Coal mining practices reduce the microbial biomass, richness and diversity of soil

*Patricia Dorr de Quadros^{1,2}, Kateryna Zhalnina¹, Austin G. Davis-Richardson¹, Jennifer C. Drew¹, Fátima B. Menezes³, Flávio A. de O. Camargo⁴, Eric W. Triplett¹

¹ Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA

² Department of Agricultural and Environmental Microbiology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

³ Department of Microbiology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

⁴ Department of Soil Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

* Corresponding author:

Department of Agricultural and Environmental Microbiology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

patiquadros11@yahoo.com.br

Phone: (51) 9545.1975, (51) 3239.8421

Abstract

Changes within the environment by mining processes include increased soil acidification, compaction, erosion, and air and water pollution. As it affects directly the soil microbial community, it may trigger severe impact on the biogeochemical cycles. The objectives of this study were to evaluate how the soil microbial community changes over time after mining and soil reconstruction at the largest coal mine in Latin America. To address this question, we compared soils from undisturbed native forest and grassland to five different sites at various stages of recovery post coal mining. We compared and correlated the soils' chemical features to the soil microbial biomass, soil enzyme activity, and prokaryotic phylogeny and diversity. 42 samples were collected from the seven sites and the V4 region of the 16S rRNA gene was sequenced on the Illumina HiSeq platform. Reads were classified to identify 525 genera from Bacteria and Archaea domains. The composition of the microbial community of post-mined sites was significantly different from the undisturbed sites. Soil from the reconstructed sites showed a significantly lower microbial diversity and biomass, and lower activity of the dehydrogenase and β -glucosidase enzymes when compared to undisturbed soils. The most abundant genera found in the reconstructed soils were *Thiobacillus*, *Sphingomonas*, *Novosphingobium*, *Acinetobacter*, and *Variovorax*. *Bradyrhizobium* was the most abundant genus in the undisturbed forest site, and *Bacillus* in the undisturbed grassland site. Microbial diversity increased as sites recuperated, but the microbial composition and abundance of post mined soils remained changed.

Keywords: microbial ecology, microbial diversity, coal mining, constructed soils, disturbed soils.

1. Introduction

Candiota, located in south Brazil, is the biggest coal mine in Latin America. The coal extracted from Candiota, an opencast mine, is situated within two adjacent layers that are 200 centimeters thick each constituting a rate of 4 cubed meters of coal "in situ" by square meter of land mine, a rare occurrence among the coals. Each year, 1.7 million tons of coal is extracted from its 3 billion ton reserve supplying the Property Union CGTEE Thermoelectric Plant President Médici, which generates 440 megawatts of energy (CGTEE, 2015).

Coal mining contributes to both the social and economic development of the region (Koppe and Costa, 2002); however, mining large areas of land results in drastic structural and biological changes, triggering many environmental concerns (Potter et al., 1988; Kampf et al., 2000; Sydnor and Redende, 2002; Wong, 2003; Sheoran et al., 2008; Chaubey et al., 2012). After the coal is mined, the mining companies are obligated to restore the landscape in order to avoid land degradation in a process called soil reconstruction. This process includes reallocation of the original soil layers over the craters followed by the stabilization of the soil chemical properties and then reestablishment of vegetation. The final form is, called 'reconstructed soil' (Koppe and Costa, 2002; Gaivizzo et al., 2002). Typically, after mining, the soil surface layers become contaminated with pyrite (FeS_2) that later oxidizes to sulphuric acid when exposed to oxygen and water, which results in high levels of soil acidification (Fanning and Fanning, 1989). The loss of soil structure, decrease of organic matter, increase in bulk density ($\text{BD} > 1.6 \text{ g cm}^{-3}$), and low rate of water infiltration into the soil ($R < 5 \text{ cm h}^{-1}$), leads to runoff and soil erosion (Kampf et al., 1997; Guebert and Gardner, 2001). Minimizing environmental damages when forming a constructed soil is of utmost importance. However, without strict implementation of the landscape reconstruction procedure and soil management, soil and biome degradation is often

1 irreversible with substantial loss of microbial and plant diversity.

2 Microbial activity is a key factor affecting the functioning of all terrestrial systems. The
3 microbial functional stability increases with the availability of soil resources (McGuire and
4 Treseder 2010; Shade et al., 2012; Griffiths and Philippot, 2013; Pagaling et al., 2013; Tardy et
5 al., 2014). The soil organic matter (SOM) is a soil resource that nourishes the microbial
6 community and its content is usually deficient in post-mined soils, due the high disturbance of
7 the topsoil. It may decrease the microbial diversity, as well the soil functional stability. Predictive
8 maps of global microbial diversity have being generated by integrating environmental data and
9 molecular analyses of the bacterial community (Larsen et al., 2012; Fierer and Ladau, 2012), but
10 the resilience of soil microbial diversity following disturbance remain unknown.

11 We hypothesized that the soil microbial community from post-mined soils may present
12 different composition and diversity comparing to the original microbial community. Than, in this
13 study we compared the soil microbial community of disturbed (after coal mining processes) and
14 undisturbed (native forest and grassland) soils from the same edaphoclimatic area. We used
15 multiple approaches, such as 16S rRNA next generation sequencing (NGS), quantification of
16 microbial biomass and enzymatic activity, and soil chemical feature analysis to assess the overall
17 processes occurring within the soils. Our goal was to detect the magnitude of these changes
18 within the soil microbial community in Candiota's coal mine, Brazil.

20 **2. Materials and Methods**

21 **2.1 Environmental characterization**

22 The studied area is used by the mining company Rio Grande do Sul (CRM), and it is
23 located in Candiota at -31°33'55"S and -53°43'30"W with an altitude of 230 m. According to
24 Köppen climate classification, the region has a humid subtropical climate. The average annual

1 temperature is 17.2 °C, and the average annual rainfall is 1,262 mm. The soil is classified as an
2 Acrisol according to the FAO system (Driessen et al., 2001). After the coal extraction, the mining
3 company restored the landscape, replacing the soil layers that were previously removed, included
4 A horizon) and applying limestone to raise the pH to 6.0. To complete the process, they seeded a
5 blend of grasses on the site. In this study, forest and grassland areas were treated as controls, and
6 sites where mining had occurred were treated as the experimental condition.

8 **2.2 Sampling**

9 The samples were collected in the Companhia Rio-Grandense de Mineração-CRM, Brazil
10 that has a total area of 20,000 ha. The 42 individual soil samples (seven sites x six replicates
11 each) represent five disturbed sites (after coal mining) with different ages of restoration: 3, 4, 5, 6
12 and 19 years of reconstruction, and two sites of undisturbed soils (controls, from the same
13 geographic region): native grassland (with diverse native grasses and legumes composing the
14 plant community), and native forest (subtropical vegetation with 2 to 5 m trees), both preserved
15 sites located from less than 1,000 m of the coal mine. Each of the 42 samples is a composite of
16 ten subsamples (collected within 0.5 ha of each site) pulled from 1-10 centimeters deep after the
17 superficial vegetation was removed.

19 **2.3 Chemical and physical characterization**

20 A neutral solution of KCl mol L⁻¹ was used to extract Calcium (Ca), Magnesium (Mg),
21 and Aluminum (Al). Levels of Ca and Mg were determined by atomic absorption
22 spectrophotometry, and Al concentration was determined by neutralization with Sodium
23 hydroxide (NaOH) titration (Tedesco et al., 1995). Potassium was extracted by using solution of
24 Sulfuric acid 0.05 mol L⁻¹ + HCl 0.05 mol L⁻¹ and determined by flame photometry (Tedesco et

al., 1995). Total acidity (H + Al) was extracted using calcium acetate solution ($0.5 \text{ mol}^{-1} \text{ L}$) buffered to pH 7.0 and quantified by titration with NaOH neutralization (Tedesco et al., 1995). The sum of bases (S), base saturation (V%), and cation exchange capacity (CEC) at pH 7.0, were than calculated. Organic carbon was analyzed according to Tedesco et al. (1995). Cadmium (Cd), Copper (Cu), Nickel (Ni), and Lead (Pb) were extracted using H_2SO_4 0.05 mol L^{-1} + HCl 0.05 mol L^{-1} solution, and their concentrations were determined by plasma spectrometry according to Tedesco et al. (1995). The pH levels were determined in water and KCl mol L^{-1} with potentiometer.

2.4 Microbial biomass C, respiration C, and enzymatic characterization of the soil

Carbon (C) from the microbial biomass was extracted using the chloroform fumigation and extraction method (Vance et al., 1987). For this, aliquots of 25g of soil (dry weight based) were subjected to 48 h fumigation with chloroform in a glass desiccator. Triplicates of each sample from fumigated and non-fumigated soils were shaken for 1h at 180 strokes per minute with 0.5 M of K_2SO_4 at a ratio of 5:1 (weight of extractant to dry soil weight) and filtered. Soluble carbon levels were determined for both fumigated and non-fumigated soils. Dissolved organic carbon (DOC) from the soil solution was extracted through potassium dichromate digestion, following the Jenkinson and Polson method (1976). The amount of soluble C from the fumigated and non-fumigated soil extracts was calculated using the following equation:

$$[\text{Biomass C} = (\text{C}_f - \text{C}_{\text{nf}})/\text{K}_{\text{ec}}]$$

where C_f is C in fumigated extract, C_{nf} is C in non-fumigated extract, and K_{ec} is the proportion of the microbial C that is extracted from the soil.

The level of C from microbial respiration was estimated by measuring the amount of CO_2 released from the soil during 40 days of incubation in accordance to Stotzky and Pramer's

methodology (1972). Three replicates of 200g from each soil sample (dry weight based) were incubated for 40 days at 25°C in closed, airtight 1L bottles with small beakers containing NaOH 0.5 N solution, which retains the CO₂ resulting from microbial biological activity, through forming Na₂CO₃ in the solution. Every 5 days (totaling eight measurements), CO₂ from the solution was measured. For this, 10 ml of 0.2 N BaCl₂ was added to the beakers containing NaOH in order to precipitate the carbonate and avoid its interference with the titration. Titration with HCl was performed using phenolphthalein as an indicator. Metabolic coefficient was determined according to Anderson and Domsch (1993). The concentrations of β-glucosidase and dehydrogenase enzymes were evaluated in the soil samples according to Tabatabai (1994).

2.5 DNA extraction and PCR

For each sample, DNA was extracted from 0.8 g of soil using the MoBio PowerSoil™ DNA Isolation Kit (Carlsbad, CA, USA) in accordance with the manufacturer's protocol. All genomic DNA concentration and purity levels were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). The primers 515F and 806R (Caporaso et al., 2010) were used to target the V4 region (*length*, ca. 250 bp) of the 16S rRNA gene with the addition of a barcoded sequence and the required Illumina adapters. PCR was performed at an initial denaturation temperature of 94°C for 3 min, followed by 20 cycles of 94°C for 45 sec, 53°C for 30 sec, and 65°C for 90 sec. A final elongation step at 65°C was run for 10 minutes. PCR products were purified using the Qiagen™ PCR purification kit following the manufacturer's protocol with the exception of eluting in sterile water (Qiagen, Valencia, CA, USA). The samples were quantified in the Qubit 2.0 Fluorometer (Invitrogen, NY, USA).

2.6 Illumina HiSeq sequencing of 16S rRNA genes

The sequencing was performed on an Illumina GAIIx (Illumina, Inc., CA, USA) with two paired-end read cycles. The sequence analysis and the OTUs identification were based on the methods of Giongo et al. (2010) and Fagen et al. (2012). The reads were trimmed to remove low quality bases and to remove the first 11 bases corresponding to the primer region by a script based on Trim2 (Huang et al., 2003; Trim2_Illumina.pl. Available online: <https://gist.github.com/1006830>). Reads were filtered by quality using 70% as the minimum percentage of bases with good quality, with a score of 20 as the minimum quality score in Phred + 33 encoding. Paired reads were mapped using CLC Assembly Cell v3.0.2b (CLC Bio, QIAGEN) to the reference Ribosomal Database Project (RDP). Full taxonomic descriptions based on the NCBI taxonomy database (National Center for Biotechnology Information–NCBI, available online: <http://www.ncbi.nlm.nih.gov>) were generated for the entries in the RDP database using TaxCollector (Giongo et al., 2010). Matches were filtered at 80% length fraction and classified at least 80% (domain and phylum), 90% (class, order and family), 95% (genus), and 99% (species) similarity. The total number of paired reads matching 16S rRNA sequences in the database at each level of similarity created an OTU abundance matrix for each taxonomic rank across each sample. Paired reads that did not match to the same sequence in the RDP database were annotated according to their Last Common Ancestor (LCA), and paired reads that did not have an LCA, or any match in the RDP database, were considered to be unclassified. To normalize for varying sequencing depths, the OTU abundance matrices for each sample were divided by the total number of pairs after trimming.

2.7 Statistical analysis

The Tukey test, PCA, and Spearman correlations were performed using XLSTAT-Pro 2014 (Addinsoft's core software). To integrate Spearman correlations analysis, only the OTUs

with relative abundance equal or higher than 0.05% of total reads (cut-off) were used. Microbial diversity was estimated for each sample to genus level, using the Shannon diversity index (SDI).

3. Results

The undisturbed forest soil presented twice the clay content, twice the organic matter content, and about seven-fold higher cation exchange capacity (CEC) than the post-mined and grassland soil (Table 1). Soil from three year-old plots showed the highest S concentration.

3.1 Soil biological characterization

The soils from three to six year-old showed the lowest microbial biomass (Figure 1a-b). The forest soil showed the highest microbial biomass, as well as the highest OM content, followed by the Grassland and 19 year-old soil (Figure 1a). All reconstructed soils showed lower microbial respiration rates than the undisturbed soil (Figure 1b). The five year-old soil showed the highest metabolic coefficient (qCO_2) indicating that the microorganisms in those samples have a lower efficiency of C metabolism. qCO_2 is an index that express the rate of microbial respiration per unit of microbial biomass. Than, the smaller the loss of C- CO_2 for tissue incorporation means greater efficiency, and consequently, the lower the qCO_2 .

In the constructed soils, microbial biomass was shown to gradually increase over time; however, it did not return to the original levels, suggesting irreversible loss of microorganisms due to the mining practices.

The soil enzymes β -glucosidase and dehydrogenase were also evaluated in these soils, and the results are represented in the Figures 2a-b. The β -glycosidase enzyme assists in the hydrolysis of glycosidic bonds in complex sugars, such as cellulose and hemicellulose. The activity of this enzyme was greatest in the forest soil compared to the other sites. The

dehydrogenase enzyme activity in the soil reflects the total oxidative activity of the microbes, and its concentration was equally high on six and 19 year old constructed soils, as well as in the forest and grassland soils, indicating that the activity of the microbial community increases with time since disturbance.

3.2 Diversity of microbial community

The microbial diversity index was performed to the genus level (Figure 3). As expected, lower microbial diversity was found in the constructed soils, but diversity increased with time since the soil reconstruction. The soils from the native forest and grassland showed significantly higher microbial diversity compared to the reconstructed soils. The soil with the highest diversity was the forest soil, and the lowest microbial diversity was detected in the four year-old soil.

3.3 Composition and relative abundance of the microbial community

Over twenty million 16S rRNA reads (20,516,155) were obtained with Illumina sequencing from all constructed soils and the two control sites, which averaged 455,759 sequences per sample, after the trimming. Bacterial and Archaeal domains were distributed within 24 phyla, 46 classes, 95 orders, 191 families and 525 genera. The composition and relative abundance of Bacteria and Archaea among the samples varied at phylum level (Figure 4). In the three year-old soil, members from six different phyla were detected (Bacteroidetes, Firmicutes, Proteobacteria, Planctomycetes, Verrucomicrobia, and Actinobacteria). In samples from soils with four, five, six, and 19 years of reconstruction, members from eight to ten different phyla were detected. In the native forest and native grassland soils, members from 20 and 22 different phyla were detected, respectively.

In all studied soils, the most abundant phyla were Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia. Proteobacteria was the most well distributed and abundant phylum in constructed soils. The abundance of Verrucomicrobia increased with time while the abundance of Firmicutes decreased, indicating some succession pattern. The archaeal phylum Crenarchaeota was detected only in the constructed soils. Crenarchaeota are very metabolically diverse and grow in extreme environmental conditions such as a high temperature and salinity. Euryarchaeota, Deinococcus-Thermus, Chlorophyta, Spirochaetes, Synergistetes, Tenericutes, Bacillariophyta, Cyanobacteria, Elusimicrobia, Nitrospirae, Euglenida, and Fusobacteria phyla were detected only in forest and grassland soils.

Firmicutes members were more abundant (Tukey test, $\alpha < 0.01$) in soils with 3 and 4 years after soil reconstruction. It was possible to detect a gradient increase of Firmicutes in reconstructed soil when compared to the undisturbed soils. Verrucomicrobia was more abundant in Grassland soil. Bacteroidetes was highly abundant on reconstructed soils.

To visualize overall microbial community differences between soil types, PCA analysis was performed to genus-level data using the 20 most abundant taxa, soils' enzymes' activity and soil chemical features through PCA analysis (Figure 5). The contribution of each observation was grouped within F1 and F2 components (largest square cosines). Genera found in forest and grassland soils were correlated and clustered with microbial diversity, dehydrogenase and β -glycosidase activities, CO₂-C microbial respiration, CEC, and OM.

The genus *Novosphingobium* clustered with Cr concentration in soil, and *Ralstonia* and *Bacteroidetes* clustered with Mg concentration, indicating that these microorganisms may use those metals as electron acceptors.

The Table 2 shows the 20 most abundant genera (only classified, with relative abundance higher than 0.1%) from each soil category. One category included genera from three, four, five,

1 and six-year old sites. The microorganisms from the 19 year-old reconstructed soil, the forest,
2 and the grassland, were grouped into independent categories. The results showed that the
3 microbial communities from reconstructed soil, forest and grassland soil are quite different. Only
4 *Sphingomonas* (Proteobacteria) was detected in all soils. *Thiobacillus* (Proteobacteria) was the
5 most abundant genus found in areas of three and six years old, with an average of 8.5% of the
6 total reads. *Bradyrhizobium* was the most abundant genus detected in forest site (6.4% of the
7 total reads) and second most abundant in grassland (4.7% of the total reads). *Bacillus* was the
8 most abundant genus found in grassland (4.8% of the total reads).

10 **4. Discussion**

11 In this study we used a combination of high throughput sequencing approaches along with
12 chemical, physical, and biological methods, in order to investigate microbial communities in
13 reconstructed soils after coal mining. We detected a higher microbial respiration and biomass,
14 and higher enzyme activity and microbial diversity in the undisturbed soils (native grassland and
15 forest) when compared to post-mined soils. Also, over time (from three to 19 years from soil
16 reconstruction after mining) the microbial biomass, enzyme activity, and even microbial diversity
17 have just slightly increased, and not reached the original level of the original land use – native
18 forest. The low SOM content in those disturbed soils is probably the main cause of this. During
19 the mining process the soil lost quality (lost physical structure, changed chemical features, lost
20 organic matter) and the original microbial biomass and diversity decreased. But we also detected
21 in parallel, throughout next generation sequencing, that microorganisms from rare biosphere
22 were recruited to maintain some basic soil function, once even in a base level we detected
23 microbial biomass and activity.

24 **4.1 Soil features**

1 The SOM was much lower in the constructed soils compared to the undisturbed soils, and
2 was also highly correlated with microbial diversity, microbial biomass, and respiration-C. After
3 soil undergoes different land uses, such as mining, conventional intensive agriculture, or even
4 overcrowding of animals in pasture, the soil system may degrade irreversibly without proper
5 human management, such as addition of nutrients and liming, consistent establishment of plants,
6 increasing the organic matter, use of conservative agricultural system (no-tillage), and erosion
7 control. It is known that SOM takes decades to increase with great management practices
8 (Grandy and Robertson, 2007; Beare, 2014; Liu et al., 2014; Castro et al. 2015; Liao et al., 2015).
9 SOM is responsible for biogeochemical processes and organic compound turnover. Also, SOM is
10 the major binding agent that aggregates soil particles and provides soil structuring (Oades, 1984;
11 Six et al., 1998). The interactions among SOM, plant roots, soil mesofauna, and microorganisms
12 helps to keep soil particles aggregated (Six et al., 2004). Additionally, soils with a high
13 percentage of organic matter can form complexes with toxic elements and metals reducing their
14 availability and toxicity in a chemical process called complexation (Cabaniss, 2009).

15 In the post-mined soil with deficient management and high erosion rates such as observed
16 in the studied post-mined sites, SOM decrease with time, and the management to increase SOM
17 is directly related to re-vegetation process, which is seriously deficient in the studied site. Once
18 the vegetation process is established, it carries out other soil structuring processes providing
19 organic matter to the soil (Grayston et al., 2004). Vegetation also contributes to the soil
20 microorganism community's stability and structure through the variety of roots that release
21 exudates with cementing action and carbon sources to the soil microbiota (Butler et al., 2003).
22 We observed an irreversible lost of microbial diversity with tentative of soil reconstruction in
23 Candiota mine, Brazil.

4.2 Microbial biomass and activity (CO₂ and enzymes)

We detected lower microbial biomass, enzymatic activity, and microbial diversity in the constructed soils when compared to forest soil (original land use). The microbial biomass is considered the most active and living part of organic matter and it is composed of microorganisms that participate in different soil's biogeochemical processes. During the mechanical processes of soil withdrawal, coal extraction, and soil layers' reallocation, the soil horizons are frequently inverted or mixed, which considerably reduces the A-horizon - that holds the organic matter and its associated microbiota. The environmental impact of coal mining causes severe physical, chemical, and biological disturbances (Dong and Liu, 2005; Shi and He, 2012). These disruptions alter the availability of nutrients and consequently affect the surrounding microbial ecosystems. Our study shows that, after the soil reconstruction, some of the chemical features of soil may be recovered, such as pH, as well as macro and micronutrients with liming and fertilization. However, the recovery of the SOM and its biological components is more complex, and microbial community re-establishment depends on time and physical stabilization throughout human management.

4.3. Composition and diversity of microbial communities

Lower microbial diversity was observed in the studied disturbed soils. Native soils have usually stable microbial communities, which have taken millions of years to form. These microbial communities play an important role in the regulation of the biogeochemical cycles (Torsvik and Ovreas, 2002; Hooper et al., 2005; Reed and Martiny, 2007; Prosser et al., 2007; Allison and Martiny, 2008; Keiser et al., 2011; Griffiths and Philippot et. al., 2013; Philippot et. al., 2013; Jurburg and Salles, 2015). On the other hand, anthropogenic soil disturbances (such as agriculture, mining, disposal of residues or waste, etc.) shift the ecosystem equilibrium forcing

1 microorganisms to adapt to the new habitat (Wohl et al., 2004; Pagaling et al., 2013; Jurburg and
2 Salles, 2015), and some of those post-disturbing microorganisms were detected in this work.
3 These microorganisms probably had arisen from the rare biosphere and although under lower
4 diversity and biomass, they are helping to maintain some basic ecological processes in the
5 changed environment.

6 In the forest soil (5.4% of SOM), the most abundant genus found was *Bradyrhizobium*, a
7 well-known nitrogen fixer. In the three years post-reconstruction soil (1.9% of SOM) the most
8 abundant genera detected was *Thiobacillus*, a well-known sulphur oxidizer, which is frequently
9 found in degraded soils (Singleton et al, 2012; Bosch et al., 2012). The three years post-
10 construction soil presented almost six fold more sulphur than in the forest soil. When this feature
11 is combined with the low SOM, the sulphur is released to the soil solution acidifying the soil.
12 This observation is consistent with the detection of a high abundance (8.5% of total reads) of the
13 sulphur-reducing bacteria *Thiobacillus*. In the control soils, the forest and grassland sites, which
14 presented with a higher percentage of SOM, a higher percentage of nitrogen-fixing
15 microorganisms, approximately 18.6% and 11.45% of total reads, was detected. These
16 microorganisms play an important role in the N cycling and in the SOM recycling. Furthermore,
17 the SOM has a capability of forming complexes with heavy metals and sulphur, which makes
18 them unavailable to the soil matrix complex. The coal mining process followed by the soil
19 construction usually reduces the SOM, which can facilitate the soil degradation. The loss of SOM
20 and, consequently, the decrease of the microbial population, affect the soil's physical restoration
21 as well as the re-vegetation process (Grayston et al., 2004). The SOM, the microorganisms, and
22 the plants form a very connected and dynamic system. If one of them is degraded in the soil, the
23 other two factors are affected also. For this reason, in order to avoid the soil degradation, the

constructed soils should be appropriately recovered and re-vegetated. Thus, over time, the soil gradually recovers its SOM content as well its physical and biological stability.

When we were comparing the twenty most abundant genera among the studied soils, we observed just a few genera in common among them. Between the forest and grassland soils' twenty most abundant genera, sixteen of them were shared. However, the constructed soils shared a different set of genera implying that constructed soils may provide different niches to host specific microbial communities that can persist in the changed environment. This result agrees with the chemical characterization of those soils. Genera with biodegradation and bioremediation capabilities were detected in soils from sites within three to six years after construction. The high content of pyrite and sulphur in recently constructed soils may explain the high abundance of microorganisms such as *Thiobacillus*, *Sphingomonas*, *Novosphingobium*, *Acinetobacter*, and *Variovorax*. These bacteria have enzymes that can degrade various compounds such as polycyclic aromatic hydrocarbons, sulphur (Prasada et al., 2014), and 2-metilphenanthrene (Nimatuzahroh et al., 1999). Yuanhu et al. (2012) isolated *Sphingomonas* from coal mine soil able to degrade ground tire rubber by desulfurization. This genus may be stimulated to grow in soil with coal residues and high sulphur content. The genus *Novosphingobium* is able to degrade aromatic compounds such as phenol, nitrobenzene, and phenanthrene (Sohn et al., 2004; Liu et al., 2005). *Acinetobacter* is able to grow in very high concentration of cadmium, and *Variovorax* tolerates high concentrations of heavy metals (Chien et al., 2008). We have observed a drastic decrease of microbial diversity and presence of the microbial taxa that are able to survive under the conditions after coal mining.

Soil is a non-renewable resource within the human life perspective. The capacity of an ecosystem to tolerate disturbance without collapsing should be observed, in order to preserve the ecosystem's biodiversity and avoid land degradation. The importance of finding suitable criteria

1 to assist land managers in decision-making and the implementation of corrective management
2 inputs is essential to the mining industry (Bardgett and Shine, 1999). We strongly suggest the
3 monitored re-vegetation in soils after mining, in order to increase the SOM and the soil stability
4 and quality, and we emphasize that just seeding the sites is not sufficiently, but the constant
5 supervising to guarantee the success of the ecosystem reclamation.

7 **4.1 Conclusions**

8 The disturbance caused by mining and, subsequently, soil construction drastically alters
9 the soil microbial community for at least 20 years. Although the pH levels, heavy metals
10 concentration, and macro/micronutrients were stabilized over the time period through the liming
11 and fertilization processes, and although microbial biomass and diversity increased with time
12 after soil restoration, those attributes have not reached the original levels that were found in the
13 forest and grassland ecosystems. The microbial community from disturbed soils is less complex
14 than that from undisturbed soils and presents a few dominant organisms. It is clear that the
15 characteristics of the microbial population are sensitive enough to differentiate the quality of
16 these soils.

17 In this paper we demonstrate a succession of the biological properties in a heavily
18 disturbed soil after coal mining process. In the time period over 20 years of restoration practices
19 altered ecosystem partially regained lost biological properties. However, the system did not reach
20 the level of the native soils (forest or grassland). Using the knowledge obtained in this study,
21 better restoration processes can be designed.

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Table 1 Chemical and physical characterization of the constructed soil samples, forest, and grassland soil samples. The numbers 3, 4, 5, 6, and 19, represent the years since mining of the reconstructed soils. The forest and grassland samples were taken from adjacent sites not subject to coal mining. Means that differ significantly are indicated by different lower-case letters (Tukey's test, $P < 0.01$).

Soil sample*	Clay	pH	$\text{Al}^{3+} + \text{H}^+$	P	K	OM	CEC	Sat CEC bases	Sat CEC Al^{3+} (m)
	%		$\text{cmol}_c/\text{dm}^3$	mg/dm^3	mg/dm^3	%	$\text{cmol}_c/\text{dm}^3$	%	%
3	35 b	4.7 b	13.7 c	7.4 b	82 bc	1.9c	16.2 c	15 d	51.6 b
4	29 b	5.6 a	3.9 d	4.4 c	85 bc	2.3 bc	11.1 d	65 b	0 d
5	25 b	5.6 a	3.1 d	4.1 c	119 a	2.6 b	18.1 c	83 a	0 d
6	18 c	4.1 c	21.8 b	6.8 b	79 c	2.4 b	22.7c	4 e	85.6 a
19	21 b	4.1 c	30.7 b	15.0 a	98 b	4.9 a	33.2 b	7 e	63.6 b
Forest	48 a	4.1 c	61.2 a	2.8 d	92 b	5.4 a	63.1 a	3 e	78.9 a
Grassland	22 b	4.9 b	6.2 cd	4.5 c	127 a	2.9 b	9.2 d	33 c	24.5 c

Soil sample	S	Zn	Cu	B	Mn	Cd	Ni	Cr	Pb
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/g	mg/kg	mg/kg	mg/kg
3	226 a	1.9 d	0.9 b	0.7	29 a	< 0.2	8 b	8 bc	4 c
4	18 e	0.7 e	0.1 b	1	15 b	< 0.2	6 b	7 bc	11 b
5	66 c	3.6 b	0.7 b	0.6	5 c	< 0.2	14 a	21 a	11 b
6	96 b	1.6 d	1.2 b	0.4	4 c	< 0.2	7 b	10 b	11 b
19	34 d	5.5 a	0.1 b	0.6	8 c	< 0.2	4 c	6 bc	9 a
Forest	40 d	2.2 c	4.4 a	1.2	12 b	< 0.2	4 c	4 c	8 a
Grassland	13 e	1.7 d	0.8 b	0.6	28 a	< 0.2	5 bc	4 c	10 a

**Quality reference value	-	60	35	-	-	< 0.5	13	40	17
**Prevention value	-	300	60	-	-	1.3	30	75	72
**Agriculture intervention	-	450	200	-	-	3	70	150	180

* Numbers in this column represent years after soil construction. Native forest and grassland are considered controls.

** Values of reference for heavy metals according the Technological Center of Sanitation from State of Sao Paulo (CETESB) (29), Brazil.

- $\text{Al}^{3+} + \text{H}^+$ = Potential acidity of the soil

- OM = organic matter content

- CEC = cation exchange capacity ($(\text{Ca}^{2+} + \text{Mg}^{2+} + \text{K}^+ + \text{Na}^+) + \text{H}^+ + \text{Al}^{3+}$)

- Sat CEC bases = saturation of CEC by changeable bases ($\text{Ca}^{2+} + \text{Mg}^{2+} + \text{K}^+ + \text{Na}^+$)

- Sat CEC Al = saturation of the CEC by Aluminum (m), where: $m < 1\%$ = very low; m between 1 and 10% = low; m between 10 and 20% = medium; $m > 20\%$ = high.

Table 2 Table showing the top 20 most abundant (all those relative abundance higher than 0.1%) genera (classified genera, in alphabetic order) from soils between three and six years old, 19 years old, forest and grassland. Values in grey show that the genus it is not between the 20 most abundant genera at the study site. Underlined values are the most abundant genera of that soil category.

Genus	Relative abundance of 16S rRNA (% of total reads)			
	3-6 years*	19 years	Forest	Grassland
<i>Acidovorax</i>	0.55	0.00	0.10	0.04
<i>Acinetobacter</i>	0.50	0.00	0.00	0.01
<i>Actinoallomurus</i>	0.00	0.00	0.76	0.68
<i>Actinomadura</i>	0.00	0.00	0.49	0.42
<i>Actinoplanes</i>	0.06	0.00	0.65	0.15
<i>Aetherobacter</i>	0.06	0.20	0.21	0.15
<i>Afipia</i>	0.45	0.60	0.08	0.05
<i>Bacillus</i>	0.00	0.00	5.74	4.79
<i>Bacteroides</i>	1.21	0.00	0.00	0.02
<i>Bradyrhizobium</i>	0.00	0.00	6.37	2.75
<i>Burkholderia</i>	0.25	0.40	1.59	1.08
Ca. <i>Koribacter</i>	0.06	1.19	0.45	0.56
Ca. <i>Solibacter</i>	0.00	0.70	0.17	0.19
<i>Chthoniobacter</i>	0.00	0.40	0.26	0.73
<i>Desulfuromonas</i>	1.15	0.00	0.00	0.00
<i>Devosia</i>	0.55	0.20	0.04	0.03
<i>Flavisolibacter</i>	0.50	0.30	0.22	0.15
<i>Flavobacterium</i>	0.42	0.10	0.05	0.42
<i>Gardnerella</i>	0.77	0.00	0.00	0.00
<i>Geobacter</i>	1.13	0.10	0.31	0.23
<i>Legionella</i>	0.06	0.20	0.05	0.09
<i>Leptothrix</i>	0.87	0.20	0.09	0.18
<i>Lysobacter</i>	0.00	0.40	0.03	0.01
<i>Mesorhizobium</i>	0.00	0.00	0.49	0.28
<i>Methylobacterium</i>	0.19	0.00	0.27	0.02
<i>Methylocystis</i>	0.74	0.00	0.00	0.00
<i>Micromonospora</i>	0.00	0.10	0.54	0.25
<i>Mucilaginibacter</i>	0.06	0.20	0.13	0.04
<i>Mycobacterium</i>	0.00	0.01	1.61	0.66
<i>Niastella</i>	0.00	0.30	0.06	0.07
<i>Nocardioides</i>	0.38	0.00	0.27	0.05
<i>Novosphingobium</i>	3.84	0.00	0.00	0.00
<i>Opitutus</i>	0.17	0.30	0.02	0.11
<i>Paenibacillus</i>	0.83	0.10	4.41	2.55
<i>Paucimonas</i>	0.00	0.00	0.33	0.00
<i>Pelomonas</i>	1.38	0.10	0.00	0.00
<i>Phenylobacterium</i>	0.00	0.50	0.56	0.37
<i>Planosporangium</i>	0.00	0.00	0.97	0.79
<i>Pseudomonas</i>	1.09	0.10	0.01	0.06
<i>Ralstonia</i>	1.02	0.00	0.04	0.03
<i>Rhodoplanes</i>	0.00	1.09	1.28	0.94
<i>Solobacterium</i>	1.15	0.00	0.00	0.00
<i>Sphingomonas</i>	3.24	0.79	2.72	2.27
<i>Steroidobacter</i>	0.17	0.50	0.02	0.02
<i>Streptomyces</i>	1.15	0.00	0.83	0.27
<i>Terrimonas</i>	0.00	0.30	0.07	0.11
<i>Thiobacillus</i>	8.50	0.00	0.01	0.01
<i>Variovorax</i>	0.55	0.60	0.18	0.06

* The soils between three and six years old showed very similar results and their abundances were averaged for each genus. Unclassified genera averages were respectively 21% (3-6 y.a.s.r); 43% (19 y..a.s.r); 37% (Forest) and 45% (Grassland).

Figure 1**Microbial biomass****a** **C-CO₂ - Microbial respiration****b** **Metabolic coefficient - qCO₂****c**

Figure 1 C-microbial biomass (1a), microbial respiration (1b), and metabolic coefficient (1c) of constructed soil for 3, 4, 5, 6, and 19 years after coal mining, compared to controls from the forest and grassland (y.a.s.r. = years after soil reconstruction). Means that differ significantly indicated by different lower-case letters (Tukey's test, $P < 0.01$).

Figure 2

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Figure 2 β -glucosidase (2a) and Dehydrogenase (2b) enzymes concentration per gram of dried soil for 3, 4, 5, 6, and 19 years after coal mining, compared to controls from the forest and grassland (y.a.s.r. = years after soil reconstruction). Means that differ significantly indicated by different lower-case letters (Tukey's test, $P < 0.01$).

Figure 3
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Figure 3 Shannon Diversity Index (SDI) at genus level (average between six replicates from each sample), across disturbed and undisturbed soils. Means that differ significantly indicated by different lower-case letters (Tukey's test, $P < 0.01$).

Figure 4

* 8 Phyla with relative abundance lower than 0.1%
y.a.s.r. = years after soil reconstruction

Figure 4 All phyla detected (100% of reads were classified to Phylum level) in soils from 3 to 19 years after soil reconstruction, as well as in native forest and grassland soils. Means that differ significantly indicated by different lower-case letters (Tukey's test, $P < 0.01$).

Figure 5

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Figure 5 PCA representing the samples from sites from 3 to 19 years after the soil construction and the two control sites, clustered according to microbial communities similarities (16S rRNA gene's relative abundance of each genus), enzymatic activity, and soil features.