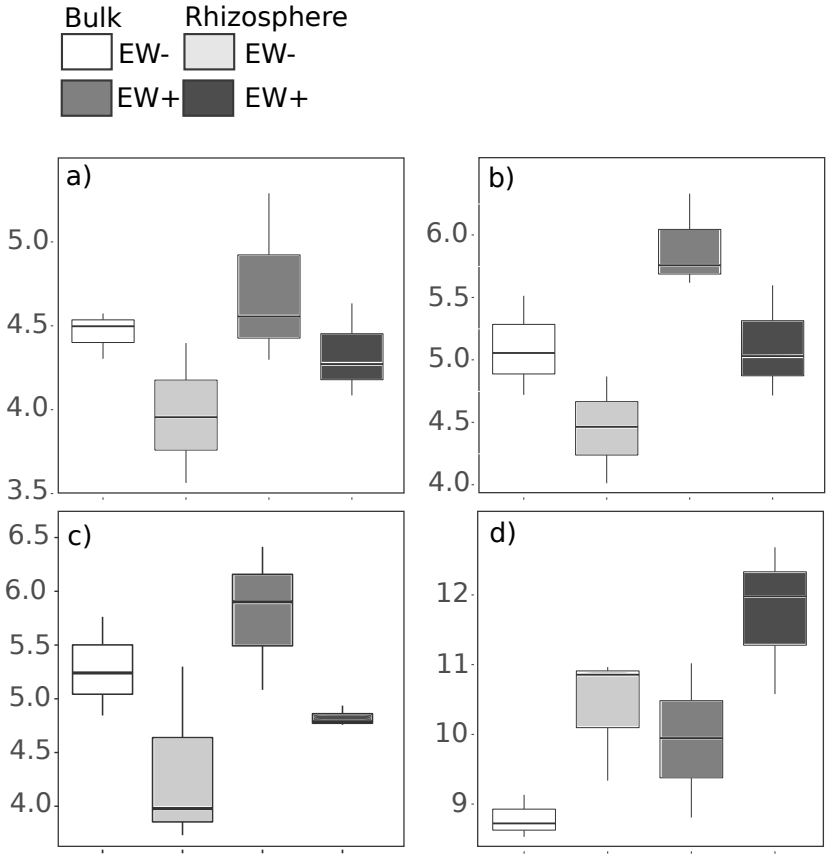


Figure 3.10 - Dynamics of the relative abundance of microbial taxonomical groups. Each plot represents one group and its variance across the different in situ conditions: bulk soil (white), bulk soil with earthworms (gray 20%), rhizosphere (gray 50%) and rhizosphere with earthworms (gray 70%). Y-axis indicates the percentage of the relative abundance of taxonomical groups. The read counts were normalized to the smallest number of reads according algorithm disposed in MEGAN6. The order of the plots follows the order that they were cited in the manuscript: a) Gemmatimonadetes; b) Deltaproteobacteria; c) Planctomycetales; d) Rhizobiales. (ANOVA, F>0.05).

3.6. Discussion

Resistance genes are expected to be present in all environmental samples, especially soil, which is known to harbor a great diversity of ARGs and appears to be the biggest reservoir of these genes (NESME; SIMONET, 2015). The dataset analyzed here (Table 3.1) demonstrates that the prevalence of resistance genes can be comparable with results reported before (NESME et al., 2014). The authors reported inferior proportion of ARGs then detected in the present study. As demonstrated in Figure 3.4, the richness of ARGs was almost entirely covered in all the samples after the screening of 40% of the gene pool. Therefore, soil metagenome reported in the present study was detected to harbor a high density of ARGs.

Earthworm presence influenced changes in the profile of ARGs associated with distinct antimicrobial compounds (Figure 3.6). In clinical studies microbes exposed to one source of antibiotic are capable of employing several resistance mechanisms. For example, isolates from the genera *Escherichia, Morganella, Proteus, Salmonella* and *Shigella* were found to harbor more than 50 different mechanisms of resistance involving up to 19 genes conferring resistance against aminoglycosides (MILLER et al., 1995). Under environmental conditions, where possibly more than one source of antibiotic is being synthetized at the same time, the number of genes and mechanisms of resistance employed are expected to be highly diverse and prevalent.

The ecological interactions predicted by the network models demonstrate that negative correlations were increased in EW+ compared to EW-. Negative correlations indicate ecological interactions associated with competition (FAUST; RAES, 2012). Strong and significant negative relationships between microbial taxa and ARGs suggest that ARGs participate directly on competition interactions within the microbial community. Still, the change in the level of importance of the nodes in EW+ compared to EW- (DBC) supports that the increased negative correlations are associated with a reconfiguration of the model, where less important players in EW- increase their importance in EW+ (Figure 3.8; Table 3.2). Thus, it can be hypothesized that earthworms contribute to competition between microbial groups in rhizosphere, consequently impacting the production of antibiotics and the resistome profile. The decrease in BC values verified in EW+ models, specially in rhizosphere, compared to the BC values found in EW- (APPENDICES section Table 3.3), indicates that the EW+ models were under less influence of dominant players, reinforcing that competition was increased in the microbial communities from EW+.

The anoxic organic carbon rich environment in the earthworm gut provides *in situ* conditions that favor accelerated anaerobic microbial processes during gut passage (DRAKE; HORN, 2007). The abundance of detectable fermenters and denitrifiers is higher in the gut than in pre-ingested soil, and many studies have demonstrated that microbes in the gut are capable of fermentation, denitrification during the gut passage (MATTHEIS et al., 1999; DRAKE; HORN, 2007; WÜST et al., 2009a; 2009b; 2011; DEPKAT-JAKOB et al., 2012; 2013). Recently, Schulz et al. (2015) demonstrated that diverse anaerobic process can be concomitantly augmented in gut contents of the methane (CH_4)-emitting earthworm *Eudrillus eugeniae*, yielding several products such as CH_4, H_2, CO_2, formate, acetate, ethanol, lactate, succinate and propionate. Earthworms may take advantage of the products of fermentation and probably have a physiological need for them. Nevertheless, some of the products of these

complex microbial and trophic interactions in the gut are inevitably egested to the soil as castings, resulting in new input of carbon-derived nutrients to the soil microbiome.

The rhizosphere is also an environment where high amounts of carbon-derived nutrients are released to the microbial soil community. Earthworms have also been shown to influence C rhizodeposition (HUANG et al., 2015). *P. corethrurus* activities increased rhizodeposited-C in the gut, bulk soil and aggregates. These findings imply that the rhizosphere of sugarcane in the present experiment had unique *in situ* conditions that exerted selective pressures on the soil microbial communitiy.

The high input of carbon sources increases pressure on uptake of other nutrients in soil by microbes. The easily available C can increase activity and growth of microbial cells in rhizosphere, increasing their demand for other nutrients. This pressure for nutrient uptake is intensified by competition with the plant. Roots are constantly taking up the same nutrients also needed by microbial cells. In the rhizosphere *in situ* available P and N are probably the most limiting nutrients (KUZYAKOV; XU, 2013). The severe competition between organisms in the rhizosphere is associated with the production of potent antibiotics in this soil compartment (RAAIJMAKERS; MAZZOLA, 2012). In this context, it is expected that earthworms increase competition between microbes in the rhizosphere. Microbes have more easily available C but they also face greater depletion of limiting nutrients such as N for example, since *in situ* conditions of the earthworm activities favor denitrification and increase the loss of N as N_2O and N_2 (DEPKAT-JAKOB et al., 2012; 2013; LUBBERS et al., 2013).

The hypothesis of resistance genes increasing fitness of microbial groups was discribed in Nesme and Simonet (2015) and the results presented here support that some mechanisms of resistance might be explaining the abundance of specific groups, as verified in the RDA analysis (Figure 3.9). Depkat-Jakob et al. (2012) found members of the Rhizobiales group as the dominant denitrifiers in earthworm guts. Here, this group had the proportions considerably increased in the rhizosphere influenced by earthworms (Figure 3.10d). The variance of the relative abundance of Rhizobiales could be explained by the variance of the relative abundance of a resistance gene associated to protect against tetracycline (Figure 3.9). This result indicates that resistance against tetracycline was important for Rhizobiales during the niche colonization of rhizosphere.

However, it is not possible to conclude about antibiotics production since it was not determined by the experiment proposed. Additionally, resistome genotypes must also be distinguished from resistant phenotypes (DANTAS; SOMMER, 2012). For this, more refined experiments must be performed, though it will still be difficult to overcome the bias of

detecting natural antibiotics concentrations in soils and distinguish phenotypes, considering the highly dynamic life style of prokaryiotic cells and the limits of cultivation methods. Nevertheless, the results presented here provide evidence to support the genetic enrichment of ARGs as a response to biological factors (rhizosphere and earthworms), and according to the ecological interaction predicted by the network models the most probable explanation for the observed phenomenon supports the 'arms-shield' race hypothesis. Antibiotic production is increased by competition, and ARGs are essential to favor fitness, so that the observation of an enrichment of one type of resistance gene is probably the result of one group outcompeting the other group.

Further, worth noting that ARGs appear to be important regardless of antibiotic concentration. Gullberg et al. (2014) verified that lower concentrations of antimicrobial molecules (150 fold lower than the necessary for growth inhibition of cells) can cause enough harm to the cells that the fitness costs of the maintenance of resistance genes are overcome. There must be a selective advantage for microbes that carry enriched ARGs, otherwise the cost for keeping them in the genome would reduce fitness.

Much of the knowledge generated around ARGs was obtained by clinical studies limited to very specific environmental conditions, and as in many other areas of microbiology they were also limited by culture-dependent methods. Recent technological advances in DNA sequencing revealed that ARGs are part of the nature of prokaryotic existence. They can be found in samples of ancient DNA (>30,000 years) and also in environments never before exposed to humans (D'COSTA et al., 2011; BHULLAR et al., 2012). The results presented here demonstrate that the structure of the community of indigenous ARGs found in soil resistome can be (re)configured by natural processes occurring in soils, such as rhizosphere depositions and earthworm activities.

The effects of diverse enrichment of ARGs connected with the taxonomical changes, and the increased negative correlations and reconfiguration of importance of the nodes in the network models, as a response to the presence of earthworms in bulk and rhizosphere, are in accordance with the idea that soil resistome is under the effect of distinct concentrations of diverse antibiotics and constant competition for nutrients. Experiments in the past demonstrated that selection of ARGs depends on concentration of antibiotics and low concentrations of antibiotics are responsible for the selection of a high diversity of resistant mutants. *In vitro* conditions confirmed that a narrow concentration range can provide a strong selection for a particular resistant genotype (BAQUERO; NEGRI, 1997).

3.7. Conclusion

Soil is considered the major reservoir for ARGs and the results presented here demonstrate that soil microbial communities naturally exposed to biotic forces such as plant roots and earthworms interactions can significantly change the abundance of ARGs. With that it is demonstrated that changes in ARGs might be relevant for fitness of microbial groups. Disentangling the ecology and evolution of soil resistome is of great concern to resolve the mechanisms employed by rhizosphere microbes to cope with soil-born pathogens capable of causing plant disease. For instance, Chapelle et al. (2015) evaluated transcripts (mRNA) from sugar beet rhizosphere upon fungal invasion, and surprisingly, the gene expression associated to resistance to fluoroquinolones was drastically reduced under disease conditions. Further, unrevealing the natural dynamics of ARGs within soil resistome may contribute to understand the mechanisms of maintenance of resistance in microbial communities, a question of great concern for human health.

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APPENDICES

Table 2.1 Soil chemical parameters determined in the bulk soil (217th day)

	EW-		EW+	
B	0.4	±0.1	0.3	±0
Cu	0.6	0	0.6	±0.1
Fe	19.3	±0.6	18.3	±1.5
Mn	10.6	±1.7	10.2	±1.1
Zn	8	±6.3	4.6	±1.5
Na	116.3	±19.5	113.7	±13.1
P	10.3	±0.6	9.3	±0.6
S-SO4	40	±33	23	±1.7
K	5.8	±2	3.6	±1.4
Ca	60.3	±4.5	58.7	±3.8
Mg	21	±1	22.7	±0.6
Al	<1		<1	
H+Al	20.7	±1.2	19.3	±1.2
SEB	87.1	±5.6	84.9	±5.4
CEC	107.8	±4.6	104.2	±6.1
pH	6.2	±0.2	6.3	±0.1

SEB: sum of exchangeable bases; CEC: cation exchangeable capacity.
The values for K, Ca, Mg, Al, H+Al, SEB and CEC are represented according the following unit: mmolc.dm-3; for all the others except pH, the unit represented is: mg.dm-3, pH unit is CaCl2 0.01 mol/L. All the variables were statistically tested following the methods as described in the main text. No significant differences were detected.

Table 3.1 – Mechanisms of resistance, mode of action and type of antibiotics according to the family of resistance genes (RESFAM)

RESFAM ID	Antibiotics	mode of action	mechanism of resistance
RF0004	aminoglycosides	inhibition of protein synthesis	enzymatic modification of aminoglycosides
RF0012	aminoglycoside	inhibition of protein synthesis	enzymatic modification of aminoglycosides
RF0021	multidrug	diverse	RND efflux pump
RF0030	aminoglycosides	inhibition of protein synthesis	enzymatic modification of aminoglycosides
RF0030	aminoglycoside	inhibition of protein synthesis	enzymatic modification of aminoglycosides
RF0055	B-lactam	interference with the cell wall synthesis	enzymatic degradation of B-lactams
RF0065	multidrug	diverse	MFS efflux pump
RF0079	multidrug	diverse	efflux punps RND
RF0104	multidrug	diverse	MFS efflux pump
RF0105	B-lactam	interference with the cell wall synthesis	enzymatic degradation of B-lactams
RF0105	B-lactam	interference with the cell wall synthesis	enzymatic degradation of B-lactams
RF0107	multidrug	diverse	ABC transporter
RF0109	multidrug	diverse	MFS efflux pump
RF0128	tetracycline	inhibition of protein synthesis	MFS efflux pump
RF0130	tetracycline	inhibition of protein synthesis	MFS efflux pump
RF0131	tetracycline	inhibition of protein synthesis	MFS efflux pump
RF0146	5-fluorouracil	interference of nucleic acid synthesis	enzymatic inactivation
RF0148	B-lactam	interference with the cell wall synthesis	enzymatic degradation of B-lactams
RF0154	vancomycin	inhibition of cell wall synthesis	production of enzymes that modify the vancomycin-binding target
RF0155	vancomycin	inhibition of cell wall synthesis	production of enzymes that modify the vancomycin-binding target
RF0164	multidrug	diverse	RND efflux transporter MFP
RF0168	tetracycline	inhibition of protein synthesis	enzymatic degradation of tetracycline
RF0173	aminoglycoside	inhibition of protein synthesis	enzymatic modification of aminoglycosides

Table 3.2 – Contrasting the change in level of importance of a node across the models by differential betweenness centrality (DBC)

Bulk				Rhizosphere			
Nodes	**EW-**	**EW+**	**DBC***	**Nodes**	**EW-**	**EW+**	**DBC**
Xanthomonadales	0	0.04377104	0.04377104	Alteromonadales	0	0.0416074	0.0416074
uncultured-archaeon	0.01866029	0.04377104	0.02511075	unclassified-Planctomycetes	0	0.0416074	0.0416074
environmental-Crenarchaeota	0.01866029	0.04377104	0.02511075	Planctomycetia	0	0.0416074	0.0416074
uncultured-bacterium-'To-T-020-P12'	0.01866029	0.04377104	0.02511075	Xanthomonadales	0	0.0416074	0.0416074
uncultured-bacterium-lac160	0.01866029	0.04377104	0.02511075	Deltaproteobacteria	0.01206612	0.0416074	0.02954128
bacterium-SM23-57	0	0.02257873	0.02257873	Candidatus-Saccharibacteria	0.01206612	0.0416074	0.02954128
Alteromonadales	0	0.02257873	0.02257873	bacterium-YEK0313	0.01206612	0.0416074	0.02954128
uncultured-bacterium-270	0	0.01902928	0.01902928	uncultured-bacterium-'To-T-020-P12'	0.01206612	0.0416074	0.02954128
Planctomycetia	0	0.01902928	0.01902928	uncultured-bacterium-70	0.01206612	0.0416074	0.02954128
uncultured-bacterium-BAC10-4	0	0.01902928	0.01902928	RF0018	0.00147059	0.02527778	0.02380719
uncultured-bacterium-F39-01	0	0.01902928	0.01902928	RF0163	0.00147059	0.02527778	0.02380719
bacterium-YEK0313	0.02773109	0.04377104	0.01603995	RF0013	0.0018457	0.02527778	0.02343208
Acidimicrobiia	0.02773109	0.04377104	0.01603995	RF0100	0.0018457	0.02527778	0.02343208
Anaerolineae	0.02773109	0.04377104	0.01603995	RF0021	0.0018457	0.02527778	0.02343208
RF0009	0.00201149	0.01113173	0.00912024	RF0134	0.0018457	0.02527778	0.02343208
RF0054	0.00201149	0.01113173	0.00912024	RF0016	0.0018457	0.02527778	0.02343208
RF0092	0.00201149	0.01113173	0.00912024	RF0035	0.0018457	0.02527778	0.02343208
RF0095	0.00201149	0.01113173	0.00912024	RF0025	0.0018457	0.02527778	0.02343208
RF0093	0.00201149	0.01113173	0.00912024	uncultured-bacterium-Rifle-16ft-4-37862	0	0.01866826	0.01866826
RF0079	0.00201149	0.01113173	0.00912024	uncultured-bacterium-66	0	0.01866826	0.01866826
RF0016	0.00201149	0.00935829	0.0073468	uncultured-bacterium-lac193	0	0.01866826	0.01866826
RF0127	0.00201149	0.00935829	0.0073468	RF0088	0.0093617	0.02527778	0.01591608
RF0171	0.00201149	0.00935829	0.0073468	RF0039	0.0093617	0.02527778	0.01591608
RF0113	0.00201149	0.00935829	0.0073468	RF0079	0.0093617	0.02527778	0.01591608
RF0024	0.00201149	0.00935829	0.0073468	uncultured-Acidobacteria-A12	0	0.01571429	0.01571429

Bulk				Rhizosphere			
Nodes	**EW-**	**EW+**	**DBC***	**Nodes**	**EW-**	**EW+**	**DBC**
RF0050	0.00201149	0.00935829	0.0073468	bacterium-SM23-31	0	0.01571429	0.01571429
RF0012	0.00639731	0.01113173	0.00473442	uncultured-bacterium-W5-102b	0	0.01571429	0.01571429
RF0164	0.00639731	0.01113173	0.00473442	uncultured-archaeon	0	0.01571429	0.01571429
RF0112	0.00639731	0.01113173	0.00473442	uncultured-Acidobacteria-p2H8	0.01206612	0.01866826	0.00660214
RF0074	0.00639731	0.01113173	0.00473442	Clostridia	0.01206612	0.01866826	0.00660214
RF0103	0.00639731	0.01113173	0.00473442	uncultured-marine-bacterium-105	0.01206612	0.01866826	0.00660214
RF0134	0.00639731	0.01113173	0.00473442	uncultured-bacterium	0.01206612	0.01866826	0.00660214
RF0144	0.00695187	0.01113173	0.00417986	Deinococci	0.01206612	0.01866826	0.00660214
RF0017	0.00695187	0.01113173	0.00417986	Burkholderiales	0.01206612	0.01866826	0.00660214
bacterium-JKG1	0.01866029	0.02257873	0.00391844	uncultured-bacterium-5G12	0.01206612	0.01866826	0.00660214
Candidatus-Entotheonella	0.01866029	0.02257873	0.00391844	environmental-Crenarchaeota	0.01206612	0.01866826	0.00660214
uncultured-Acidobacteria-p2H8	0.01866029	0.02257873	0.00391844	uncultured-bacterium-lac160	0.01206612	0.01866826	0.00660214
uncultured-bacterium-lac121	0.01866029	0.02257873	0.00391844	uncultured-bacterium-BAC10-10	0.01206612	0.01866826	0.00660214
Clostridia	0.01866029	0.02257873	0.00391844	bacterium-UASB270	0.01206612	0.01866826	0.00660214
RF0102	0.00639731	0.00935829	0.00296098	uncultured-bacterium-F39-01	0.01206612	0.01571429	0.00364817
RF0065	0.00639731	0.00935829	0.00296098	Anaerolineae	0.01206612	0.01571429	0.00364817
RF0039	0.00639731	0.00935829	0.00296098	Rhizobiales	0.01206612	0.01571429	0.00364817
RF0022	0.00639731	0.00935829	0.00296098	Bacteroidetes	0.01206612	0.01571429	0.00364817
RF0148	0.00639731	0.00935829	0.00296098	Actinobacteria	0.01206612	0.01571429	0.00364817
RF0089	0.00639731	0.00935829	0.00296098	Candidatus-Entotheonella	0.01206612	0.01571429	0.00364817
RF0025	0.00639731	0.00935829	0.00296098	Ktedonobacteria	0.01206612	0.01571429	0.00364817
RF0038	0.00639731	0.00935829	0.00296098	RF0048	0.00147059	0.00431619	0.0028456
RF0014	0.00695187	0.00935829	0.00240642	RF0020	0.00147059	0.00431619	0.0028456
RF0088	0.00695187	0.00935829	0.00240642	RF0011	0.00147059	0.00431619	0.0028456
RF0147	0.00695187	0.00935829	0.00240642	RF0168	0.00147059	0.00431619	0.0028456
RF0142	0.00695187	0.00935829	0.00240642	RF0141	0.00147059	0.00431619	0.0028456
RF0143	0.00695187	0.00935829	0.00240642	RF0050	0.00147059	0.00431619	0.0028456
RF0094	0.00695187	0.00935829	0.00240642	RF0065	0.00147059	0.00431619	0.0028456

Bulk				Rhizosphere			
Nodes	**EW-**	**EW+**	**DBC***	**Nodes**	**EW-**	**EW+**	**DBC**
RF0021	0.00201149	0.00334637	0.00133488	RF0121	0.0018457	0.00431619	0.00247049
RF0067	0.00201149	0.00334637	0.00133488	RF0103	0.0018457	0.00431619	0.00247049
RF0032	0.00201149	0.00334637	0.00133488	RF0067	0.0018457	0.00431619	0.00247049
RF0013	0.00201149	0.00334637	0.00133488	RF0144	0.0018457	0.00431619	0.00247049
RF0085	0.00201149	0.00334637	0.00133488	RF0014	0.0018457	0.00431619	0.00247049
RF0153	0.00201149	0.00334637	0.00133488	RF0153	0.0018457	0.00431619	0.00247049
RF0020	0.00201149	0.00334637	0.00133488	RF0104	0.0018457	0.00431619	0.00247049
RF0058	0.00201149	0.00334637	0.00133488	RF0094	0.0018457	0.00431619	0.00247049
RF0018	0.00201149	0.00334637	0.00133488	RF0143	0.0018457	0.00431619	0.00247049
RF0168	0.00201149	0.00334637	0.00133488	RF0115	0.0018457	0.00431619	0.00247049
RF0125	0.00201149	0.00334637	0.00133488	RF0125	0.0018457	0.00431619	0.00247049
RF0104	0.00201149	0.00334637	0.00133488	RF0023	0.0018457	0.00431619	0.00247049
Sphingomonadales	0.01866029	0.01902928	0.00036899	RF0058	0.0018457	0.00431619	0.00247049
uncultured-marine-bacterium-105	0.01866029	0.01902928	0.00036899	RF0086	0.00147059	0.00347716	0.00200657
Deltaproteobacteria	0.01866029	0.01902928	0.00036899	RF0007	0.00147059	0.00347716	0.00200657
uncultured-Acidobacteria	0.01866029	0.01902928	0.00036899	RF0145	0.00147059	0.00347716	0.00200657
Rhizobiales	0.01866029	0.01902928	0.00036899	RF0022	0.00147059	0.00347716	0.00200657
Burkholderiales	0.01866029	0.01902928	0.00036899	RF0140	0.00147059	0.00347716	0.00200657
bacterium-UASB270	0.01866029	0.01902928	0.00036899	RF0092	0.00147059	0.00347716	0.00200657
Bacteroidetes	0.01866029	0.01902928	0.00036899	RF0036	0.0018457	0.00347716	0.00163146
Deinococci	0.01866029	0.01902928	0.00036899	RF0164	0.0018457	0.00347716	0.00163146
Cyanobacteria	0.01866029	0.01902928	0.00036899	RF0012	0.0018457	0.00347716	0.00163146
RF0106	0.00639731	0.00334637	-0.00305094	RF0106	0.0018457	0.00347716	0.00163146
RF0048	0.00639731	0.00334637	-0.00305094	RF0038	0.0018457	0.00347716	0.00163146
RF0163	0.00639731	0.00334637	-0.00305094	RF0009	0.0018457	0.00347716	0.00163146
RF0090	0.00639731	0.00334637	-0.00305094	RF0054	0.0018457	0.00347716	0.00163146
RF0121	0.00639731	0.00334637	-0.00305094	RF0136	0.0018457	0.00347716	0.00163146
RF0060	0.00639731	0.00334637	-0.00305094	RF0107	0.0018457	0.00347716	0.00163146

Bulk				Rhizosphere			
Nodes	**EW-**	**EW+**	**DBC***	**Nodes**	**EW-**	**EW+**	**DBC**
RF0035	0.00639731	0.00334637	-0.00305094	RF0127	0.0018457	0.00347716	0.00163146
RF0154	0.00639731	0.00334637	-0.00305094	RF0171	0.0018457	0.00347716	0.00163146
RF0115	0.00639731	0.00334637	-0.00305094	RF0095	0.0018457	0.00347716	0.00163146
RF0008	0.00639731	0.00334637	-0.00305094	RF0154	0.0018457	0.00347716	0.00163146
RF0011	0.00639731	0.00334637	-0.00305094	RF0102	0.0093617	0.00431619	-0.00504551
RF0165	0.00639731	0.00334637	-0.00305094	RF0060	0.0093617	0.00431619	-0.00504551
RF0023	0.00639731	0.00334637	-0.00305094	RF0090	0.0093617	0.00431619	-0.00504551
RF0140	0.00695187	0.00334637	-0.0036055	RF0010	0.0093617	0.00431619	-0.00504551
RF0141	0.00695187	0.00334637	-0.0036055	RF0017	0.0093617	0.00431619	-0.00504551
RF0145	0.00695187	0.00334637	-0.0036055	RF0112	0.0093617	0.00431619	-0.00504551
RF0077	0.00695187	0.00334637	-0.0036055	RF0093	0.0093617	0.00431619	-0.00504551
RF0100	0.00695187	0.00334637	-0.0036055	RF0089	0.0093617	0.00431619	-0.00504551
RF0156	0.00695187	0.00334637	-0.0036055	RF0156	0.0093617	0.00431619	-0.00504551
RF0135	0.00695187	0.00334637	-0.0036055	RF0148	0.0093617	0.00431619	-0.00504551
RF0086	0.00695187	0.00334637	-0.0036055	RF0032	0.0093617	0.00431619	-0.00504551
RF0155	0.00695187	0.00334637	-0.0036055	RF0113	0.0093617	0.00431619	-0.00504551
RF0007	0.00695187	0.00334637	-0.0036055	RF0024	0.0093617	0.00431619	-0.00504551
RF0107	0.00695187	0.00334637	-0.0036055	RF0165	0.0093617	0.00431619	-0.00504551
RF0010	0.00695187	0.00334637	-0.0036055	RF0077	0.0093617	0.00347716	-0.00588454
RF0036	0.00695187	0.00334637	-0.0036055	RF0085	0.0093617	0.00347716	-0.00588454
RF0136	0.00695187	0.00334637	-0.0036055	RF0147	0.0093617	0.00347716	-0.00588454
Ktedonobacteria	0.02773109	0.02257873	-0.00515236	RF0135	0.0093617	0.00347716	-0.00588454
Actinobacteria	0.02773109	0.02257873	-0.00515236	RF0155	0.0093617	0.00347716	-0.00588454
bacterium-UASB14	0.02773109	0.02257873	-0.00515236	RF0142	0.0093617	0.00347716	-0.00588454
uncultured-bacterium-70	0.02773109	0.02257873	-0.00515236	RF0008	0.0093617	0.00347716	-0.00588454
uncultured-bacterium	0.02773109	0.02257873	-0.00515236	RF0074	0.0093617	0.00347716	-0.00588454
uncultured-bacterium-BAC10-10	0.02773109	0.01902928	-0.00870181	bacterium-UASB14	0.05315615	0.0416074	-0.01154875
uncultured-bacterium-5G12	0.02773109	0.01902928	-0.00870181	Thermomicrobia	0.05315615	0.0416074	-0.01154875

Bulk				Rhizosphere			
Nodes	**EW-**	**EW+**	**DBC***	**Nodes**	**EW-**	**EW+**	**DBC**
Candidatus-Saccharibacteria	0.02773109	0.01902928	-0.00870181	bacterium-JKG1	0.05315615	0.01866826	-0.03448789
bacterium-SM23-31	0.02773109	0.01902928	-0.00870181	Chloroflexia	0.05315615	0.01866826	-0.03448789
uncultured-bacterium-lac193	0.02773109	0.01902928	-0.00870181	Rhodospirillales	0.05315615	0.01866826	-0.03448789
unclassified-Planctomycetes	0.02773109	0	-0.02773109	uncultured-Acidobacteria	0.05315615	0.01866826	-0.03448789
Solibacterales	0.09064039	0.04377104	-0.04686935	Solibacterales	0.05315615	0.01866826	-0.03448789
Thermomicrobia	0.09064039	0.02257873	-0.06806166	Acidimicrobiia	0.05315615	0.01571429	-0.03744186
Chloroflexia	0.09064039	0.02257873	-0.06806166	Cyanobacteria	0.05315615	0.01571429	-0.03744186
uncultured-bacterium-lac127	0.09064039	0.02257873	-0.06806166	Bacilli	0.05315615	0.01571429	-0.03744186
Rhodospirillales	0.09064039	0.01902928	-0.07161111	bacterium-SM23-57	0.05315615	0	-0.05315615
uncultured-bacterium-W5-102b	0.09064039	0.01902928	-0.07161111	uncultured-bacterium-lac127	0.25735294	0.0416074	-0.21574554
Bacilli	0.09064039	0.01902928	-0.07161111	uncultured-bacterium-lac121	0.25735294	0.01866826	-0.23868468
				Sphingomonadales	0.25735294	0.01866826	-0.23868468

*DBC = differential betweenness centrality (DBC = $n\mathrm{BC}^{EW+} - n\mathrm{BC}^{EW-}$).

Table 3.3 – Betweenness centrality determined in the network models with (EW+) and without earthworms (EW-).

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
Bacilli	0.09064039	Xanthomonadales	0.04377104	uncultured-bacterium-lac121	0.25735294	uncultured-bacterium-70	0.0416074
Chloroflexia	0.09064039	uncultured-bacterium-lac160	0.04377104	uncultured-bacterium-lac127	0.25735294	Alteromonadales	0.0416074
Rhodospirillales	0.09064039	uncultured-bacterium-'To-T-020-P12'	0.04377104	Sphingomonadales	0.25735294	uncultured-bacterium-lac127	0.0416074
Solibacterales	0.09064039	uncultured-archaeon	0.04377104	bacterium-JKG1	0.05315615	uncultured-'To-T-020-P12'	0.0416074
Thermomicrobia	0.09064039	Solibacterales	0.04377104	bacterium-UASB14	0.05315615	bacterium-YEK0313	0.0416074
uncultured-bacterium-lac127	0.09064039	environmental-Crenarchaeota>	0.04377104	Acidimicrobiia	0.05315615	Deltaproteobacteria	0.0416074
uncultured-bacterium-W5-102b	0.09064039	bacterium-YEK0313	0.04377104	Cyanobacteria	0.05315615	unclassified-Planctomycetes	0.0416074
Acidimicrobiia	0.02773109	Anaerolineae	0.04377104	Chloroflexia	0.05315615	Planctomycetia	0.0416074
Actinobacteria	0.02773109	Acidimicrobiia	0.04377104	Bacilli	0.05315615	Thermomicrobia	0.0416074
Anaerolineae	0.02773109	uncultured-bacterium-lac127	0.02257873	Rhodospirillales	0.05315615	bacterium-UASB14	0.0416074
bacterium-SM23-31	0.02773109	uncultured-bacterium-lac121	0.02257873	Thermomicrobia	0.05315615	Candidatus-Saccharibacteria	0.0416074
bacterium-UASB14	0.02773109	uncultured-bacterium-70	0.02257873	uncultured-Acidobacteria	0.05315615	Xanthomonadales	0.0416074
bacterium-YEK0313	0.02773109	uncultured-bacterium	0.02257873	bacterium-SM23-57	0.05315615	RF0013	0.02527778
Candidatus-Saccharibacteria	0.02773109	uncultured-Acidobacteria-p2H8	0.02257873	Solibacterales	0.05315615	RF0039	0.02527778
Ktedonobacteria	0.02773109	Thermomicrobia	0.02257873	Deltaproteobacteria	0.01206612	RF0163	0.02527778
unclassified-Planctomycetes	0.02773109	Ktedonobacteria	0.02257873	uncultured-bacterium-F39-01	0.01206612	RF0016	0.02527778
uncultured-bacterium	0.02773109	Clostridia	0.02257873	Anaerolineae	0.01206612	RF0018	0.02527778
uncultured-bacterium-5G12	0.02773109	Chloroflexia	0.02257873	Candidatus-Saccharibacteria	0.01206612	RF0025	0.02527778
uncultured-bacterium-70	0.02773109	Candidatus-Entotheonella	0.02257873	uncultured-Acidobacteria- p2H8	0.01206612	RF0088	0.02527778
uncultured-bacterium-BAC10-10	0.02773109	bacterium-UASB14	0.02257873	Clostridia	0.01206612	RF0100	0.02527778
uncultured-bacterium-lac193	0.02773109	bacterium-SM23-57	0.02257873	uncultured-marine-bacterium-105	0.01206612	RF0134	0.02527778
bacterium-JKG1	0.01866029	bacterium-JKG1	0.02257873	uncultured-bacterium	0.01206612	RF0021	0.02527778
bacterium-UASB270	0.01866029	Alteromonadales	0.02257873	bacterium-YEK0313	0.01206612	RF0079	0.02527778
Bacteroidetes	0.01866029	Actinobacteria	0.02257873	Rhizobiales	0.01206612	RF0035	0.02527778
Burkholderiales	0.01866029	uncultured-marine-bacterium-105	0.01902928	Deinococci	0.01206612	uncultured-Acidobacteria	0.01866826

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
Candidatus-Entotheonella	0.01866029	uncultured-bacterium-W5-102b	0.01902928	uncultured-bacterium-'To-T-020-P12'	0.01206612	Sphingomonadales	0.01866826
Clostridia	0.01866029	uncultured-bacterium-lac193	0.01902928	Burkholderiales	0.01206612	bacterium-UASB270	0.01866826
Cyanobacteria	0.01866029	uncultured-bacterium-F39-01	0.01902928	uncultured-bacterium-5G12	0.01206612	uncultured-bacterium-lac160	0.01866826
Deinococci	0.01866029	uncultured-BAC10-4	0.01902928	environmental-Crenarchaeota>	0.01206612	bacterium-JKG1	0.01866826
Deltaproteobacteria	0.01866029	uncultured -BAC10-10	0.01902928	Bacteroidetes	0.01206612	uncultured-Acidobacteria-p2H8	0.01866826
environmental-samples-<Crenarchaeota>	0.01866029	uncultured-bacterium-5G12	0.01902928	Actinobacteria	0.01206612	uncultured-Rifle-16ft-37862	0.01866826
Rhizobiales	0.01866029	uncultured-bacterium-270	0.01902928	uncultured-bacterium-lac160	0.01206612	uncultured-bacterium-5G12	0.01866826
Sphingomonadales	0.01866029	uncultured-Acidobacteria-bacterium	0.01902928	Candidatus-Entotheonella	0.01206612	uncultured-bacterium-105	0.01866826
uncultured-Acidobacteria-bacterium	0.01866029	Sphingomonadales	0.01902928	uncultured-bacterium-70	0.01206612	uncultured-BAC10-10	0.01866826
uncultured-Acidobacteria-p2H8	0.01866029	Rhodospirillales	0.01902928	Ktedonobacteria	0.01206612	Rhodospirillales	0.01866826
uncultured-archaeon	0.01866029	Rhizobiales	0.01902928	uncultured-bacterium-BAC10-10	0.01206612	Environmental-Crenarchaeota>	0.01866826
uncultured-bacterium-'To-T-020-P12'	0.01866029	Planctomycetia	0.01902928	bacterium-UASB270	0.01206612	Chloroflexia	0.01866826
uncultured-bacterium-lac121	0.01866029	Deltaproteobacteria	0.01902928	RF0088	0.0093617	Solibacterales	0.01866826
uncultured-bacterium-lac160	0.01866029	Deinococci	0.01902928	RF0102	0.0093617	Clostridia	0.01866826
uncultured-marine-bacterium-105	0.01866029	Cyanobacteria	0.01902928	RF0060	0.0093617	uncultured-bacterium-lac121	0.01866826
RF0007	0.00695187	Candidatus-Saccharibacteria	0.01902928	RF0077	0.0093617	uncultured-bacterium	0.01866826
RF0010	0.00695187	Burkholderiales	0.01902928	RF0090	0.0093617	uncultured-bacterium-66	0.01866826
RF0014	0.00695187	Bacteroidetes	0.01902928	RF0010	0.0093617	uncultured-bacterium-lac193	0.01866826
RF0017	0.00695187	bacterium-UASB270	0.01902928	RF0017	0.0093617	Deinococci	0.01866826
RF0036	0.00695187	bacterium-SM23-31	0.01902928	RF0085	0.0093617	Burkholderiales	0.01866826
RF0077	0.00695187	Bacilli	0.01902928	RF0112	0.0093617	uncultured-Acidobacteria-A12	0.01571429
RF0086	0.00695187	RF0164	0.01113173	RF0093	0.0093617	bacterium-SM23-31	0.01571429
RF0088	0.00695187	RF0144	0.01113173	RF0039	0.0093617	Ktedonobacteria	0.01571429
RF0094	0.00695187	RF0134	0.01113173	RF0147	0.0093617	Bacteroidetes	0.01571429
RF0100	0.00695187	RF0112	0.01113173	RF0089	0.0093617	Actinobacteria	0.01571429
RF0107	0.00695187	RF0103	0.01113173	RF0156	0.0093617	uncultured-W5-102b	0.01571429
RF0135	0.00695187	RF0095	0.01113173	RF0148	0.0093617	Acidimicrobiia	0.01571429
RF0136	0.00695187	RF0093	0.01113173	RF0135	0.0093617	uncultured-archaeon	0.01571429

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
RF0140	0.00695187	RF0092	0.01113173	RF0155	0.0093617	Anaerolineae	0.01571429
RF0141	0.00695187	RF0079	0.01113173	RF0032	0.0093617	Cyanobacteria	0.01571429
RF0142	0.00695187	RF0074	0.01113173	RF0079	0.0093617	Bacilli	0.01571429
RF0143	0.00695187	RF0054	0.01113173	RF0142	0.0093617	uncultured-bacterium-F39-01	0.01571429
RF0144	0.00695187	RF0017	0.01113173	RF0113	0.0093617	Candidatus-Entotheonella	0.01571429
RF0145	0.00695187	RF0012	0.01113173	RF0008	0.0093617	Rhizobiales	0.01571429
RF0147	0.00695187	RF0009	0.01113173	RF0024	0.0093617	RF0048	0.00431619
RF0155	0.00695187	RF0171	0.00935829	RF0165	0.0093617	RF0024	0.00431619
RF0156	0.00695187	RF0148	0.00935829	RF0074	0.0093617	RF0112	0.00431619
RF0008	0.00639731	RF0147	0.00935829	RF0036	0.0018457	RF0023	0.00431619
RF0011	0.00639731	RF0143	0.00935829	RF0164	0.0018457	RF0153	0.00431619
RF0012	0.00639731	RF0142	0.00935829	RF0013	0.0018457	RF0102	0.00431619
RF0022	0.00639731	RF0127	0.00935829	RF0012	0.0018457	RF0032	0.00431619
RF0023	0.00639731	RF0113	0.00935829	RF0100	0.0018457	RF0011	0.00431619
RF0025	0.00639731	RF0102	0.00935829	RF0121	0.0018457	RF0065	0.00431619
RF0035	0.00639731	RF0094	0.00935829	RF0103	0.0018457	RF0148	0.00431619
RF0038	0.00639731	RF0089	0.00935829	RF0106	0.0018457	RF0156	0.00431619
RF0039	0.00639731	RF0088	0.00935829	RF0038	0.0018457	RF0094	0.00431619
RF0048	0.00639731	RF0065	0.00935829	RF0021	0.0018457	RF0090	0.00431619
RF0060	0.00639731	RF0050	0.00935829	RF0009	0.0018457	RF0115	0.00431619
RF0065	0.00639731	RF0039	0.00935829	RF0067	0.0018457	RF0168	0.00431619
RF0074	0.00639731	RF0038	0.00935829	RF0134	0.0018457	RF0020	0.00431619
RF0089	0.00639731	RF0025	0.00935829	RF0054	0.0018457	RF0017	0.00431619
RF0090	0.00639731	RF0024	0.00935829	RF0144	0.0018457	RF0121	0.00431619
RF0102	0.00639731	RF0022	0.00935829	RF0014	0.0018457	RF0050	0.00431619
RF0103	0.00639731	RF0016	0.00935829	RF0016	0.0018457	RF0104	0.00431619
RF0106	0.00639731	RF0014	0.00935829	RF0153	0.0018457	RF0103	0.00431619
RF0112	0.00639731	RF0168	0.00334637	RF0104	0.0018457	RF0141	0.00431619

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
RF0115	0.00639731	RF0165	0.00334637	RF0094	0.0018457	RF0144	0.00431619
RF0121	0.00639731	RF0163	0.00334637	RF0035	0.0018457	RF0113	0.00431619
RF0134	0.00639731	RF0156	0.00334637	RF0143	0.0018457	RF0093	0.00431619
RF0148	0.00639731	RF0155	0.00334637	RF0136	0.0018457	RF0058	0.00431619
RF0154	0.00639731	RF0154	0.00334637	RF0115	0.0018457	RF0125	0.00431619
RF0163	0.00639731	RF0153	0.00334637	RF0107	0.0018457	RF0165	0.00431619
RF0164	0.00639731	RF0145	0.00334637	RF0127	0.0018457	RF0089	0.00431619
RF0165	0.00639731	RF0141	0.00334637	RF0025	0.0018457	RF0014	0.00431619
RF0009	0.00201149	RF0140	0.00334637	RF0125	0.0018457	RF0060	0.00431619
RF0013	0.00201149	RF0136	0.00334637	RF0171	0.0018457	RF0143	0.00431619
RF0016	0.00201149	RF0135	0.00334637	RF0023	0.0018457	RF0010	0.00431619
RF0018	0.00201149	RF0125	0.00334637	RF0095	0.0018457	RF0067	0.00431619
RF0020	0.00201149	RF0121	0.00334637	RF0058	0.0018457	RF0107	0.00347716
RF0021	0.00201149	RF0115	0.00334637	RF0154	0.0018457	RF0077	0.00347716
RF0024	0.00201149	RF0107	0.00334637	RF0086	0.00147059	RF0054	0.00347716
RF0032	0.00201149	RF0106	0.00334637	RF0007	0.00147059	RF0009	0.00347716
RF0050	0.00201149	RF0104	0.00334637	RF0048	0.00147059	RF0095	0.00347716
RF0054	0.00201149	RF0100	0.00334637	RF0018	0.00147059	RF0145	0.00347716
RF0058	0.00201149	RF0090	0.00334637	RF0020	0.00147059	RF0086	0.00347716
RF0067	0.00201149	RF0086	0.00334637	RF0011	0.00147059	RF0155	0.00347716
RF0079	0.00201149	RF0085	0.00334637	RF0168	0.00147059	RF0135	0.00347716
RF0085	0.00201149	RF0077	0.00334637	RF0145	0.00147059	RF0142	0.00347716
RF0092	0.00201149	RF0067	0.00334637	RF0163	0.00147059	RF0038	0.00347716
RF0093	0.00201149	RF0060	0.00334637	RF0141	0.00147059	RF0074	0.00347716
RF0095	0.00201149	RF0058	0.00334637	RF0022	0.00147059	RF0106	0.00347716
RF0104	0.00201149	RF0048	0.00334637	RF0050	0.00147059	RF0147	0.00347716
RF0113	0.00201149	RF0036	0.00334637	RF0140	0.00147059	RF0007	0.00347716
RF0125	0.00201149	RF0035	0.00334637	RF0092	0.00147059	RF0085	0.00347716

Bulk				Rhizosphere			
EW-		EW+		EW-		EW+	
RF0127	0.00201149	RF0032	0.00334637	RF0065	0.00147059	RF0136	0.00347716
RF0153	0.00201149	RF0023	0.00334637			RF0036	0.00347716
RF0168	0.00201149	RF0021	0.00334637			RF0171	0.00347716
RF0171	0.00201149	RF0020	0.00334637			RF0012	0.00347716
		RF0018	0.00334637			RF0022	0.00347716
		RF0013	0.00334637			RF0092	0.00347716
		RF0011	0.00334637			RF0164	0.00347716
		RF0010	0.00334637			RF0154	0.00347716
		RF0008	0.00334637			RF0008	0.00347716
		RF0007	0.00334637			RF0140	0.00347716
						RF0127	0.00347716