FLÁVIA LOUZEIRO DE AGUIAR SANTIAGO

ASSESSMENT OF BIOLOGICAL AND BIOCHEMICAL PROPRIETIES IN SOIL AND TAILING FROM IRON ORE MINING OPERATIONS UNDER REHABILITATION

LAVRAS-MG
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Orientador
Prof. Dr. Marco Aurélio Carbone Carneiro

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Á Deus por sempre preparar o melhor para mim,
Á minha mãe Jacilane, pela compreensão e exemplo diário,
Á minha irmã Bruna, pela sua ternura e amizade,
Ao meu irmão Júnior pelo carinho e companheirismo
Ao meu pai Adênio (in memoria) que se faz presente em todos os momentos da minha vida

"Foi, é e será sempre por vocês"

DEDICO

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RESUMO

Atividades de mineração têm crescido em todo o mundo para atender às demandas industriais e ao desenvolvimento tecnológico. No entanto, o processo de extração mineral exerce impactos negativos à biodiversidade, além de gerar rejeitos que ficam vulneráveis à acidentes e que podem ser fontes de contaminação ambiental. Assim, por lei, faz-se necessário a reabilitação do ecossistema local. Para isso, a revegetação têm proporcionado bons resultados na reabilitação das funções do ecossistema, assim como alguns microrganismos do solo, à exemplo dos fungos micorrízicos arbusculares que além da ampla distribuição espacial, estão envolvidos no processo de agregação do solo, maior tolerância das plantas, absorção de água e nutrients e produção de glomalina. Nesse sentido, o objetivo do primeiro capítulo foi avaliar a reabilitação das áreas após mineração de ferro, através dos atributos biologicos e bioquímicos do solo. Para isso, foi avaliado em laboratório, atividades de enzimas envolvidas nos ciclos biogeoquímicos do C, N e P, carbono e nitrogênio total, carbono da biomassa, respiração microbiana e atividade metabolica bacteriana do solo. Os resultados mostraram que os atributos bioquímicos do solo (carbono e nitrogênio da biomassa, FDA, uréase e fosfatase ácida) das áreas em reabilitação foram incrementados a curto prazo em relação as áreas de solo exposto, sugerindo que a reabilitação está ocorrendo progressivamente e que, em curto à médio prazo, as funções ecológicas do solo podem ser totalmente recuperadas. No segundo capitulo, objetivou-se investigar o status das micorrízas arbusculares e sua contribuição no estoque de carbono nas áreas em reabilitação no Complexo Mineral Urucum. Os resultados mostraram que a riqueza de espécies foi reduzida em 50% com atividade de mineração, entretanto, observou-se que o número de esporos e colonização micorrízica aumentaram no period de dois e três anos de revegetação. Os teores de proteína do solo relacionados à glomalina, facilmente extraíveis e totais, foram aumentando com a idade de revegetação. Nosso conjunto de dados indicam que as melhorias no status das FMAs, responsáveis pelo fornecimento de conteúdo da GRSP, estão positivamente relacionadas com o estoque de carbono do solo e recuperação de funções ecológicas nas áreas em reabilitação. No terceiro capítulo objetivou-se avaliar como os rejeitos de mineração de ferro, em Mariana, afetaram a composição das comunidades bacterianas, além da ação condicionante do biochar na composição e diversidade das comunidades bacterianas.

Palavras-chave: mineração de ferro, reabilitação de áreas mineradas, comunidades bacterianas ambientais, glomalina, bioindicadores, carbono do solo.

ABSTRACT

Mining activities have grown worldwide to meet industrial demands and technological development. However, the process of mineral extraction has negative impacts on biodiversity, besides generating tailings that are vulnerable to accidents and which can be sources of environmental contamination. Thus, by law, rehabilitation of the local ecosystem is necessary. For this, revegetation has provided good results in rehabilitation of ecosystem functions, as well as some soil microorganisms, such as arbuscular mycorrhizal fungi that besides the wide spatial distribution, are involved in process of soil aggregation, higher plant tolerance, water and nutrient absorption and glomalin production. In this sense, the objective of the first chapter was evaluate rehabilitation of areas after iron mining through biological and biochemical attributes of soil. For this, it was evaluated in the laboratory, enzyme activities involved in the C, N and P biogeochemical cycles, carbon and total nitrogen, biomass carbon, microbial respiration and soil bacterial metabolic activity. The results showed that soil biochemical attributes (carbon and nitrogen from biomass, ADF, urea and acid phosphatase) of areas under rehabilitation were increased in the short term over exposed soil areas, suggesting that rehabilitation is occurring progressively and that, In the short to medium term, the ecological functions of the soil can be fully restored. In the second chapter, the objective was investigate the arbuscular mycorrhizal fungi and their contribution to carbon stock in areas under rehabilitation in Urucum Mineral Complex. The results showed that species richness of mycorrhizal fungi reduced by about 50% with mining activity. However, with the revegetation progress it was observed that spores number and mycorrhizal colonization increased in period of two and three years of revegetation. The easily extractable and total glomalin-related soil protein content increased with the age of revegetation. Our data set indicates that improvements in status of FMAs responsible for providing GRSP content are positively related to soil carbon stock and restoration of ecological functions in rehabilitation areas. In third chapter the objective was evaluate how the iron mining tailings in Mariana affected the composition of bacterial communities, as well as the conditioning action of biochar under different pH conditions on activity, composition and diversity of bacterial communities.

Keywords: iron mining, rehabilitation of mined areas, environmental bacterial communities, glomalin, bioindicators, soil carbon.

SUMÁRIO

PRIMEIRA PARTE

1. INTRODUÇÃO GERAL

1.1. Mineração de Ferro

O ferro é um metal disponível na natureza e como material de origem um substrato litológico constituído por rochas ferruginosas, como formações ferríferas bandadas (BIF) ou itabiritos, cangas, jaspilitos e filitos ferruginosos. Estes geossistemas datam origem no Arqueano (2,7 - 2,6 Ga) e Paleoproterozoico (2,5 Ga - 540 Ma), assim como suas paisagens formadas por litologias com cerca de 40 Ma. Estes geossistemas apresentam particularidades na flora, fauna e recursos hídricos (MESSIAS & CARMO; VASCONCELOS & HOFFMANN, 2015).

Assim como todos os metais, a extração do minério de ferro é dependente de sua distribuição geológica, do tipo de minério e dos rejeitos produzidos (WAHSHA et al., 2017). Neste caso, geograficamente, o minério de ferro comercial concentra-se principalmente em países como a Austrália, Brasil, Rússia, Índia e China. Estes países também são os maiores produtores de minério de ferro, representando, em conjunto, 81% da produção mundial (CARVALHO et al., 2014).

No Brasil, as reservas de minério de ferro encontram-se em três principais regiões: no Estado de Minas Gerais na Região do Quadrilátero Ferrífero (72,5% das reservas, com teor próximo de 46,3% de Fe), em Mato Grosso do Sul no Complexo Mineral do Urucum (13,1% das reservas e teor próximo de 55,3% de Fe) e Pará na Serra de Carajás (10,7% das reservas e teor próximo de 64,8% de Fe) (JESUS, 2014). De maneira geral, há uma predominância de formações ferríferas itabirito (20 – 54% de Fe), embora seja encontrada diferentes tipos de minério nestes complexos.

Em 2017 o Brasil ficou na segunda posição na produção e exportação anual do minério de ferro e seus produtos, com 419,17 e 347, 62 milhões de tonelada, respectivamente. Para 2019, com base no desempenho das empresas inseridas na atividade mineradora, estima-se que o Brasil ocupe primeiro lugar no *ranking* da produção mundial de minério de ferro, com 691 milhões de toneladas (CARVALHO et al., 2014).

No entanto, apesar da atividade mineradora contribuir diretamente com o desenvolvimento econômico e social de muitos países (MARTINEZ et al., 2018), também exerce efeitos negativos nos ecossistemas onde elas ocorrem (GU, 2018), com perda da

biodiversidade (MEIRA et al., 2016; MIRANDA et al., 2016), pulverização do solo (RENJAN et al., 2015), perturbação dos processos biológicos e bioquímicos (CARNEIRO et al., 2008; JOZÉFOWSKA et al., 2017; NARENDRULA-KOTHA et al., 2017; CHAE et al., 2017), perdas dos estoques de carbono (STUMPF et al., 2016; AHIRWAL et al., 2017; 2018), produção de rejeitos que normalmente apresentam teores de metais pesados elevados (CIARKOWSK, et al., 2014; RHODES et al., 2018; CHAE et al., 2018; KHAN et al., 2018; LIU et al., 2018), além de estar susceptível a protagonizar grandes catástrofes que a curto, médio e longo prazo, podem comprometer a saúde ambiental e humana, (PORTO et al.,; MIRANDA et al.,; MEIRA et al.,; SEGURA et al., 2016; HATJE et al., 2017).

1.2.Reabilitação das áreas mineradas: técnicas e desafios futuros

A Constituição Federal brasileira, estabelece no Artigo 225 (capítulo VI), parágrafo 2º, que "aquele que explorar recursos minerais fica obrigado a recuperar o meio ambiente degradado, de acordo com solução técnica exigida pelo órgão público competente, na forma da lei". O Decreto 97.632, de 10/04/1989, regulamenta a Lei 6.938/86, no que se refere à reabilitação de áreas degradadas pela atividade de mineração (IBAMA, 1990). No atual contexto, um dos grandes desafios da atividade de mineração é atender as demandas por minerais e implementar programas de reabilitação eficientes e sustentáveis concomitante a cada etapa do processo de extração, assim como também o monitoramento, interdisciplinaridade e aprimoramento das técnicas frequentemente utilizadas (MUKHOPADHYAY et al., 2013; GASTAUER et al., 2018).

Os resíduos remanescentes do processo de mineração encontram-se com suas propriedades degradadas e muitas vezes inadequadas para o crescimento de plantas (SILVA et al., 2018a). Assim, o uso do solo original (*Topsoil*), removido antes de iniciar a mineração, pode ser uma importante estratégia e fonte de propágulos vegetais, microrganismos do solo, reserva de C e nutrientes para o crescimento inicial da vegetação (FOWLER et al., 2015; BULOT et al., 2016). Vários estudos mostraram melhorias no desenvolvimento vegetal quando foi utilizado o *Topsoil,* (RIBEIRO et al., 2018; SANTOS et al., 2016; SILVA et al., 2018a; ANTONELI et al., 2018), o que consequentemente, pode promover a reabilitação das áreas em menos tempo.

O rápido estabelecimento da cobertura vegetal nas áreas mineradas é importante para a proteção e retomada das funções bioquímicas do solo, já que a revegetação reinicia o processo fotossintético e entrada de carbono no solo, com fluxo contínuo, diversificado e sustentável.

Esse processo também pode ser conhecido por re-carbonização (RATTAN et al., 2018). Atrelado ao sequestro de carbono, a re-carbonização pela revegetação em áreas de mineração promove ciclagem de nutrientes, melhorias nos atributos físicos do solo, aumento da biodiversidade, fonte de energia para biomassa microbiana, retomada dos processos bioquímicos e de importantes serviços ecossistêmicos do solo (RENAJAN et al., 2015; BORGES et al., 2016; LEI et al., 2016; AHIRWAL et al., 2017; 2018; YUAN et al., 2017; FERNANDES et al., 2018; SÁ et al., 2018; SILVA et al., 2018b; KUMAR et al., 2018).

Além da diversidade vegetal e outros fatores ambientais, o tempo deve ser extremamente considerado na reabilitação das áreas mineradas, assim como compreender o perfil dinâmico dos atributos do solo sob estas condições (AHIRWAL & MAITI, 2017; GASTAUER et al., 2018). No estudo realizado por AHIRWAL et al. (2018) foi possível verificar que muitos atributos do solo só foram recuperados após 16 anos de revegetação. Durante esse período, houve aumentos nos atributos químicos, tais como recuperação de 81% de carbono orgânico do solo 125% de N disponível, 160% de P disponível, e 61% de K trocável.

Uma pesquisa conduzida em áreas de minas de carvão na Índia, com três estágios de revegetação 2, 8 e 14 anos, mostrou aumentos no estoque de C de 30%, 63% e 7% respectivamente, em função ás idades da vegetação. Entretanto, a taxa média de C após 14 anos foi de 6.4 Mg C ha^{-1} ano^{-1}, considerada muito baixa quando comparada a área de referência (floresta) com um total de C 273 Mg C ha^{-1}, aproximadamente três vezes o valor encontrado nos sítios revegetados. Os pesquisadores estimaram ser necessário 42 anos para alcançar os estoques de C similar a área de floresta e atribuem a lenta recuperação, ao tempo que alguns atributos necessitam para se restabelecer no sistema e ás condições climáticas da região (AHIRWAL & MAITI, 2017).

1.3.Biochar

O biochar é um composto produzido a partir da pirólise (condições de elevadas temperaturas e baixo teor de oxigênio) de biomassa (LEHMANN, 2007), normalmente subprodutos comerciais que seriam descartados ou mesmo subutilizados, como por exemplo, a casca de café, particulados de eucalipto, cama de frango, casca de arroz, resíduo de açaí, castanha-do-brasil e bagaço de cana-de-açucar (LUSTOSA FILHO et al., 2017; DOMINGUES et al., 2017; SOUZA et al., 2019). Contudo, por ser um composto alternativo e bastante recalcitrante no solo é importante conhecer a composição mineral da biomassa produzida para

que seus efeitos não venham a ser contrários ao objetivo empregado (ENDERS & LEHMANN, 2012).

O biocarvão é uma tecnologia promissora para o sequestro de carbono, melhora na fertilidade do solo, aumento no rendimento das culturas e adsorção de poluentes (LEHMANN e JOSEPH, 2009; BEESLEY et al., 2011; RODRIGUEZ-VILA et al., 2016; KAN et al., 2016). Além disso, a interação organo-mineral é eficiente na proteção física da matéria orgânica contra a degradação microbiana do solo (LEHMANN et al., 2011; ZIMMERMAN et al., 2011) e pode, direta ou indiretamente, influenciar as comunidades microbiológicas (AMELOOT et al. , 2013; THIES et al., 2015; ZHENG et al., 2016; SHENG e ZHU, 2018). Todas essas características potencializam a ideia de que o biocarvão pode ser uma importante proposta com ação condicionante para a estabilização desses resíduos nas áreas afetadas pela mineração.

A diversidade microbiológica é bastante sensível as alterações no ambiente e por serem os principais mediadores de processos funcionais no solo, as técnicas de reabilitação que favoreçam a biota são bastante incentivadas. O uso do biochar afeta a estrutura e diversidade das comunidades microbianas (WATZINGER et al., 2013; THIES et al., 2015), embora ainda sejam necessários muitos estudos a respeito das interações ecológicas. Além disso, os efeitos do biochar é dependente do processo no qual é produzido, da natureza do material orgânico e de sua composição química final (TSAI et al., 2009; CHEN et al., 2013; QUILLIAN et al., 2013; FORJÁN et al., 2017).

O estudo conduzido por MOORE et al. (2018) mostraram forte influência do biocarvão sobre a física-química (pH) e microbiológica do solo. Os autores observaram que o biocarvão produzido em baixa temperatura aumentou a respiração, a riqueza de espécies e a diversidade, devido à fatores, como a disponibilidade de nutrientes e criação de micro-habitats que protegem os microrganismos do solo da contaminação. Além disso, observaram melhorias no crescimento das plantas em até três vezes quando se aplicou o biocarvão em relação ao controle, sem o biocarvão.

Os efeitos positivos da aplicação de biochar de palha de trigo, pelo período de 4 anos, foram verificados nas propriedades físico-químicas e nas comunidades microbiológicas, principalmente fúngicas. Houve aumento na biomassa microbiana, menor quocientes metabólicos e atividades enzimáticas (β-glicosidase e desidrogenase). Os autores atribuem as mudanças na composição da comunidade microbiana ao aumento do pH e carbono orgânico do solo quando induzido pela adição de biochar (ZENG et al., 2016). Esta pesquisa evidencia, mais uma vez, o efeito condicionador do biochar e como esse material pode afetar a composição das comunidades microbiológicas e recuperar importantes processos bioquímicos do solo.

Estudo publicado recentemente, a partir de solo contaminado por metal pesado, mostrou que a aplicação de 5% (w/w) de biochar aumentou tanto a emissão de CO_2 quanto a biomassa microbiana ao final de 45 dias de incubação. Além disso, contribuiu na diversidade nas comunidades de bacterianas ($Gran^+$) e fungos, e reduziu a biodisponibilidade dos elementos tóxicos (XU et al., 2018).

No Brasil, o uso de biochar na reabilitação de áreas mineradas, principalmente seus efeitos sobre as comunidades microbiológicas, ainda se encontra bastante limitado. Contudo, recentemente, foi publicado um estudo com solos de mina da Serra de Carajás, no Pará, usando biochar produzido a partir de resíduos de açaí e castanha-do-brasil. O estudo mostrou redução de absorção de Pb e Ba pelas plantas, assim como aumentos da imobilização de Cu e Ni após a adição do biochar. Os autores indicaramm o uso dos materiais em programas de fitoestabilização de elementos tóxicos em áreas reabilitação após a mineração (SOUZA et al., 2019).

1.4. Indicadores biológicos e bioquímicos da qualidade do solo e o potencial para monitoramento de áreas em reabilitação

A qualidade do solo, segundo DORAN and PARKIN (1994), é definida como "capacidade do solo funcionar, dentro dos limites do ecossistema manejado ou natural, para sustentar a produtividade biológica, manter a qualidade ambiental e promover a saúde vegetal e animal". Contudo, devido as alterações abruptas que ocorrem durante a atividade de mineração, inevitavelmente, a qualidade e funções do solo ficam comprometidas e precisam ser recuperadas (GU, 2018).

Na prática, o monitoramento da reabilitação da qualidade do solo após a mineração, pode ser realizada pela análise de atributos físicos, químicos e biológicos uma vez que consistem em atributos mensuráveis e capazes de indicar a condição atual do solo (SILVA et al., 2018b; SCHIMMER and van DEVENTER, 2018; SÁ et al., 2018).

No entanto, por se tratar do componente vivo, os atributos biológicos e bioquímicos são mais sensíveis ao manejo e reflete, em menos tempo, a dinâmica dos processos que ocorrem no solo quando comparado aos demais atributos. Este comportamento foi observado no estudo conduzido por CARNEIRO et al. (2008) onde a revegetação das áreas mineradas estimulou a recuperação total dos teores de carbono da biomassa microbiana e atividades enzimáticas, no primeiro de reabilitação. Já os teores de C orgânico e N total, os autores estimaram ser necessário um período de 18 anos. Esse comportamento mostra que os atributos biológicos são

eficientes em sinalizar mudanças com a reabilitação a curto prazo, uma característica importante para sua aplicação em programas de monitoramento.

1.4.1. Biomassa microbiana (BM)

A biomassa microbiana é a fração viva do solo e corresponde de 1% a 5% do carbono orgânico (ANDERSON & DOMSCH, 1989; JENKINSON & LADD, 1981). Representa a principal fonte de enzimas e é considerada a força motora para realização de processos bioquímicos no solo (NANNIPIERI et al., 1983; TAO-LI et al., 2009; CARNEIRO et al., 2013; STEINWEG et al., 2013; WANG et al., 2016).

A biomassa microbiana é considerada um ótimo indicador da saúde do solo, isso por que é bastante sensível a mudanças ambientais (SINGH et al., 2018) tais como tipo de vegetação, temperatura, conteúdo de água no solo, clima, tempo, disponibilidade e qualidade de substrato orgânico (SPARLING, 1992; SARATHCHANDRA et al., 1989; YANG et al., 2003; BASILIKO et al., 2005; TSAI et al., 2007; RAVINDRAN et al., 2015; AGBOOLA et al., 2018).

Na reabilitação de áreas contaminadas por Zn, Cu, Pb e Cd, após anos de implementação, foi possível verificar que o carbono da biomassa microbiana refletiu os efeitos negativos e aumentou quando houve redução do teor de metais devido ao uso do solo superficial. Os autores observaram um aumento de 100% na biomassa microbiana em relação aos sítios com contaminação intermediária e até 240% em relação ao sitio de maior contaminação (SANTOS et al., 2016).

1.4.2. Respiração basal do solo (CO_2) e quociente metabólico (qCO_2)

A respiração basal do solo traz informações sobre a atividade biológica no solo. A emissão de CO_2 expressa o comportamento da biomassa microbiana frente decomposição de compostos orgânicos disponíveis. Contudo, seu comportamento deve ser avaliado com bastante critério e em conjunto com outros atributos, pois valores elevados podem indicar tanto que a biomassa encontra-se metabolicamente ativa, como também refletir condições estressantes, já que ambas situações resulta em maior emissão de CO_2 (BUJALSKY et al., 2014).

O quociente metabólico consiste na relação da emissão de CO_2 e o carbono incorporado na biomassa microbiana. Assim, se o valores de qCO_2 são mais baixos em ecossistemas mais estável ou em ambientes mais próximos do equilíbrio (ANDERSON 1982; SOUZA et al., 2006;

2010). Entretanto, em áreas não-consolidadas e com alteração antrópica, a biomassa microbiana aumenta seu metabolismo, utilizando as fontes de carbono pouco lábeis para garantir a manutenção da população, mesmo na presença de estresse ambiental (CUNHA et al., 2012).

Em áreas de mina de carvão revegetada, o fluxo anual de CO_2 no solo foi mais alto na floresta natural (53 Mg CO_2 $há^{-1}$ ano^{-1}) seguida da área de revegetação mais avançada (42 Mg CO_2 há-1 ano-1) > intermediado (33 Mg CO_2 $há^{-1}$ ano^{-1}) > locais com vegetação mais jovens (11 Mg CO_2 $há^{-1}$ ano^{-1}). Os autores atrelam o aumento do fluxo de CO_2 no solo juntamente com a idade de revegetação, ao aumento no conteúdo SOC que apresentou o mesmo comportamento (AHIRWAL et al., 2017).

Em estudo avaliando a qualidade do solo de diferentes fitofisionomias, incluindo áreas em reabilitação após a mineração de ferro, SILVA et al. (2018b) reportaram que a área minerada apresentou os menores valores de CBM (321.69 e 272.98 mg g^{-1}) e emissões de CO_2 (48.76 e 12.68 mg C-CO_2 kg^{-1} h^{-1} de solo) e os maiores valores de qCO_2 (155.15 e 48.39 mg h^{-1} C-CO_2 $mg^{-1}C$), nas época chuvosa e seca, respectivamente. Esse comportamento reflete uma condição estressante para a biomassa microbiana, e necessidade de maior disponibilidade de carbono para sua manutenção. Neste caso a análise do qCO_2 foi determinante conhecer o verdadeiro status a área em reabilitação.

1.4.3. Enzimas do solo

A maioria das enzimas do solo são produzidas por microrganismos (endocelulares ou extracelulares), participam dos ciclos biogeoquímicos, ciclagem de nutrientes, transformação do material orgânico (MUSCOLO et al., 2014) e suas atividades afetadas por fatores como temperatura e umidade (STEINWEG et al., 2012; 2013), pH (ACOSTA-MARTINEZ, 2000), manejo e tipo de solo (CARNEIRO et al., 2013; STURSOVÁ et al., 2011) uso de insumos agrícolas e elevadas concentrações de metais pesado.

Mensurar a atividade enzimática foi considerado um bom caminho para avaliar a qualidade e saúde do solo (DICK 1992; VISSER and PARKINSON 1992; BRZEZIŃSKA et al., 1998; NA and KIM, 2009; CHAE et al, 2018) e funcionalidade dos ciclos biogeoquímicos, como por exemplo o do C, N e P. Um estudo piloto em áreas de mineração de ouro, mostrou que a baixa atividade da fosfatase ácida e alcalina, urease, dehydrogenase e β-glucosidase estava relacionada a ausência de substrato orgânico e baixo conteúdo de carbono da biomassa das áreas mineradas, já que são indispensáveis para atividade e manutenção das enzimas no solo (SCHIMMER and van DEVENTER, 2018).

As atividades do diacetato de fluoresceína (FDA), por exemplo, não correspondem a ação especifica, mas a um conjunto de enzimas (lipases, proteases, esterases) (BALOTA et al., 2013) capazes de fornecer informações sobre a atividade das comunidades microbianas como um todo. Dessa maneira, sua determinação é valiosa, principalmente no monitoramento da reabilitação de áreas pós-mineração.

A β-glucosidase é uma enzima bastante comum e persistentes no solo. Atua na hidrolise final da celulose e outros oligossacarídeos disponibilizando como produto final a glicose, uma importante fonte de C para os microrganismos (TABATABAI, 1994). Estudos mostrando a eficiência dessa enzima como indicador do status de saúde do solo em áreas de reabilitação foram bem conduzidos (LAMMIRATO et al., 2010; PAZ-FERREIRO et al., 2012; PATHAN et al., 2017; CHAE et al., 2017; WAHSHA et al., 2017; MORENO-BARRIGA et al., 2017).

A urease atua no ciclo do nitrogênio, catalisando a hidrólise da molécula de ureia em amônia e gás carbônico. No solo, a amônia produzida é transformada rapidamente em amônio que pode ser volatilizado, parte absorvida pelas plantas ou nitrificada. É considerada uma enzima vital, pois é responsável pelo suprimento de N às plantas. (TABATABAI et al., 1971; TABATABAI, 1994; BALOTA, et al, 2013). Estudos recentes conduzidos por CIARKOWSKA et al. (2014) and MEDEIROS et al. (2017) mostraram o potencial desta enzima como bom indicador da saúde do solo.

As fosfatases são hidrolases que catalisam substratos fosfoésteres em fósforo solúvel (SINSABAUGH et al., 2008; NANNIPIERI et al., 2011) e são classificadas em ácidas, neutras e alcalinas, dependendo do seu pH de atuação. Assim como as outras enzimas as fostasese são influenciadas por vários fatores, como cobertura vegetal, propriedades do solo (pH, matéria orgânica, concentração de metal) e comunidade microbiana (ACOSTA-MARTINEZ e TABATABAI, 2000; HAGMANN et al., 2015).

Os resultados de NARENDRULA-KOTHA e NKONGOLO, (2017a) mostraram que maiores atividades das fosfatases foram observadas em locais não contaminados por metais pesados e com a aplicação de calcários. O estudo conduzido por SANTOS et al. (2016) também mostrou a mesma tendência em áreas de reabilitação. Registros na literatura relacionam a produção de fosfatase alcalina as bactérias enquanto a atividade da fosfatase ácida é de bactérias, fungos e plantas (Nannipieri et al., 2011). Os fungos micorrízicos arbusculares, por exemplo, são destaque na liberação de fosfatases (WEINTRAUB, 2011; NARENDRULA-KOTHA e NKONGOLO, 2017b, 2017c).

1.4.4. Diversidade de FMAs e protéinas do solo relacionadas a glomalina

Os fungos micorrízicos arbusculares (FMAs), pertencente ao filo Glomeromycota, estão amplamente distribuídos nos ecossistemas (BAREA et al., 1983). Realizam diversas funções no ambiente edáfico: aumentam a estabilidade dos agregados do solo através das hifas e produção de glomalina (KUMAR et al., 2018; JI et al., 2019), favorece a nodulação e fixação biológica de nitrogênio (JESUS et al., 2005; CARVALHO & MOREIRA, 2010), aumenta a absorção de nutriente com baixa mobilidade no solo e maior tolerância das plantas a estresses hídricos e por metais pesados (SIQUEIRA et al., 1999).

Mais de 80% das espécies de plantas fazem simbiose mutualística com fungos micorrízicos arbusculares (FMA), característica que as ajudam a crescer e se estabelecer muito bem em áreas contaminados e em reabilitação (BONFANTE e ANCA, 2009; JEFFRIES et al., 2003; KHAN, 2005; MIRANSARI, 2010; MEIER et al., 2012). *Glomus sp*. são os gêneros mais abundantes encontrados em áreas contaminadas ou mineradas (SILVA et al., 2005; ZAREI et al., 2008).

Em áreas de mineração no Quadrilátero Ferrífero, as áreas de Canga apresentaram grande diversidade de FMAs e o maior potencial de inoculo em relação as demais áreas estudadas. Os autores ressaltam que esse resultado positivo pode conferir maior resiliência e facilitar a reintrodução de especies vegetais durante a reabilitação. Neste estudo os gêneros *Glomus* e *Aucalospora* foram considerados generalistas e mais abundantes (TEXEIRA et al., 2017).

A glomalina é uma glicoproteína produzida pelos FMAs, de natureza complexa e recalcitrante. Operacionalmente, é definido como proteínas do solo relacionadas à glomalina (PSRG) e pode ser determinado a partir do solo como facilmente extraível (PSRG-EE) e total (PSRG-T) (SINGH et al., 2013). Esta substância pode representar em mais de 30% do teor de C do solo, 3 a 5% N; 0,03 a 0,1% de P (NICHOLS e WRIGHT, 2006; SCHINDLER et al. 2007; TRESEDER e TURNER, 2007; ZHONG et al., 2017) e 0,8 a 8,8% de Fe (RILLIG et al. 2001), considerando-a como reserva nutricional a longo prazo e mecanismo importante de sequestro de solo C (RILLIG et al., 2004; 2006; MALEKZADEH et al., 2016; XU et al., 2017).

Além disso, muitos estudos mostram efeitos positivos da glomalina na estabilidade dos agregados do solo (BORIE et al., 2006; WRIGHT et al., 2007; WILSON et al., 2009; RILLIG, 2014; VILELA et al., 2014; CARNEIRO et al., 2015; YANG et al., 2017) e complexação de elementos tóxicos (por exemplo, Pb, Cu, Zn, Cd e Cr) (GONZALEZ-CHAVEZ et al., 2004; GIL-CARDEZA et al. 2014; WU et al., 2014), características importantes para o uso da glomalina como indicador durante o processo de reabilitação de áreas mineradas.

Fatores bióticos e abióticos podem influenciar a produção e persistência da glomalina no solo. Pesquisas mostraram correlação negativa entre concentrações de glomalina e aumento de pH (RILLIG et al., 2003), possivelmente pelos fungos se desenvolverem melhor em ambientes ácidos. Outros estudos mostram que a diversidade de FMAs, e a idade da vegetação exercem forte influência na produção de glomalina (SOUSA et al., 2011; SILVA et al., 2012; SUI et al., 2017).

Resultados relatados por YANG et al. (2017) mostraram que a colonização micorrízica, densidade do comprimento da hifa, GRSP, foi inibida pelo Pb comparado ao Zn a 0 ± 20 cm de profundidade do solo, indicando que o metal pesado teve efeitos inibitórios significativos no crescimento da FMA. Além disso, a colonização micorrízica e a densidade do comprimento da hifa tiveram correlação significativamente positiva com GRSP, SOM e SOC, sugerindo que a FMA desempenhou um papel essencial na acumulação de Carbono em GRSP, SOM e SOC em metais pesados. Os autores indicam que práticas adequadas de manejo devem ser desenvolvidas para garantir o máximo benefício da simbiose planta-AMF durante a restauração ecológica.

1.4.5. Atividade metabólica de comunidades bacterianas do solo

A análise do Perfil Fisiológico ao Nível-Comuinitário (CLPP) é frequentemente utilizada para avaliar o efeito de alterações ambientais sobre a atividade bioquímica, além disso, pode ser uma ferramenta poderosa para entender contextos ecológicos (STEFANOWICZ, 2006). Consiste em uma análise rápida, fácil e capaz de distinguir alterações espacial e temporal de vários ambientes (CHOI e DOBBS, 1999; TIQUIA, 2010; FRAC et al., 2012).

Esta analise usando EcoPlates foi originalmente descrito por GARLAND e MILLS, (1991). Eles descobriram que inoculando microplacas com uma população mista de microrganismos poderiam caracterizar o perfil metabolico das comunidades microbianas. Seguindo essa linha, a análise da CLPP foi aprimorada por INSAM (1997), que desenvolveu uma microplaca de 96 poços com 31 novos substratos e mais controle (EcoPlate), cada um em três repetições. As fontes de C da placa Ecoplate estão divididas em seis grupos: carboidratos, aminoácidos, fenóis, carboxílicos, polímeros e aminas.

Embora na literatura seja possível encontrar questionamentos sobre a aplicação e interpretação da análise CLPP (PRESTON-MAFHAM et al., 2002) muitas pesquisas mostram a capacidade do teste bioquímico Ecoplate em refletir as mudanças no catabolismo a partir de alterações ambientais (BUSSE et al., 2001; WALKER et al., 2001; MARCHAND et al., 2002; FLIESSBACH e MADER, 2004; ROS et al., 2006; MIKI et al., 2018).

FRAC et al. (2012) e LEON et al. (2012) mostrando relação entre a análise CLPP e diversidade microbiana a partir de técnicas moleculares. Resultados que tornam a aplicação do teste ainda mais consistente. Recentemente, o teste EcoPlate também foi utilizado no monitoramento de diferentes ecossistemas no Quadrilátero Ferrífero, MG, Brasil, incluindo áreas em reabilitação. O potencial de degradação foi correspondente aos resultados de diversidade das áreas. Os pesquisadores indicam as análises como ferramenta para monitorar áreas em reabilitação (FERNANDES et al., 2018).

1.4.6. Análise metagenomica do solo

A metagenômica torna-se uma importante e poderosa ferramenta, capaz de acessar rapidamente a biodiversidade do solo e melhorar a compreensão dos processos e serviços ecossistêmicos. Além disso, permite conhecer microrganismos com elevado potencial biotecnológico, que podem ser utilizados tanto no setor ambiental, no caso da biorremediação, como em diversos setores industriais (Muller et al. 2013; Ahsan et al 2017; Vestergaard et al., 2017; Hussain et al., 2018; Haleyur et al., 2018).

A técnica consiste inicialmente na extração de DNA a partir de amostras ambientais, neste caso, amostras de solo. Em estudos das comunidades bacterianas, a região RNAr 16S do material genético é amplificado pela reação da cadeia polimerase (PCR) e submetido a análise de sequenciamento (Sanger, Pirosequenciamento), que é determinado conforme o objetivo do estudo. Nesta etapa, obtêm-se sequências nucleotídicas que serão processadas em análises de bioinformática e comparadas em bancos de dados (Pessoa Filho et al., 2010).

Esse tipo de abordagem, a respeito da composição das comunidades microbianas do solo, é extremamente importante para estratégias de reabilitação de áreas degradadas, pois com esse banco de dados é possível associar informações com outros atributos do solo e traçar estratégias eficientes para acelerar a reabilitação das áreas, já que os micróbios do solo são considerados a força motora dos ciclos biogeoquímicos (Jing et al., 2017; Meena et al., 2017). Outro ponto relevante a ser considerado, é que através da metagenômica pode-se mensurar, de forma ampla, o impacto das intervenções antrópicas sobre os microrganismos (Navarrete et al., 2015; Carvalho et al., 2015; Cao et al., 2017).

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - Revegetation of mined areas influences physiological profile of bacterial communities and improves the biochemical functions of soil

Article under review in Ecological Enginnering

Revegetation of mined areas influences physiological profile of bacterial communities and improves the biochemical functions of soil

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Abstract - Mining activities have been growing worldwide in order to meet industrial demands and technological development. However, mining activities have a negative impact on the environment, which needs to be repaired. Thus, this study was aimed at monitoring the rehabilitation process of the areas after iron mining, using biochemical soil attributes. The experimental design consisted of recently mined areas, areas at 2 and 3 years after revegetation and areas with native vegetation. Analyses were made of carbon and nitrogen of microbial biomass, soil basal respiration, metabolic quotient, acid phosphatase activity, fluorescence diacetate hydrolysate, urease and β-glucosidase, as well as metabolic activity of soil bacterial communities. There was average gain of 46% of Cmic in areas with 2 and 3 years of rehabilitation, in comparison to exposed soil area. Area 3A showed an 86% increase of microbial biomass nitrogen in comparison to exposed soil area, and recovery of 39% Nmic was found in the reference area. In FDA activity, there were significant increases corresponding to the recovery of 53% and 68% activity observed in the reference area in only 2 and 3 years rehabilitation, respectively. For urease, there were increases of 52% and 66% in comparison to value observed in exposed soil area, with 2 and 3 years of rehabilitation respectively. Acid phosphatase was also increased as function of rehabilitation time, with area 3A increasing by 90% in comparison to exposed soil area, and a recovery of 28% in comparison to the reference area. The results indicate that recovery is occurring positively and that, in the short to the medium term, the ecological functions of the soil can be totally recovered. Addition, the present study highlights the positive effects of carbon input into the soil, by revegetation, on resumption of biogeochemical cycles and soil health, particularly, of areas affected by mining activities.

Keywords: rehabilitation of mined areas, environmental monitoring, bioindicators, soil carbon

1. Introduction

Population growth promotes greater demand for industrial products manufactured from raw metals, mainly iron ore. However, this activity causes a negative impact on the ecosystems where they take place (Gu, 2018), because of removal of native vegetation, soil disintegration and interruption of soil biochemistry, a process which is essential for proper functioning of natural ecosystems. Thus, recovery programs have to be implemented during and after mining activities in mined areas to reduce the impact on the environment (Gastauer et al., 2018). During this period, it is necessary monitor the vegetation reintroduced in the areas, their evolution and similarity with reference areas, or if interventions are needed to improve rehabilitation programs.

Although revegetation is a challenging and complex technique (Gasteur et al., 2018), it works as input of organic matter into the soil (Medeiros et al., 2017), decomposition and nutrient cycling (Ranjan et al., 2015), In addition, it keeps cover soil and reduces microbial losses by oxidation (Sá et al., 2018). In Brazil, in the last few years, revegetation programs in mining areas have had important advances (Ribeiro et al., 2018; Santos et al., 2016; Silva et al., 2018) in terms of increased implementation, concomitant each mining stage, thus promoting the return of ecosystems services.

Although there have been advances, there are still many gaps to be filled, for example, monitoring of rehabilitation programs adopted in the area, evaluation of biochemistry processes realized by soil microorganisms (important agents for input of biomass), decomposition of organic compost, mineralization and nutrient cycling (Fang et al., 2018; Fujii et al., 2018; Jing et al., 2017). Furthermore, soil microorganisms establish a symbiotic relationship with plants, which contribute to nutrition (Behie and Bidochka, 2014; Borges et al., 2016) and phytosanity (Gourion et al., 2015; Meena et al., 2017; Pinto-Carbó et al. 2018).

Microbial biomass is the most labile fraction of organic carbon (Santos et al., 2016) and the main source of soil enzymes, which in turn are negatively influenced by some factors, including removal of vegetation and tailing deposition with high metal content, a common practice in mining activities. The activity of B-glucosidase, FDA, urease and phosphatase is directly related to the cycle of carbon, nitrogen and phosphor, respectively, in addition to total organic matter cycling (Chae ae al., 2017; Ciarkowska et al., 2014; Sharma et al., 2015), and ecosystem service maintenance. In this context, biochemical attributes are great bioindicators of the status of rehabilitation areas, since they are very sensitive to management and reflect, in the short term, the dynamics of important processes that occur in the soil (Klerk et al., 2016).

Studies conducted in bauxite mining, gold extraction and zinc processing areas, showed that microbial activity of soil is sufficiently stimulated by vegetation, with considerable increases that indicate adaptability of edaphic microbiota to the conditions to which they are exposed during evolution of rehabilitation process (Carneiro et al., 2008; Santos et al., 2016). However, there are few studies conducted in iron mining areas (Fernandes et al., 2018; Silva et al., 2018).

Thus, this study aimed (a) monitor the rehabilitation process of the areas after iron mining, using the biochemical soil attributes (b) and evaluate behavior soil bacterial metabolic activity with reintroduction vegetation in the mined areas.

2. Material and Methods

2.1. Characterization of study areas

Soil samples were collected from a mineral complex in the city of Corumbá, Mato Grosso do Sul, Brazil. These areas are included in the Cerrado biome, Canga ecosystem, characterized by a ferruginous surface and rupestrian fields. The climate in region is Aw

according to Köppen Classification, with dry winters and rainy summers. The average annual temperature is 25.4 °C, and average annual rainfall is 1074 mm.

Three mining areas were sampled, representing different stages: recently mined (without vegetation), with 2 and 3 years after revegetation and areas with native vegetation. In all areas, the same recovery plan was carried out, consisting of base fertilization, topsoil layer deposition, sowing and planting seedlings of native plant species, as authorized by the competent authorities. The main vegetable families used were *Poaceae, Fabaceae, Bignoneaceae, Lauraceae, Asteraceae, Melastomataceae* and others.

Data collection occurred in 2016. At each stage, three samples were collected; they were composed of soil at different points, with minimum distance of 50m between them. Each sample was collected from five points homogeneously distributed within a 10x10m parcel, at the 0-20 cm layer.

Subsequently, the samples were stored in sterile plastic bags, refrigerated and sent to the Laboratory of Microbiology and Biochemical Processes of Soil at the Federal University of Lavras, MG. The samples were sieved in a 4 mm mesh and stored in a cold room at 4 °C for further biological and biochemical analyses. Part of the material was separated to determine soil physicochemical attributes (Embrapa, 2009), as shown in Table A1.

2.2. Soil carbon and nitrogen

Carbon (Ct) and total nitrogen (Nt) of soil were determined by elemental analysis. The samples were placed in tin capsules, and the material was digested in a combustion chamber at a temperature of approximately 975 °C. The gases were detected and converted into percent carbon and nitrogen. The equipment used was a CHNS/O universal elemental analyzer (Vario Micro Cube model). Organic carbon (Corg) was determined by oxidation with potassium dichromate in the presence of H_2SO_4, followed by titration of excess dichromate in the sample

with ammoniacal ferrous sulfate and diphenylamine (1%) as an indicator (Walkley and Black, 1934).

2.3. Soil biological and biochemical attributes

Microbial Carbon (Cmic) and microbial nitrogen (Nmic) of soil were determined by the fumigation/extraction method (Vance et al., 1987) using 20 g of soil, and a potassium sulphate solution (0.5 mol L-1) was used as an extractor. Cmic was determined by oxidation with potassium dichromate (0.066 mol L^{-1}) and addition of H_2SO_4 and H_3PO_4 acids, and the mixture was brought to the hot plate to a boil. After cooling, the solution was titrated with ammoniacal ferrous sulfate (0.033 mol L^{-1}) and diphenylamine (1%) as an indicator. Nmic, after extraction, was determined by the Kjeldahl method.

Soil basal respiration (CO_2) was quantified by incubating 10 g of soil in closed pots and in a dark environment for 72 hours at 28 °C. Released CO_2 was (0.5 mol L^{-1}), which was then titrated with HCl (0.5 mol L^{-1}) using phenolphthalein (1%) as an indicator (Alef and Nannipieri, 1996). The metabolic quotient (qCO_2) was calculated by the CO_2/Cmic ratio.

The activities of β-glucosidase and acid phosphatase were determined according to Dick et al. (1996). Briefly, 1g of soil, 4 ml of universal buffer (MUB) at pH 6.0 and 1 ml of substrate solution ρ-nitrophenyl-β-D-glucoside (0.025M in MUB buffer) were added for β-glucosidase; and 1g of soil, 4 ml of universal buffer (MUB) at pH 6.5, and 1 ml of ρ-nitrophenyl phosphate solution (0.05 M in MUB buffer) were added for acid phosphatase. The pots were shaken and incubated in the dark at 37 °C for 1 hour. Enzyme activity was stopped by adding 1 ml of $CaCl_2$ (0.5 M) and 4 ml of Tris buffer (pH 10) for β-glucosidase activity and 1 ml of $CaCl_2$ (0.5 M) and 4 ml of NaOH (0.5 M) for acid phosphatase. All solutions were filtered and activities were determined using a spectrophotometer at wavelength of 410 nm.

Total enzyme activity in the soil was determined by the activity of fluorescein diacetate (FDA) using 2 g of moist soil. In brief, 1 mL of fluorescein diacetate solution and 4 mL of potassium phosphate buffer (pH 7.0) were added in falcon tubes. The samples were then incubated at 37ºC in the dark for 1 hour, under constant stirring at 60 rpm. After the incubation period, 5 mL of acetone (50%) was added to stop the activity. To separate the supernatant, the tubes were centrifuged at 1500 rpm for 15 minutes and subsequently filtered. Absorbance was measured at a wavelength of 490 nm (Adam and Duncan, 2001).

Urease activity was determined using 5.0 g of soil, 9 ml of Tris THAM buffer (pH 9) and 1 ml of urea solution (0.2 M), incubated at 37 ° C for 2 hours. Then, 40 ml of KCl-Ag_2 SO_4 (2.5 M, 100 mg L^{-1}) was added to stop the reaction. After that, 20 ml of supernatant solution was transferred to digestion tubes, added with 0.2 g of MgO and taken to the distillation process. Subsequently, it was titrated with a H_2SO_4 solution (0.005 M) (Tabatabai and Bremner, 1972).

2.4. Soil bacterial community metabolic activity

Metabolic activity was evaluated by different substrates by the bacterial community in the soil, applying the biological test Biolog EcoPlate; 10 g soil was suspended in 90 ml 0.85% sterile NaCl solution and stirred at 22 °C for 30 min at 150 rpm. After 10 minutes in settling, 1 ml of supernatant was diluted in 9 ml of a sterile 0.85% NaCl solution. After dilution, 125 µl of this suspension was pipetted into the wells of the microplate, and then incubated at 25 °C in the dark. Absorbance was measured with Dialab EL800 Microplate Reader by the incubation period of 72 hours, every 24 hours, at 590 nm optical density. Average color development of each well (AWCD) was calculated for the sources with the following equation: AWCD = ΣODi/n, where ODi is the corrected OD value of each substrate, to remove the effects of inoculum density is the number of substrates, in this case n = 31.

2.5. Statistical analysis

The Shapiro-Wilk test was used to determine the normality of data. After meeting the normality and homoscedasticity criteria, the linear mixed effects model (LMMs) was applied, using the 'lme4' package, where the mining areas were determined as a fixed factor, the rehabilitation stages in each area as a random factor and the attributes biological and biochemical responses as variables. The averages of the biological and biochemical attributes were compared by the Scott-knott test at $p < 0.05$. The chemical characterization and soil biochemical attributes of the areas under rehabilitation were summarized by a principal component analysis (PCA) in the correlation matrix, using the 'FactoMineR' package. For the graph, the 'biplot' function was used.

For the analysis of metabolic activity, mean color development values for the wells (AWCD) (Table A2) were used in each microplate. Then, a heat map was designed using a the 'heatmap' function. Pearson's correlation was determined between biological and biochemical attributes using the 'Hmisc' package. All analyses were performed in R, version 3.4.1 (R Development Core Team, 2017).

3. Results and Discussion

3.1. Carbon and Nitrogen in Soil

The areas with two (2A) and three (3A) years of rehabilitation had increased total carbon (Ct) of 54.23% and 80% in comparison to exposed soil area (SE), respectively, although it is still below the value found in the reference area (Table 1). For the levels of organic carbon (Corg) and total nitrogen, there were no significant increases with revegetation of the areas.

In the case of Corg, where areas 2A and 3A showed similar content to the SE area, it is still important to use the soil layer of origin (*Topsoil*) in the revegetation process, because this

soil, initially, behaves as a reserve of organic compounds and nutrients that will be mineralized and become available to plants and microbial biomass. Recently, studies have shown that use of *Topsoil* in the rehabilitation of mined areas improves the revegetation process, hence this practice is indicated whenever it is technically feasible (Ribeiro et al., 2018; Silva et al., 2018). However, input of C has to be continuous. in this case, by the effective restoration of vegetation in order promote carbon maintenance of soil and rehabilitation consolidation.

In addition, the low increase found in Corg can be considered as normal in the initial stages of rehabilitation, since Corg represents the fraction of soil carbon that is the most susceptible to decomposition by microbial biomass (Christensen et al., 2001; Singh et al. al., 2016). Thus, in addition to the fact that the soil is depleted of carbonic reserves and presents favorable physical-chemical attributes to make it a true C sink (Zhang et al., 2019), the period of rehabilitation of the areas is short.

C sequestration by revegetation consists of the transfer of atmospheric CO_2 to the vegetal biomass through photosynthesis and conversion of the vegetal biomass to organic carbon of the stable soil through the formation of organo-mineral complexes. Additionally, with the decomposition process, inorganic carbon is formed from bicarbonates and carbonates, which is associated with total soil carbon (FAO et al., 2017). All this process is also known as biosphere re-carbonization (soil and vegetation) (Lal et al., 2018).

Biosphere re-carbonization is an important strategy to mitigate environmental changes and improve ecosystem services (FAO et al., 2017; Lal et al., 2018) which, in this case, were disrupted by mining activities. This process involves the creation of a positive C balance in the soil through adoption of better management practices (Smith, 2016; Tang et al., 2017) that will have consequences for maintenance of microbial biomass (Yuan et al., 2017) and lead to an increase in total soil carbon stocks (Chen et al., 2018). It should be stressed that microbial

biomass not only promotes rotation of organic carbon in the soil, but is also directly involved in stabilization in the system (Zang et al., 2019).

In a study conducted by Kumar et al. (2018), in areas with different stages of rehabilitation after coal mining, there was lower particulate organic carbon content in comparison to no-particulate carbon in the first years (1, 2, 5 and 9 years). However, there was greater balance was between organic fractions with the advanced years of rehabilitation and accumulation of organic residues. The authors pointed as the main factor for low initial content of Corg the rapid decomposition and immobilization of the organic material by microbial biomass. Thus, with the advancement of vegetative development in the areas of the present study, it is believed that the levels of residues will increase and there will be greater equilibrium in the forms of carbon in the soil, as has also been reported in other studies (Bartuška et al., 2015; Zhang et al., 2019).

3.2. Soil biochemical attributes

The results of microbial biomass carbon (Cmic) did not differ between areas 2A and 3A; however, there were improvements as a result of revegetation (Table 2), with mean increase of 46% in comparison to the SE area, and recovery of approximately 81% of the Cmic was found in the reference area (RF). Nitrogen from microbial biomass (Nmic) in area 2A did not show a difference in comparison to the SE area; however, with vegetation advancement and rehabilitation time, area 3A showed an increase of 86% in comparison to the SE area, and there was recovery of 39% of the Nmic in the RF area.

Increases of Cmic in areas 2A and 3A show how revegetation of degraded areas and, consequently, input of carbon into the soil, was able to stimulate recovery of biochemical processes, because Cmic content represents the size of microbial biomass, the main mediators of the processes that occur in the soil (Fang et al., 2018; Fujii et al., 2018; Grebner et al., 2013).

This behavior indicates that C flux has been occurring satisfactorily in the rehabilitation areas, through plant residues and via rhizosphere deposition, and, in the short run, this attribute may be similar to the Cmic content found in the RF area.

Areas in rehabilitation 2A and 3A presented lower values of qCO_2 when compared to the value found in the SE area, positively reflecting advances in the recovery of the mined areas (Table 2), since lower values of metabolic quotient (qCO_2) indicate more stable edaphic conditions or environments that have almost reached a state of equilibrium. For CO_2 emission, no significant differences were found between the revegetated areas and the SE area.

The positive correlation of CO_2 emission with Corg and the reductions of qCO_2 are interpreted as positive aspects in the rehabilitation process, since they indicate present and active biomass, in addition to more balanced conditions in the ecosystem. The values found in the present study for these attributes were similar to results reported for CO_2 and qCO_2 emissions in the Canga ecosystem, with 14.08 mg CO_2 kg^{-1} h^{-1} and 29.44 mg h^{-1} CO_2 mg^{-1}, respectively (Silva et al. 2018).

In the activity of fluraceine diacetate (FDA), there were significant increases (Table 3), corresponding to a recovery of 53% and 68% of the FDA activity observed in the RF area in only 2 and 3 years of rehabilitation, respectively. Increased urease activity in areas 2A and 3A, and acid phosphatase and FDA in area 3A, as a function of rehabilitation time, indicate that there were improvements in the biochemical attributes of the soil, as well as resumption of important biogeochemical cycles in ecosystem soil.

The results of the present study are in line with results reported recently by Medeiros et al. (2017). The authors found increases in the activity of enzymes such as urease and acid phosphatase, and emphasized their potential as very sensitive tools for monitoring rehabilitation of environments. Pearson's correlation analysis showed that the activity of the study enzymes

improved in detriment to increases in C and N contents in the soil and growth of microbial biomass, a behavior also reported in other studies (Ciarkowska et al. 2014; Wahsha et al., 2017).

There were no significant increases for β-glucosidase activity (Table 3). Low β-glucosidase activity in the mined areas is due, in part, to a short period of rehabilitation and limited development of vegetation, since this enzyme is directly relational to carbon cycle and presence of organic material in soil. However, other factors may affect soil enzyme activity, such as temperature and humidity (Steinweg et al., 2012; Zhou et al., 2013), nutrient availability (Weand et al., 2010) and pH (Štursová and Baldrian, 2011).

A previous study in the literature about adsorption of β-glucosidase to montmorillonite as a function pH, showed that adsorption of the enzyme increased with pH reduction below 5.0 (Quiquampoix et al., 1993). This research brings relevant insights to our results regarding low β-glucosidase activity; although no data about mineralogical composition have been collected, adsorption may have occurred, since pH in the areas ranged from 4.01 to 4.45.

3.3. Soil bacterial metabolic activity

The heat map showed values of average color development and the areas were grouped according to degradation of carbon sources by soil bacterial communities (Figure 1). In ascending order, revegetated areas 2A, 3A and reference site RF presented higher rates of metabolic activity; the latter two were very similar regarding use of carbon sources. The present results for bacterial metabolic activity consist of recently published metagenomic data, and they are very similar in the taxonomic composition of the rehabilitation areas and reference sites in the Urucum massif (Gastauer et al., 2019). The behavior observed in the present study also indicates that the rehabilitation program was able to metabolically rehabilitate microbial communities.

Metabolic activity of bacterial communities, based on the use of different carbon sources, has been successfully used to characterize functional diversity in different environments (Fernandes et al., 2012; Leon et al. 2012). In the present study, all substrate groups available on the plate were used; this is an extremely important behavior in terms of diversity and metabolic activity capacity by the existing bacterial communities, mainly areas RF, 3A and 2A. The heat map showed that some carbon sources in the SE area presented low degradation by the bacterial communities, signaling limitations of the metabolism of those bacterial communities. Certainly, absence of cover plants reduces or limits the metabolic activity of these microorganisms.

Areas 3A and RF demonstrated wide use of carbon sources. The amino acids and carbohydrates of the microplate were the sources of C most used by the bacterial communities in these areas, because low nitrogen availability in comparison to carbon, as observed in Nmic and Nt contents, stimulates biomass use of more sources of organic nitrogen (Haleyur et al., 2018). Another factor that may have influenced the higher consumption of amino acids is that the study areas (Canga) are environments with limited water availability. Moreover, at the time of data collection, the dry period was ending in the region; under these conditions, bacteria usually specialize in the use of these compounds, where available, for osmoregulation process (Fierer et al., 2012).

In addition to amino acids and carbohydrates groups, in the areas 3A and RF there is also great use of carboxylic acids, for example, D-Malic Acid, which is one of the carbon sources that was not potentially degraded in the SE and 2A areas. Furthermore, the use of this organic substrate in the areas 3A and RF demonstrates the importance of plant presence and diversity for soil bacterial community metabolism (Frąc et al., 2012).

Some polymers and carboxylic acids present in the Biolog Ecoplate microplate, such as glycogen and α-ketobutyric acid respectively, were widely used in the SE area, demonstrating

that bacterial communities are specialized in metabolizing recalcitrant compounds. In this case, these compounds represent carbon reserve in the soil derived from the original soil layer (Topsoil), since there was no vegetation cover in this area. α-ketobutyric acid, for example, is an important intermediate compound that regulates the amount of proteins in the metabolism of microorganisms; also, it is used for production of amino acids (Coban and Demirci, 2017), a characteristic that indicates stimulus to the incorporation and increase of microbial biomass in this area.

3.4. Pearson's correlation between biological and biochemical attributes

Enzyme activity had positive and significant correlations (range of r = 0.63, at 0.96, $p < 0.05$), as with the other study attributes (Table 4), except for qCO_2, which did not show a significant correlation with FDA and acid phosphatase. Pearson's correlation coefficients for urease activity with organic carbon, total nitrogen and microbial biomass were 0.86, 0.70 and 0.82, respectively, significant at $p < 0.05$. Also, β-glucosidase and Corg had coefficient of 0.82 to $p < 0.05$. Nmic, Cmic and Corg showed positive correlations ($p < 0.05$) with most biological and biochemical attributes.

Correlation analysis between the biochemical attributes particularly showed a positive relationship between carbon contents and respiratory rate and enzyme activity of the soil, biochemical attributes of soil activity. That is, the contribution of organic matter positively influenced previously observed advances during a short period of rehabilitation in areas affected by mining activities.

3.5. Principal Component Analysis (PCA)

For further insights into behavior of the areas under rehabilitation, principal component analysis (PCA) was performed between the biochemical attributes and the physical-chemical

attributes, as shown in Figure 2 and Table 5. Together, components 1 and 2 (PC1 and PC2) accounted for 60.71% of variation, PC1 (horizontal axis) was responsible for the greatest variance of the attributes.

Biochemical attributes Ct, Nt, Cmic, Nmic, and higher biochemical activity of the soil evaluated by the enzymes urease, FDA, phosphatase and β-glucosidase and higher clay content, provided a better explanation for the isolation of the RF area. In area 2A, there were mainly higher percentages of the sand fraction and lower soil pH.

Area 3A showed greater proximity to the horizontal axis (PC1), where biochemical attributes were more expressive; however, still far from the RF area. The SE area showed low correlation with the soil attributes, and showed the negative effect of absence of vegetation on the biochemical attributes.

PCA showed that physico-chemical parameters that were most correlated with the areas under rehabilitation were pH and sand content, mainly in area 2A. However, the areas under rehabilitation were highly influenced by low carbon contents in the soil. These factors may potentially change the level and metabolic diversity of the soil, as found in the present study through the metabolic analysis of bacterial communities. This relationship is due to changes in soil pH in the solid phase equilibrium, nutrient availability and enzyme activity, which affect the development and composition of the plant community (Narendrula-Kotha and Nkongolo, 2017).

Furthermore, it should be noted that the initial success of revegetation was essential to continuous flow of soil organic matter, nutrient cycling and activation of microbial communities in the rehabilitation process (Silva et al., 2018a; al., 2016). In addition, monitoring studies of ecosystem services are essential, not only as a basis to future interventions, but also to make the mining activities more sustainable (Gastauer et al., 2018).

5. Conclusions

In areas 2A and 3A, the attributes Ct, Cmic, Nmic, Corg, CO_2, FDA activity, urease and acid phosphatase demonstrated greater sensitivity to soil changes as a result of the revegetation of the areas. Importantly, the study areas were submitted to rehabilitation over a period of 2 and 3 years and, despite the short period, there were improvements in soil biochemical attributes in comparison to the exposed soil area. However, a longer period is needed for biochemical attributes to fully restore or resemble native areas.

The metabolic activity of areas 2A and 3A shows that there was no loss of functional diversity; however, it indicated preferences in the use of carbon sources, a situation that reflects the metabolic activity of bacterial communities under the current conditions of the areas. As in other studies, this analysis can be considered as a good indicator in the monitoring of the mined areas, since it was shown to be sensitive to the dynamics of the metabolisms that occur in the soils and to the changes that occur during the rehabilitation process.

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Table 1. Total soil nitrogen (Nt), total carbono (Ct), organic carbon (Corg), from areas at different stages of rehabilitation after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

Area	Nt	Ct	Corg
	%	%	%
SE	0.23±0.09b	1.77±0.48c	1.82±1.36b
2A	0.24±0.10b	2.73±0.55bc	1.84±2.82b
3A	0.24±0.10b	3.20±0.35b	2.03±2.47b
RF	0.67±0.08a	8.43±0.77a	4.56±5.62a

SE: Exposed soil: no rehabilitation intervention; 2A: two years in rehabilitation; 3A: three years in rehabilitation; RF: reference area. Equal letters do not differ from each other at p <0.05 by the Skott-Knott test.

Table 2. Microbial biomass carbon (Cmic), microbial biomass nitrogen (Nmic), soil microbial respiration (C-CO2), metabolic quotient (qCO_2), from rehabilitation areas after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

Area	β -gluc	FDA	Urease	Fosfatase
	µg PNG g^{-1} h^{-1}	µg F g^{-1} h^{-1}	µg NH_4 g^{-1} h^{-1}	µg PNF g^{-1} h^{-1}
SE	21.88±3.05b	90.83±20.57c	2.07±0.42d	209.34±18.93c
2A	25.49±0.74b	154.53±44.81bc	4.00±0.36c	324.83±59.48bc
3A	27.58±4.12b	197.74±48.83b	6.09±1.45b	398.01±24.84b
RF	206.17±2.79a	291.98±25.45a	22.41±1.24a	1430.58±140.52a

SE: Exposed soil: no rehabilitation intervention; 2A: two years in rehabilitation; 3A: three years in rehabilitation; RF: reference area. Equal letters do not differ at p <0.05 by the Scott-Knott test.

Table 3. Enzymatic activity of soil: β-glucosidase, activity of fluracein diacetate hydrolyzate (FDA), urease and acid phosphatase, from areas at different stages of rehabilitation after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

Area	Cmic	Nmic	CO_2	qCO_2
	µg g^{-1}	µg g^{-1}	mg CO_2 kg^{-1} h^{-1}	mg h^{-1} CO_2 $mg^{-1}C$
SE	254.93±22.22c	1.09±0.09c	10.24±2.03b	40.63±7.72b
2A	368.61±82.22b	1.62±0.31bc	12.38±1.97b	33.58±7.58b
3A	377.68±53.68b	2.03±0.53b	13.19±0.89b	34.92±6.57b
RF	460.16±8.37a	5.21±0.25a	25.29±1.36a	55.04±3.23a

SE: Exposed soil: no rehabilitation intervention; 2A: two years in rehabilitation; 3A: three years in rehabilitation; RF: reference area. Equal letters do not differ from each other at p <0.05 by the Skott-Knott test.

Table 4. Coefficients of Pearson Correlation between biochemical attributes of rehabilitation areas after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

	Corg	Nt	Ct	CO_2	qCO_2	NBM	CBM	Ure	β - gluc	FDA	Fosf
Corg	1.00	0.82*	0.85*	0.74*	0.49*	0.82*	0.86*	0.86*	0.86*	0.55*	0.82*
Nt		1.00	0.76*	0.61*	0.55*	0.68*	0.14ns	0.70*	0.75*	0.51*	0.71*
Ct			1.00	0.79*	0.40ns	0.89*	0.55*	0.92*	0.88*	0.73*	0.93*
CO_2				100	0.68*	0.87*	0.63*	0.91*	0.88*	0.71*	0.87*
qCO_2					100	0.53*	-0.09ns	0.51*	0.53*	0.33ns	0.47ns
Nmic						1.00	0.58*	0.92*	0.91*	0.75*	0.91*
Cmic							1.00	0.61*	0.53*	0.60*	0.58*
Ure								100	0.92*	0.70*	0.96*
β-gluc									100	0.63*	0.90*
FDA										1.00	0.73*
Fosf											100

Organic carbon (Corg), total soil nitrogen (Nt); (C), microbial soil respiration (CO_2), metabolic quotient (qCO_2), microbial biomass nitrogen (Nmic), microbial biomass carbon (Cmic), urease (Ure), β-glucosidase (β-gluc), fluracein diacetate (FDA) hydrolyzate activity, and acid phosphatase (Fosf); (*) Correlation $p < 0.05$ and (ns) non-significant correlation.

Table 5. Rotated loadings on the PC1 and PC2 of the PCA

Attributes	Principal Components	
	PC1	PC2
pH_cacl	-0.4358	0.5210*
Corg	0.9031*	-0.1107
P	0.2023	0.6566*
S	0.7543*	0.1746
Ca^{2+}	0.2945	0.7974*
Mg^{2+}	0.5868*	0.4443
Al^{3+}	0.8516*	-0.3363
B	0.6548*	-14.69
Zn	0.6723*	0.3565
Mn	0.1556	0.7879*
Nt	0.7993*	-0.0200
Ct	0.9348*	-0.0398
CO_2	0.8841*	0.0753
qCO_2	0.5394*	-0.0271
Cbm	0.5379*	0.1345
Nbm	0.9132*	0.0536
urease	0.9476*	0.0527
Fosfatase	0.9264*	-0.0251
FDA	0.6954*	0.1739
β-glucosidase	0.9527*	-0.0330
areia	-0.4986	0.5921*
silte	0.6090*	-0.3111
argila	0.1065	-0.6552*

*Variables with significant projections over the principal componentes (loadings >0.05)

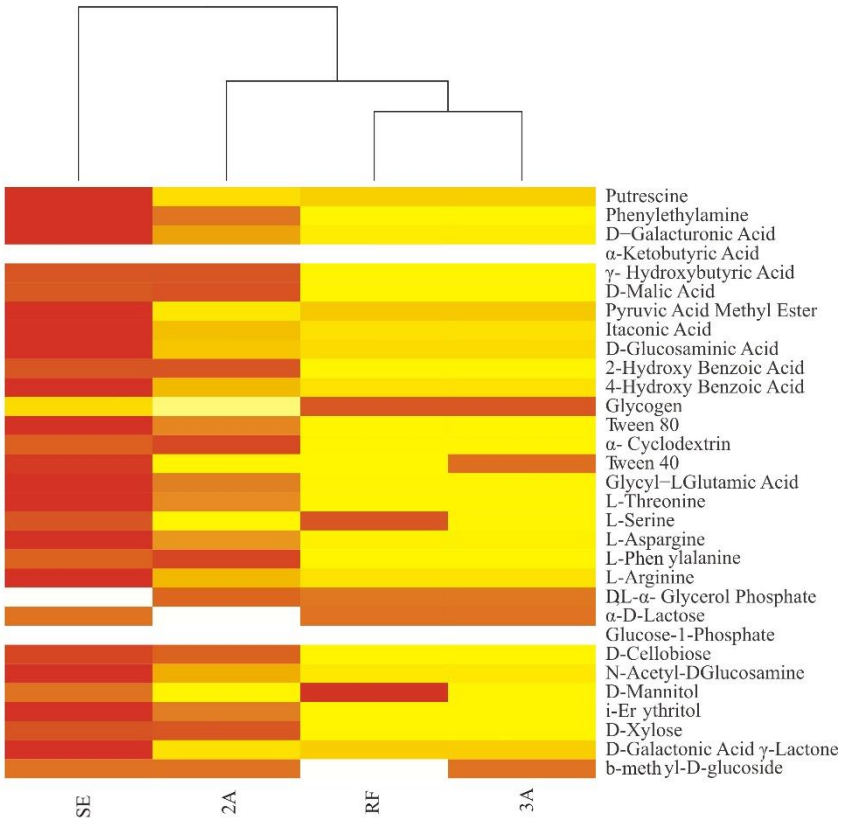

Figure 1. Heat map and hierarchical clustering the areas under study from the average values of color development of each well (AWCD) having based the use of carbon sources 31 during the 72 hours incubation of the microplate Ecoplate. RF (reference area); 3A (three years in rehabilitation); 2A (two years in rehabilitation); SE (only exposed).

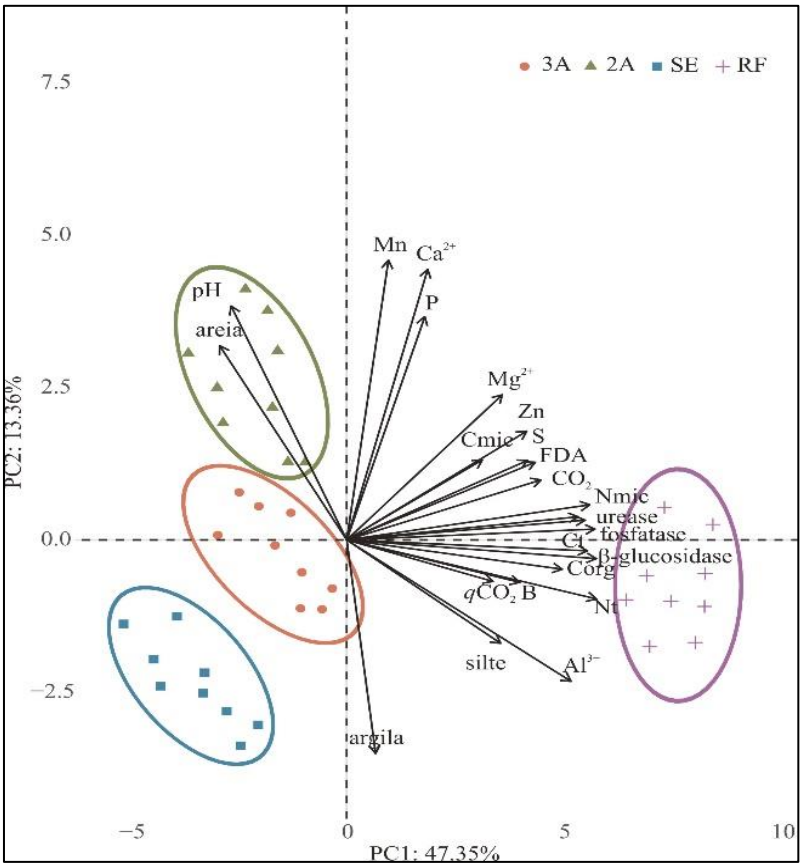

Figure 2. Principal Component Analysis (PCA) relating chemical and physical atributes and biological indicators of soils of areas in different rehabilitation stages after iron mining in Corumbá, MS, Brazil. SE (Exposed Soil); 2ª (two years rehabilitation); 3A (three years rehabilitation); RF (reference area).

Table A1. Physico-chemical characterization of rehabilitation areas after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

Area	pH	P	K^+	Ca^{2+}	Mg^{2+}	Al^{3+}	$Al+H^+$	Zn	Fe	Mn	Cu	B	Areia	Silte	Argila
	Cacl	mg dm^{-3}	----------$cmol_c$ dm^{-3}----------					----------mg dm^{-3}----------					----------%----------		
SE	4.14	2.08	0.96	0.43	0.16	0.76	7.85	0.47	65.92	40.37	0.35	0.28	46.44	21.88	31.66
2A	4.45	6.14	2.71	1.15	0.31	0.46	6.35	0.86	105.22	74.25	0.45	0.27	51.62	21.26	27.11
3A	4.34	3.68	1.75	0.82	0.21	0.38	6.86	0.62	104.32	62.63	0.42	0.26	46.85	26.58	26.55
RF	4.01	5.54	2.78	1.05	0.45	2.21	19.00	1.90	100.08	65.04	0.51	0.47	35.97	34.13	29.88

The physico-chemical characterization was determined according to the methods described by Embrapa (1997). SE: Exposed soil: no rehabilitation intervention; 2A: two years in rehabilitation; 3A: three years in rehabilitation; RF: reference area.

Table A2. Bacterial metabolic diversity of 31 carbon sources, incubated during the 72 hours period on microplates of the biological test Biolog EcoPlate.

Grupos das Fontes de C	Fontes de C	SE	2A	3A	RF
		AWCD			
Carbohydrates	b-methyl-D-glucoside	0	0	0	0.005763
	D-Galactonic Acid γ-Lactone	0.020806	0.029806	0.029312	0.029312
	D-Xylose	0.00086	0.001065	0.009172	0.009172
	i-Erythritol	0.001118	0.004645	0.008968	0.008968
	D-Mannitol	0.025333	0.040656	0.039925	0.017839
	N-Acetyl-DGlucosamine	0.006054	0.012581	0.01429	0.01429
	D-Cellobiose	0	0.003351	0.020882	0.020882
	Glucose-1- Phosphate	0	0	0	0
	α-D-Lactose	0	0.000993	0	0
	D,L-α- Glycerol Phosphate	0.004366	0.002075	0.002301	0.002301
Amino Acid	L-Arginine	0.009172	0.02871	0.03214	0.03214
	L-Phenylalanine	0.000548	0.000305	0.001839	0.001839
	L-Asparagine	0.029559	0.045172	0.053935	0.053935
	L-Serine	0.024204	0.036473	0.035656	0.024204
	L-Threonine	0.000337	0.000928	0.00143	0.00143
	Glycyl-LGlutamic Acid	0.001075	0.00443	0.008312	0.008312
Polymer	Tween 40	0.019054	0.029527	0.022129	0.030645
	α- Cyclodextrin	0.000186	0.000165	0.000312	0.000312
	Tween 80	0.016849	0.026151	0.035312	0.035312
	Glycogen	0.000649	0.000878	0.000118	0.000118
Phenolic compounds	4-Hydroxy Benzoic Acid	0.009118	0.025398	0.028054	0.028054
	2-Hydroxy Benzoic Acid	0	0	0.000667	0.000667
Carboxylic acids	D-Glucosaminic Acid	0.006462	0.023312	0.024591	0.024591
	Itaconic Acid	0.012548	0.024914	0.026559	0.026559
	Pyruvic Acid Methyl Ester	0.008376	0.009065	0.008989	0.008989
	D-Malic Acid	6.09E-05	0	0.00129	0.00129
	γ- Hydroxybutyric Acid	0	0	0.012161	0.012161
	α-Ketobutyric Acid	0	0	0	0
	D-Galacturonic Acid	0.02843	0.047022	0.054527	0.054527
Amines	Phenylethylamine	0	0.000229	0.000613	0.000613
	Putrescine	0.007398	0.02629	0.025333	0.025333

AWCD - values of the average color development of each well corrected.; SE (exposed soil, no vegetation); 2A (two years in rehabilitation);3A (three years in rehabilitation); RF (reference area, with native vegetation).

SEGUNDA PARTE – ARTIGOS

ARTIGO 2 - Arbuscular mycorrhizal fungi and glomalin contributions in carbon stocks of rehabilitation areas after iron mining

***Article written according to the rules of Soil Biology and Biochemistry**
(Versão preliminar)

Arbuscular mycorrhizal fungi and glomalin contributions in carbon stocks of rehabilitation areas after iron mining

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Abstract – Arbuscular mycorrhizal fungi (AMF) are well known in rehabilitation process of mining areas because they play important ecological functions, such as glomalin production, which acts as cementing agent, nutritional reserve and contributes significantly to carbon stocks. Thus, the aim of this study was to investigate the status of AMF and their contribution to the carbon stocks of rehabilitation areas after iron mining. Were analyzed the species richness, mycorrhizal colonization, number of spores, glomalin-related protein fractions and their contribution in the soil carbon stock. Species richness has been reduced by about 50% with mining activity. However, with the revegetation of the areas, we observed an increase in the number of AMF species. Families Glomeraceae (*Glomus sp1*.) and Acaulosporaceae (*Acaulospora mellea*) were considered dominant in the studied areas. The spore density also increased with reintroduction of vegetation. Total and easily extractable soil protein content glomalin-related was increasing with age revegetation. However, the contribution of glomalin showed reductions with greater contribution in soil carbon stocks in the newly extracted area without vegetation cover. This result reveals the important role that glomalin plays in soil, mainly as carbon reserve in environment disturbed by mining. In addition, our results indicate that improvements in AMF status are positively related to soil carbon stocks and the recovery of ecological functions in mining areas during rehabilitation.

Keywords: revegetation, environmental assessment, glomalin, carbon stocks

1. Introduction

Efficient rehabilitation of areas after mining is a requirement of supervisory agencies and essential for greater sustainability of mining activities, besides the important mission of rehabilitating local ecosystem functions. In this sense, techniques based on plant biodiversity and soil microorganisms are used (Kumar et al., 2018). Revegetation, for example, is an economically viable rehabilitation technique that promotes rapid environmental responses due to the reintroducing continuous flow of carbon in soil. In addition, directly stimulates microbial communities to resumption their soil functions.

Arbuscular mycorrhizal fungi (AMF) are well known during the mining area rehabilitation process (Smith et al., 2011; Gu et al., 2017; Yang et al., 2017) as these microorganisms are able to form mutualistic symbiosis with more than 80% of vegetables and play important ecological function, and contribute to the water and nutrients uptake inaccessible to the root system, aggregate stability and increased tolerance of many plant species in relation to the high concentrations of some metals (Smith and Read, 2010; Carrenho et al., 2018).

AFMs are responsible for production of glomalin, a stable soil glycoprotein that due to its chemical complexity is operationally defined as glomalin-related soil proteins (GRPS), and can be determined from the soil as easily extractable (EE-GRPS) and total (T-GRPS) (Singh et al., 2013). GRPS typically contain 3 to 5% of N, 30 to 60% of C, 0.03 to 0.1% of P, and 2 to 5% of Fe (Lovelock et al., 2004; Schindler et al., 2007; Singh et al., 2013) contributing approximately 5 to 10% of C content, which makes them an important mechanism for increasing soil C stocks (Rillig et al., 2004; 2006; Malekzadeh et al., 2016; Xu et al., 2017).

Among other functions, GRSP acts as biological cementing agent, which mineral particles promoting aggregation (Qin et al, 2017; Ji et al, 2019) and soil stability aggregates

(Rillig et al., 2004; Rillig, 2014; Yang et al., 2017; Wang et al., 2019). In addition, studies show their effect on complexing toxic elements (Gonzalez-Chavez et al., 2004; Gao et al., 2018b).

Recently, were reported high contributions of GRSP on total soil carbon content, 4.1 and 8.1% in areas with 1 and 2 years of revegetation, respectively (Kumar et al., 2018). The authors attributed behavior with age and vegetative development of the area, factors that stimulate symbiotic association with AMF and glomalin production.

Considering the link between soil carbon and AMF functionalities, the aim this study was to investigate the status of AMF and their contribution to rehabilitation and carbon stock after iron mining in the Urucum Mineral Complex, Corumbá, Mato Grosso do Sul, Brazil.

2. Material and Methods

2.1. Characterization of study areas

Soil samples were collected from mineral complex at Corumbá, Mato Grosso do Sul, Brazil. The climate in region is Aw according to Köppen Classification, with dry winters and rainy summers. The average annual temperature is 25.4 °C, and average annual rainfall is 1074 mm.

Three mining areas were sampled, representing different stages: recently mined (without vegetation), with 2 and 3 years after revegetation and areas with native vegetation. In all areas, the same recovery plan was carried out, consisting of base fertilization, topsoil layer deposition, sowing and planting seedlings of native plant species, as authorized by the competent authorities.

Data collection occurred in 2016. At each stage, three samples were collected; they were composed of soil at different points, with minimum distance of 50 m between them. Each sample was collected from five points homogeneously distributed within a 10 x 10 m parcel, at the 0-20 cm layer.

Subsequently, the samples were stored in sterile plastic bags, refrigerated and sent to the Laboratory of Microbiology and Biochemical Processes of Soil at the Federal University of Lavras, MG. The samples were sieved in 4 mm mesh and stored in a cold room at 4 °C for further biological and chemical analyses. Part of the material was separated to determine soil physicochemical attributes (Embrapa, 2009), as shown in Table 1.

2.2. Total soil carbon

Total soil carbon (TC) were determined by elemental analysis. The samples were placed in tin capsules, and the material was digested in a combustion chamber at a temperature of approximately 975 °C. The gases were detected and converted into percent carbon. The equipment used was a CHNS/O universal elemental analyzer (Vario Micro Cube model). Organic carbon (Corg) was determined by oxidation with potassium dichromate in the presence of H_2SO_4, followed by titration of excess dichromate in the sample with ammoniacal ferrous sulfate and diphenylamine (1%) as an indicator (Walkley and Black, 1934).

2.3. Mycorrhizal colonization

For the evaluation of mycorrhizal colonization (MC), root samples were taken from sample soil, washed several times with running tap water. The root segments were first softened with 5% KOH at 90 °C in a water bath for 40 min, bleached with fresh alkaline H_2O_2 at room temperature for 30 min, acidified with 2% HCl for 10 min, and then stained in 0.05% / v) trypan

blue solution (200 mL phenol, 0.5 g trypan blue, 250 mL lactic acid, 250 mL glycerol, and 300 mL distilled water) at room temperature for 2 h.

The stained root samples were then transferred to glycerol and incubated for 12 h at room temperature. Mycorrhizal colonization was estimated by means of the intersection method on a plaque with a 40-fold magnification binocular loupe (Giovannetti & Mosse 1980). The mycorrhizal colonization of each area was determined by the occurrence of fungal structures.

2.4.Spore density and Taxonomic identification of AMFs

AMFs spores were isolated from soil samples using wet sieving method (Gerdeman and Nicolson, 1963). Briefly, fifty grams of soil from each sample was independently suspended water, stirred with a glass stirring rods for 5 min. The suspension passed through a sequence of sieves. Spores were collected from the last two sieves with tap water, were counted and categorized live and dead, with the aid of the stereoscopic microscope. AMF spore density (SD) was expressed as number of spores in 50 g of dry soil.

Apparently viable spores were observed in a microscope, separated for areas studied and mounted on slides with polyvinyl lactoglycerol (PVLG) and Melzer reagent for observation on a microscope and taxonomic identification through spore morphology, as for example color, size, ornamentation e cell wall. AMF species were identified in comparison with the descriptions contained in the International Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, 2016) and Blaszkowski (2012).

2.5.Determination of Glomalin-Related Soil Protein - GRSP: Easily Extractable and Total Soil

The concentrations of easily extractable glomalin-related soil protein (EE-GRSP) and total glomalin-related soil protein (T-GRSP) were measured according to procedures described

by Wright and Upadhyaya. Briefly, 1 g soil sample was placed into a centrifuge tube. The EE-GRSP was incubated with 8 mL of 20 mM citrate solution (pH 7.0), autoclaved at 121ºC for 30 min, and then centrifuged at 3300,000 g for 30 min to remove residual soil particles. The T-GRSP was extracted with 8 mL of 50mM citrate solution (pH 8.0) by autoclaving at 121ºC for 60 min.

After each autoclaving cycle, the supernatant was removed by centrifugation at 3300,000 g for 30 min for sequential extraction. The extraction of a soil sample continued until the supernatant showed none of the black-brown-yellow color. Protein in the supernatant was determined by the Bradford dye-binding assay with bovine serum albumin as a standard. The contribution of GRSP to soil carbon was revealed by the GRSP/Corg and GRSP/TC ratio.

2.6.Statistical analysis

The Shapiro-Wilk test was used to determine the normality of data. After meeting the normality and homoscedasticity criteria, the linear mixed effects model (LMMs) was applied, using the 'lme4' package, where the mining areas were determined as a fixed factor, the rehabilitation stages in each area as a random factor and the attributes biological and biochemical responses as variables. The averages of the biological and chemical attributes were compared by the Scott-knott test at $p < 0.05$. Pearson's correlation was determined using the 'Hmisc' package. All analyses were performed in R, version 3.4.1 (R Development Core Team, 2017).

3. Results and Discusion

3.1. Species Richeness, Mycorrhizal colonization and AMF spore density

Total species richness was 39 species, distributed in 8 families and 14 genus of the Glomeramycota phylum (Table 2). Among the studies areas the highest richness was observed

in the reference area, and lowest in exposed soil area, with 26 and 12 species, respectively. This result shows how mining activity negatively impacted AMF diversity. Normally, anthropic interventions cause severe reductions in AMF species diversity, as reported in other studies (Oehl et al., 2010; Verbruggen et al., 2010; Xião et al., 2019).

The most abundant species in the samples are classified in Glomeraceae families (*Glomus sp1*) and Acaulosporaceae (*Acaulospora mellea*), being considered dominant in the studied areas. Our results agree with the results reported by Texeira et al. (2017) who also reported species of these families as generalists in the Iron Quadrangle mining areas.

Acaulospora morrowiae, *Dentiscutata heterogama*, *Gigaspora margarita*, *Glomus glomerulatum* and *Rhizophagus clarus* were also common in mining areas at Urucum complex (Table 2 and Figure 1). Studies conducted by Ortega-Larrocea et al. (2010) and Xu et al. (2017) in rehabilitated mining areas, observed that the most abundant AMF species in the rhizosphere were Glomus and Acaulospora genus, as well as our results.

Additionally, we also observed that the number of spores increased significantly ($p<0.05$) with the reintroduction of vegetation in areas under rehabilitation, about 3 times the number of spores found in area without vegetation cover (Figure 2). This variable is indicative of the resumption of mycorrhizal functionality in areas, since it is one of the main forms of AMF propagation.

Root colonization rate by AMFs also increased significantly ($p <0.05$) among the studied areas, being observed in area with 3 years an increase of 6% in colonization in comparison to the area with 2 years and 92% compared to exposed soil area. Low colonization in exposed soil area was already expected due the vegetation absence. In reference area, with native vegetation, fungal structures were observed in 51% of evaluated root fragments.

3.2.Glomalin-related soil protein fractions (GRSP) and contribution to soil carbon sequestration

Easily extractable glomalin-related soil protein contents (EE-GRSP) were not significantly different between the revegetation stages, however, increases of 9.13% and 20.90% were observed in 2 and 3-year-old areas, respectively, compared to exposed soil.

In contrast, there was a significant and significant increase of 41.25% in total glomalin-related soil protein content (T-GRSP) of areas under rehabilitation when compared to exposed soil area, and recovery of approximately 50% of content found in reference area (Figure 2).

The dominance and species richness of the Glomeraceae and Aucalosporaceae families in reference area may be responsible for the higher GRSP content in this area. Previous studies showed that Acaulosporaceae, Gigasporaceae and Glomeraceae species showed high levels of glomalin (Lovelok et al., 2004).

Figures 2 and 3 show that the recovery of T-GRSP contents in areas under rehabilitation is synergistic with the increased colonization rate. This result demonstrates the direct binding of GRSP to carbon sequestration, as this glycoprotein can represent up to 15% of soil C reserves (Rillig et al., 2001; 2003; Schindler et al. 2007; Fokomet al., 2012).

Reports in literature have shown that GRSP is able to contribute more than 20 times to soil carbon stocks compared to microbial biomass (Sinsabaugh et al., 2009). In addition, due to its hydrophobic bonding and formation of the “sticky wire bag” structure formed by hyphae, GRSP reduces the volume of organic carbon and increases its sequestration in terrestrial ecosystems (Rillig, 2004; Smith and Read, 2008; Lehmann and Rillig, 2015). Therefore, GRSP concentrations are considered as a sensitive indicator of soil quality (Zhang et al., 2017).

The largest T-GRSP / TC contribution was observed in exposed soil and rehabilitated areas at 2 and 3 years (Figure 3). We attribute this behavior to the fact that in these areas carbon inputs are still limited and GRSPs are one of the main sources of carbon in this period. Contrary

to what occurs in reference area, in addition to plant biomass, microbial communities are established and contributing to increases in soil carbon stocks. However, our results differ from those found by Jiang et al. (2019) who observed increasing contributions with soil age.

Additionally, the behavior of the T-GRSP / TC relationship observed in our study reinforces the importance of GRSP and, consequently, the communities of AMFs during the rehabilitation of areas. Several studies have shown high contributions from GRSP to carbon stocks, including mine rehabilitation areas (Rillig et al., 2003; Lovelock et al., 2004; Luna et al., 2016; Kumar et al., 2018; Al Maliki et al., 2018; Xiao et al., 2019).

3.3.Pearson Correlation

Positive and significant correlations between T-GRSP and CT and Corg fractions (Figure 3) found in our results have been reported in other studies (Gispert et al., 2013; Gao et al., 2017; Yang et al., 2017). This results may be related not only to the contribution to carbon stock, but also to the fact that they share similar functions in the soil, such as high contribution in aggregation of mineral particles (Bronick and Lal, 2005; Rillig et al., 2003 Wu et al., 2014; Ji et al., 2019).

T-GRSP, Corg, TC, RC, SN contents correlated negatively with sand content (Figure 3), because high levels this textural fraction affect aggregation, leaving carbonic reserves and microbial communities unprotected and exposed to external factors, such as oxidation and erosion which can slow down ecological restoration.

The positive and significant correlation between mycorrhizal colonization and Fe availability indicates that AMFs can be potentially Fe accumulators, interesting mechanisms in the initial vegetation rehabilitation process. In addition, mycorrhizal colonization showed a positive and significant correlation with T-GRSP, Corg, TC levels.

Correlations observed in our results strongly reflect the AFMs activity, because it is through the colonization of root tissues that these obligatory biotrophic microorganisms obtain energy source for the development of extraradicular mycelia, which besides contributing to carbon stocks in soil, and production of important substances such as glomalin (Driver et al., 2005; Treseder and Turner, 2007; Xu et al., 2017), directly increase nutrient and water uptake for host plants (Marulanda et al., 2003; Chen and Zhao, 2009; Javaid, 2009), essential factors for plant survival in rehabilitation areas.

Mycorrhizal activity is extremely important in rehabilitation programs, as they are fungi capable of improving soil properties, contributing directly to carbon stocks and ecological restoration (Qin et al., 2017; Yang et al., 2017; Kumar et al. al., 2018).

4. Conclusion

GRSP and CT accumulation increased with the progress of rehabilitation. These increments, among other factors, are linked with the age of revegetation and root richness and colonization by AMF.

Our data indicate high dependence of soil C on T-GRSP levels, especially from younger areas where C inputs are even more limited. Also, improvements in the status of AMFs, which provided increases in GRSP content, are a positive sign for increases in soil carbon stock and recovery of ecological functions in the short rehabilitation period after mining.

Table 1. Physico-chemical characterization of rehabilitation areas after iron mining in Corumbá, Mato Grosso do Sul, Brazil.

Area	pH	P	K^+	Ca^{2+}	Mg^{2+}	Al^{3+}	Al+H	Fe	Areia	Silte	Argila
	$CaCl_2$	mg dm^{-3}	----------$cmol_c$ dm^{-3}----------					mg dm^{-3}	----------%----------		
SE	4.14	2.08	0.96	0.43	0.16	0.76	7.85	65.92	46.44	21.88	31.66
2A	4.45	6.14	2.71	1.15	0.31	0.46	6.35	105.22	51.62	21.26	27.11
3A	4.34	3.68	1.75	0.82	0.21	0.38	6.86	104.32	46.85	26.58	26.55
RF	4.01	5.54	2.78	1.05	0.45	2.21	19.00	100.08	35.97	34.13	29.88

physico-chemical characterization was determined according to the methods described by Embrapa (1997). SE: Exposed soil: no rehabilitation intervention; 2A: two years in rehabilitation; 3A: three years in rehabilitation; RF: reference area.

Table 2. Taxonomic identification, richness (N°. of species for area) and frequency (%) of AMFs in areas under rehabilitation after iron mining. Exposed soil (SE); area with two (2A) and three years in rehabilitation; reference area (RF).

Family	Specie	SE	2 years	3 years	RF	Frequency (%)
Paraglomeraceae	*Paraglomus occultum*	+	-	-	+	6.060606061
Archaeosporaceae	*Archaeospora myriocarpa*	-	-	+	-	6.060606061
Ambisporaceae	*Ambispora leptoticha*	+	+	-	+	9.090909091
	Ambispora sp1	-	-	+	-	3.03030303
Acaulosporaceae	*Acaulospora foveata*	+	-	+	+	15.15151515
	Acaulospora mellea	+	+	+	+	87.87878788
	Acaulospora morrowiae	+	+	-	+	33.33333333
	Acaulospora spinosissima	-	+	+	+	9.090909091
	Acaulospora tuberculata	+	+	-	+	18.18181818
	Acaulospora sp1	+	+	-	+	33.33333333
	Acaulospora sp2	-	-	-	+	6.060606061
	Acaulospora sp3	-	-	+	-	3.03030303
Diversisporaceae	*Diversispora* sp1	-	+	+	+	45.45454545
	Diversispora sp2	-	-	-	-	3.03030303
	Diversispora sp3	-	-	-	+	6.060606061
	Diversispora sp4	-	-	-	+	3.03030303
Gigasporaceae	*Cetraspora pellucida*	-	+	-	+	18.18181818
	Dentiscutata biornata	-	+	-	-	6.060606061
	Dentiscutata heterogama	+	+	-	+	33.33333333
	Dentiscutata savannicola	-	-	+	-	3.03030303
	Gigaspora albida	-	+	-	+	12.12121212
	Gigaspora gigantea	-	-	+	-	3.03030303
	Gigaspora margarita	-	+	-	+	33.33333333
	Racocetra cf. *fulgida*	-	-	+	-	6.060606061
	Racocetra verrucosa	-	+	-	-	6.060606061
	Scutellospora sp1	-	+	+	-	12.12121212
Glomeraceae	*Dominikia* sp1	-	-	+	+	6.060606061
	Dominikia sp2	-	-	+	+	6.060606061
	Glomus glomerulatum	+	-	-	+	30.3030303
	Glomus microaggregatum	-	-	+	+	6.060606061
	Glomus sp1	+	-	+	+	100
	Glomus sp1	-	+	+	+	18.18181818
	Rhizophagus clarus	-	+	+	+	36.36363636
	Rhizophagus fasciculatus	-	+	+	+	15.15151515
	Rhizophagus sp1	-	+	-	+	3.03030303
	Rhizophagus sp2	-	-	-	-	3.03030303
	Sclerocystis rubiformis	+	+	-	+	6.060606061
	Septoglomus cf. *altomontanum*	-	-	-	-	15.15151515
Incertae sedis	*Entrophospora infrequens*	+	+	-	+	3.03030303
Riqueza (Nº de espécie)		12	20	18	26	

Figure 1. Photomicrographs of PVLG and PVGL / Melzer permanent AMF spore slides from rehabilitated areas after iron mining.

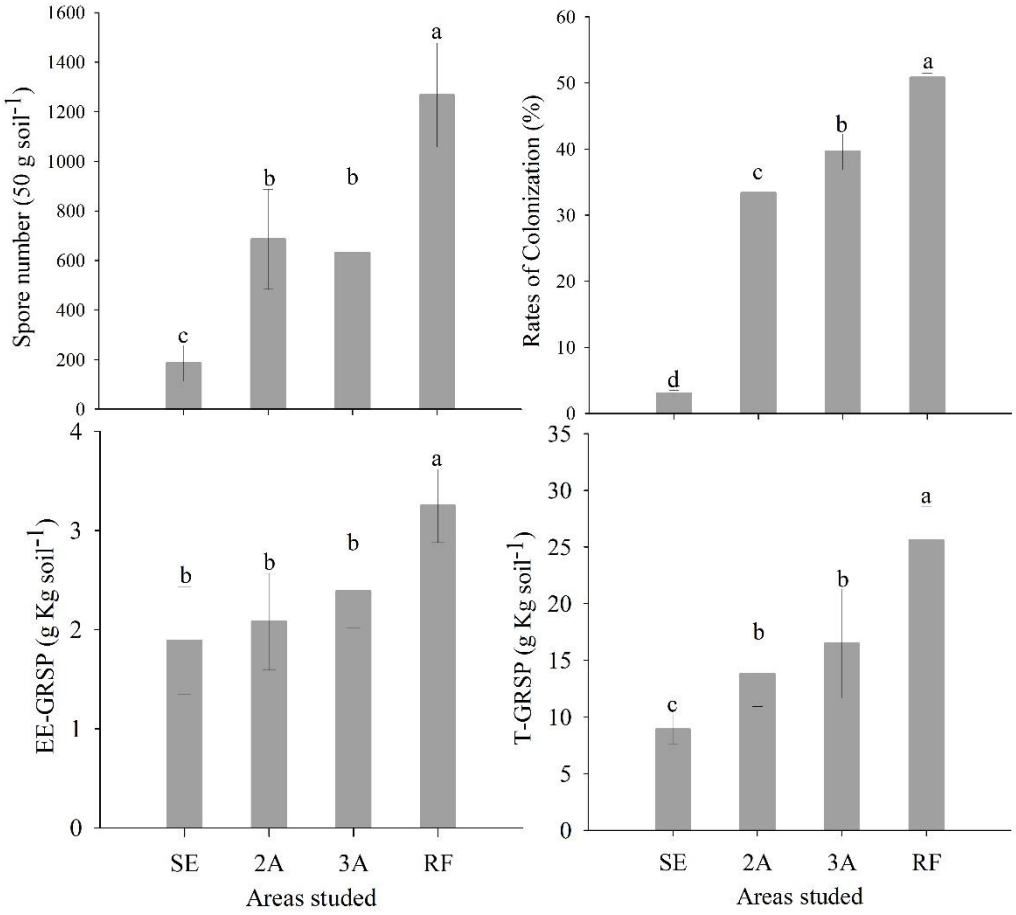

Figure 2. Spore Number (SN), micorrizal colonization (MC), easily extractable (EE-GRSP) and total (T-GRSP) glomalin-related soil proteins in rehabilitation areas after iron mining. Exposed soil (SE); area with two (2A) and three (3A) years in rehabilitation; reference area (RF). Bar corresponding to standard error.

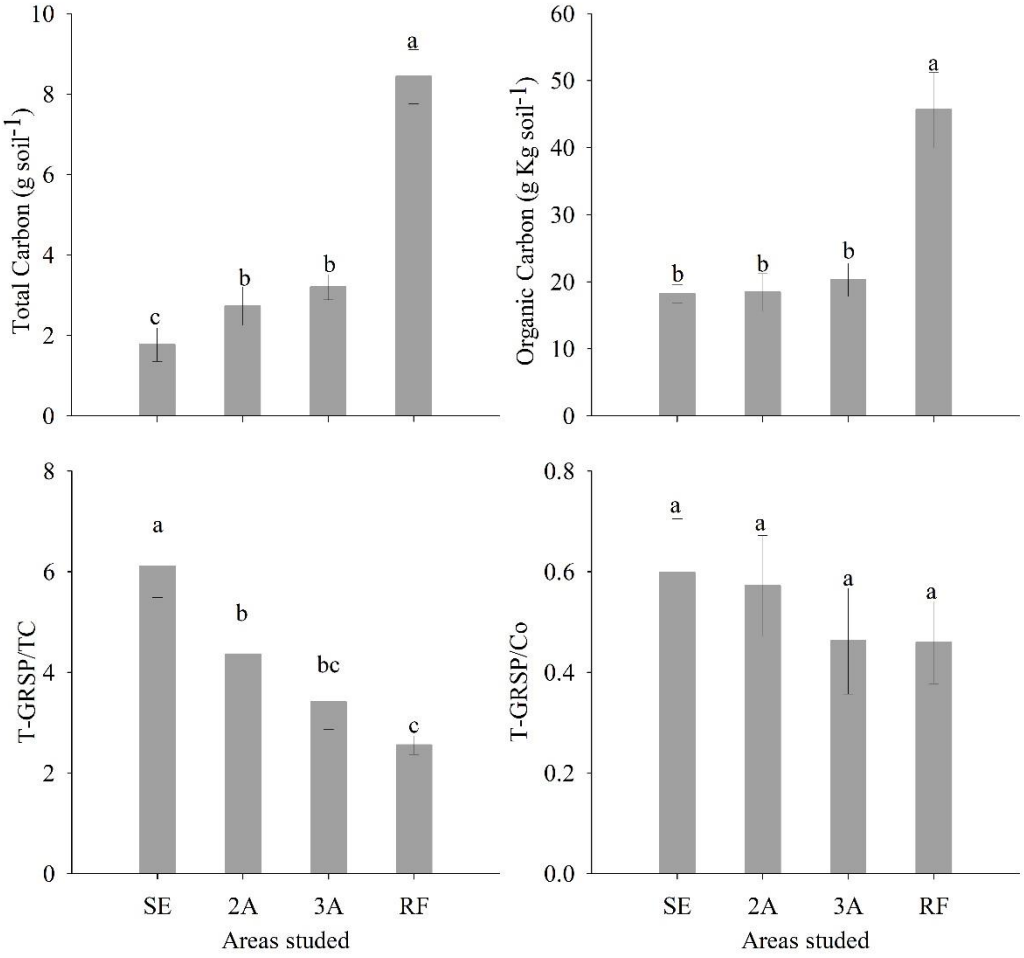

Figure 3. Total carbon (TC) and Organic carbon (Corg); T-GRSP/TC and T-GRSP/Corg ratio in rehabilitation areas after iron mining. Exposed soil (SE); area with two (2A) and three years in rehabilitation; reference area (RF). Bar corresponding to standard error.

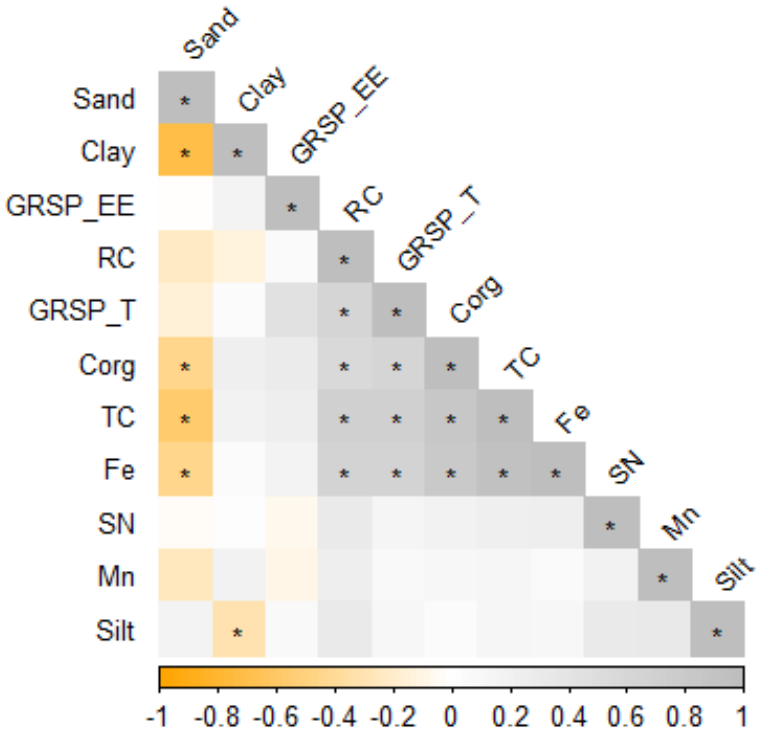

Figure 3. Person correlation between AMF activities and chemical and physical attributes of rehabilitated areas after iron mining. (*) significant correlation at $p < 0.05$.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 3 - Changes in pH, CO_2 emission and composition of bacterial communities after the addition of biochar to soil of affected area with the mining tailings in Mariana, Minas Gerais - Brazil

***Article written according to the rules of Science of the Total Environment**
(Versão preliminar)

Changes in pH, CO_2 emission and composition of bacterial communities after the addition of biochar to soil of affected area with the mining tailings in Mariana, Minas Gerais – Brazil

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Abstract - Tailings produced during the ore process are potential sources of contamination, as most of them have metals that are toxic to environmental function and health human. With the disruption of tailing dam in Mariana, many ecosystem functions were affected, including functions related to soil biological communities. Thus, the purpose this study was evaluate effect of biochar application on composition of bacterial communities under two conditions pH (6.0 and 8.0). Soil samples were incubated for 90 days. After these periods, the pH, CO_2 emission and analysis of bacterial composition and diversity were determined. Our results showed that application of biochar affected the composition soil bacterial communities, mainly phyla Acidobacteria, Actinobacteria and Proteobacteria. The reduction of pH promove changes in CO_2 emission, showing increased in rates at 1% and 2% of biochar. The alpha diversity was, in general, higher on pH 8.0 condition in the 1 and 2% of biochar rates. This result indicates, partially, adaptation by the bacterial communities to new edaphic conditions. Thus, conclude that application of biochar may be an interesting technology during rehabilitation of the areas affected by the tailings. However, this study was only a pilot for future investigations.

Keywords: iron mining, tailings, bacterial communities, environmental quality

1. Introduction

The activity of mining and your processing have been increasing in the world, in order to meet industrial demands and technological development (Gastauer et al., 2018; Ribeiro et al., 2018; Silva et al., 2018). However, negative impacts caused by mineral extraction on environment, and on important exosystemic services, are enormous (Gu, 2018). Moreover, the tailing produced during process mining are potential sources contamination (Zhuang et al., 2009), since most them present heavy metal at level toxic to environment function and human health (Porto et al., 2016; Tepanosyan et al., 2018).

In Brazil, on the November of 2015, the dam Fundão located in the Quadrilatero Ferrifero, county of Mariana, Minas Gerais, broken, releasing 35 million m^3 of tailings at along the of Doce river. The catastrophe caused consequences serious of water supply, aquatic biodiversity, terrestrial fauna and flora, local agriculture, and the deposition of metal at level concern (Porto et al., 2016; Miranda et al., 2016), which will remain at site as contamination source and environmental changes for long period (Segura et al., 2016; Hadje et al., 2017).

Results in literature indicated that concentration some heavy metals are high as example the Cr was 5-fold increased that in content found in samples collected before tailings arrival (Oliveira et al., 2017; Queiroz et al., 2018). However, although exact origin this metal not known, the search for immobilization and reduction of Cr levels and other heavy metals content in soil undisputed.

Soil contamination with heavy metal is considered big problem world which affect directly microbial structure and diversity, enzymatic activities (phosphatase, urease and xylanase), nutrients cycling and functions of soil microbiota (Zhang, 2011; Niemeyer et al., 2012; Li et al., 2015; Santos et al., 2016; Li et al., 2017; Scholz et al., 2017; Xião et al., 2017; Sun et al., 2018; Liu et al., 2018; Oka and Uchida, 2018).

Biochar is product of degradation of materials organics, in high temperature and absence oxygen (Lehmann, 2007) that have received many attention soil scientists. This material is able sequestration carbon and make nutrients available (Lehmann et al., 2011), redox potential (Xu, et al., 2018), adsorption of metals (Xu et al., 2018a; Qi et al., 2018; Meng et al., 2018; Van Poucke et al., 2018) and effects in soil biological community (Thies et al., 2015; Moore et al., 2018; Liu et al., 2018).

Study conducted for Moore et al. (2018) showed strong influence biochar on propriety physical chemistry (pH) and soil microbiological. The authors did can observed than biochar produced on low temperature increase the respiration, species richness and diversity , probably due to factors such as nutrient availability, and news micro-habitat that protects microorganisms of Cu contamination.

Kolton et al. (2011) observed increase relative abundance of members of Bacteroidetes phylum from 12 to 30% as result of biochar amendment, while that of Proteobacteria decreased from 71 to 47%. In addition, in Bacteroidetes phylum was observed greater induction of decomposer genus of chitin, cellulose and aromatic compounds, notoriety an alteration the functions soil bacterial communities.

Based several search results showing benefits of biochar on biological properties of soil in contaminated land with heavy metal (Moreno-Barriga et al., 2017; Liu et al., 2018; Oka and Uchida, 2018; Xu et al., 2018; Wang et al., 2018; Forján et al., 2018; Khan et al., 2018; Xu et al., 2019; Souza et al., 2019), we can be consider it an important ally in rehabilitation of affected areas by mining, specifically this case, of dam disruption Fundão, where was found high content of chromium.

Furthermore, this is first investigation the conditioner action of biochar on structure and diversity of communities microbial of soil, after deposition of tailing of mining in region of Mariana MG. Thus, our study sought to respond questions as: (a) anthropic activities and

mining tailings affect composition of communities microbial? (b) Biochar is able change composition and diversity microbial? (c) Reduction of pH of soil influence effects of biochar about activity, composition and diversity microbial?

2. Material and Methods

2.1. Area of study and Experimental Design

Although great benefices economic and social, activity mining cause impacts severe the environment. In 2015, rupture of dam of Fundão localized at city Mariana, became known as bigger disaster environment caused for mining in Brazil. The tailing affected the beds of important rivers of region, forming a crust on surface; limit for growth and development many species vegetable and microbial communities of soil. Furthermore, deposition of tailing increased availability of some heavy metals, that will can compromise soil health.

May of 2018, soil sample were collected in points very affected for the tailing and in areas nonaffect, with vegetation native of the Atlantic forest. Five sample been collects in layer 0 - 20 cm, to form composite sample. Then, soil samples has been homogenized and stored in sterile plastic bags under cooling of 4ºC and send to laboratory for be analyses. The parameters chemistry soil been analyzed and available in supplementary material. Some value these parameters been show in Table 1.

The study present consisted addition of five levels of biochar (0, 0.5%; 1.0%; 2.0%; 5.0%) in 25 grams soil/tailing (w/w), under two conditions of pH (6.5; 8.0). Each treatment has been represented for five replicas, totalized 50 experimental units.
To reduce pH, 0.00625g of elemental sulfur was added to each 25g of soil. This procedure was performed 4 days before soil incubation.

2.2.Characterization of the Biochar

The biochar utilized has been 2000 milligrams of wood particulates eucalyptus. The temperature was increased each 10ºC/minutes until reaches desired temperature is of 450 ºC, which maintained by 2 hours. The pH was determined by bench pH meter, elements as C, H, N, H, S has been determined for the CNHS/O Elementary and other elements minerals biochar analyzed by modified Dry Ash Method (Enders, A. and Lehmann, J., 2012). Basic characteristics of biochar were pH: 6.5; 79% of C; 2.79% of H; 0.26% of N; 0.02% of S; 69.28% of fix C; 1.0% of ashes; 35% gases; 2.17g kg-1 of K; 0.14g kg-1 of P; 1.88g kg-1 of Ca; 0.36g kg-1 of Mg; 177.12mg kg-1 of Fe; 115.92mg kg-1 of Mn.

2.3.*Incubation and CO_2 emission experiment*

The soil activity was determined by the CO_2 emission. The samples of 25 grams soil+biochar was incubated glass pots and in fully enclosed and dark place during 91 days, being the CO_2 emissions verified periodically the 1, 7, 14, 21, 28, 35, 42, 49, 56, 63, 77 and 91 days incubation. The CO_2 released was captured by the solution NaOH (0.5 mol L-1), which was then titled with HCl (0.5 mol L-1) utilized phenolphthalein (1%) as indicator (Alef and Nannipieri, 1996).

2.4.Soil pH after incubation

Soil pH was measured days after incubation. Briefly, 10 grams of soil was used in 25 ml of CaCl (0.01 mol) followed by homogenization and rest for 1 hour. After the resting period, the samples were shaken and then read with a bench pH (Embrapa, 1997).

2.5. Analysis of bacterial community composition

Total DNA was extracted from soils to characterize bacterial community composition. DNA was extracted using the DNeasy PowerSoil Kit, as per manufacturers recommendation

(QIAGEN, Germantown, MD, USA) with a bead beating treatment of 2 min at 5.5 $m \cdot s^{-1}$. Soil DNA extracts, and corresponding controls, were quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™, Thermo Fisher Scientific, Inc., Waltham, MA, USA), as per the manufacturer's instructions. Fluorescence was measured with FilterMax F5 micro-plate reader (Molecular Devices, San Jose, CA, USA) with 485 nm excitation and 535 emission.

Bacterial community composition was determined by sequencing the V4 region of the 16S rRNA gene was amplified from soil DNA extracts by polymerase chain reaction (PCR) using dual-indexed barcoded 515f/806r primers as described by Kozich *et al*. (2013). Each 25 uL PCR reaction contained (i) 2 ng of DNA template; (ii) 12.5 uL of Q5 High Fidelity, Hot Start PCR Mastermix (New England Biolab, MA, USA); (iii) 1.25 uL of 10 uM primer; and (iv) a final concentration of 0.05x Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, MA, USA). The following thermocycler conditions were used to amplify the 16S rRNA gene: initial denaturation at 95 °C for 2 minutes, then 30 cycles of 95 °C for 20 sec, 55 °C for 15 sec (annealing) and 72 °C for 10 sec (extension) with followed by a final.

PCR was performed in duplicate and pooled, purified and normalized to a standard concentration using the SequalPrep normalization kit (Invitrogen, CA, USA) prior to multiplexed sequenced on a single lane of Illumina MiSeq (2x250 paired-end) at the Biotechnology Resource Centre (Cornell University, Ithaca, NY, USA) with an 8% addition of PhiX. The sequence have been assigned into 'Amplicon Sequence Variants', which have representative sequences for.

2.6. Data analysis and statistics

Amplicon libraries were processed using QIIME2, with dependencies on DADA2, to create assign sequences to amplicon sequence variants (ASV). Taxonomic classification was performed using the QIIME2 'q2-feature-classifier' trained on the data base (nr_v132; Quast et

al., 2013). Libraries were filtered to remove ASVs present in no template controls and low abundance and sparse ASVs (minimum occurrence in three samples or at a total relative abundance > 0.01% of all samples).

The data of pH and basal respiration were processed and the graphs plotted using the ggplot2 package of the statistical program R version 3.5.1, as well as the determination of species richness (Chao1), alpha diversity (Shannon) and composition of the bacterial communities we used the package Phyloseq (McMurdie & Holmes, 2013).

3. Results and Discussion

3.1. Effect of Biochar on soil pH

At the end of 90 days of incubation, the pH of the soil samples was modified by application biochar (Figure 1), showing a mean reduction of 0.7 units in relation to the control, without application biochar. In pH 6.0 condition, rates of 0.5%, 1% and 2% promoted significant reductions in pH, while the rate 5% the reduction was lower. In condition of pH 8.0, was also possible to observe significant reductions in relation to treatment without biochar, however, among applied rates there was no difference. In general, biochar rates in the present study had effect of reducing soil pH, especially at rates of 0.5%, 1% and 2% at pH 6.0, where we can observe high effect among biochar treatments.

The effect of biochar on changes in soil pH is associated with the pyrolysis process and feedstock used, factors that directly influence chemical composition of biochar (Antal and Gronli, 2003; Lehmann, 2007; Novak et al., 2009; Sulimann et al., 2016; Meng et al., 2018), mainly percentage ashes, material that is carbonates sources and acts in correction of the acidity. Several studies have confirmed the positive correlation between ash content and pH increase (Brewer et al., 2011; Yuan et al., 2011; Liang et al., 2014; Sánchez-Monedero, 2017; Chen et al., et al., 2019). However, it was not possible to observe this behavior in our study.

The biochar used has pH 6.5, 2.79% H, 0.26% N and only 1% ash, which certainly led to reduction in pH. The study conducted by Liao and Thomas, (2019) showed that lower pH values were verified in treatments with lower ash content. Zhao et al. (2013) also reported influence of biochar ash content on soil pH changes, both of which were reduced when produced at lower temperatures.

Although biochar with low ash concentration does not provide carbonates in substantial quantities, the carbon contents of these materials are higher, which makes them a more persistent nutrient source in soil (Schmidt and Noack, 2000; Enders et al. al., 2012). In present study, where the pH of the anthropic area affected by tailings is alkaline, use of this soil conditioner is beneficial not only by pH reduction but also to implement the carbon stocks during rehabilitation of area.

3.2. Effect of Biochar on CO_2 Emission

In general, average CO_2 emission at pH 6.0 was almost twice high the treatments at pH 8.0, and in all treatments with biochar when compared to control without biochar (figure 2). In pH 6.0 higher CO_2 emissions were observed at rates 1% and 2% of biochar. Although treatment with 0.5% also has promoted reduction in soil pH, the CO_2 emission remained low, situation that partially eliminates inferences about increased microbial respiration has occurred due to reduction soil pH. In pH 8.0, we observed higher CO_2 emission in treatment with 2%, besides abrupt increase of CO_2 in rate 5% (Figure 2). Certainly, what occurred in treatment of 5% in both pH conditions is related to proportion of biochar the metabolism of microbial communities, which is notoriously more balanced after 56 days of incubation.

Possibly, higth CO2 emission is linked to able of microorganisms decompose the fraction of C labil of biochar (Lehmann et al., 2007, Ulyett et al., 2014). We believe that in more acid pH and low ash content, the biochar becomes more reactive due to the dissolution of

carbonates (Sheng and Zhu, 2018) and serves as energy for soil microorganisms in a short period (Antal and Gronli, 2003; Hamer et al., 2004). Beside, biochar maximizes adsorption of contaminants and heavy metals (Spokas, 2009; Meng et al., 2018; Xu et al., 2019), increasing aeration and its porous surface become niches to protect microorganisms (Warnock et al. al., 2007; Thies and Rillig, 2009; Quilliam et al., 2013; Silvani et al., 2017; Moore et al., 2018), characteristics that also promote improvements in soil microbiological activity.

Our results agree with reports by Sheng and Zhu (2018) that observed higher CO_2 emission when applied biochar in Ferrasol (pH 5.1) compared to Phaeozems (pH 7.81). The authors attribute this result greater degradation of biochar under more acidic conditions, and point adsorption of native organic carbon as main parameter to reduce CO_2 emission at high pH. Another factor that may have contributed to lower cumulative CO_2 emissions in high pH soils is precipitation of CO_2 as carbonate on surface of biochar, as was briefly discussed on review made by Lehamann et al. (2011).

3.3. Effect of Biochar on Composition of Bacterial Communities

Our results provide valuable information regarding composition and bacterial diversity of soil after rupture of Fundão dam in Minas Gerais, since this is first record in literature after the disaster. Additionally, this type of information allows us to think about the ecological interactions and biochemical processes that are occurring in the soil, as well as to outline future bioremediation strategies of affected areas.

From sequencing of V4 region of 16S gene, it was possible to evaluate the structure of bacterial communities of soil. In general, 262,914 readings, 3,032 valid OTUs and 1186 taxas were obtained for 11 samples. The richness by Chao1 and the Shannon diversity index were based on the number of OTUs and are presented in table 2. Both richness and diversity were altered in relation to pH conditions, and consequently by application of biochar. In both pH

conditions the richness and diversity were reduced when applied at 5% rate of biochar. However, the diversity of OTUs was higher in pH 8.0 condition at 1% of biochar. In pH 6.0, the 1% rate also provided highest values of the Shannon richness and diversity.

Twenty-two taxonomic classifications were observed at phylum level (including unclassified sequences), with *Proteobacteria* and *Actinobacteria* together showing an average abundance of 75.96% of total sequences. Only 2.32% of total sequences could not be classified. Figure 3 shows the composition of bacterial communities through relative abundance of dominant phyla.

The phylum Proteobacteria was sensitive to application of biochar and of greater abundance in all the treatments. In condition of pH 8.0, without addition of biochar there was an increase of 12.65% in the abundance in relation to reference area. However, at rates 0.5%, 1% and 2% of biochar, we observed a reduction in the abundance of this phylum of 53.43%, 51.60% and 49.54%, respectively. In pH 6.0, we observed similar behavior to previous condition, with abundance of 60.93%, 54.24%, 48.80% at rates 0.5%, 1%, 2% respectively. In the 5% rate of biochar increases were observed at both pH conditions.

The phylum *Actinobacteria* was increased from 20.76% to 30.57% as a function of biochar rates in pH 6.0, mainly rates 1%, 2% and 5%. *Clorofex, Bacteroidetes, Gemmatimonadetes* and *Plactomycetes* were present in area affected by the tailings, although abundance last three was reduced with increase of biochar rate. The *Acidobacteria* phylum showed higher abundance at pH 8.0 and although there were no observed increases between applied rates, there was increase in abundance relation to treatment without application biochar.

The bacterial communities of phylum *Acidobacteria* are among most frequent in soils and are strongly influenced by pH conditions, being more abundant acidic conditions (Jones et al., 2009; Rousura et al., 2010; Kuramae et al., 2011), and nutrient availability (Zhao et al., 2014). However, we observed present study, greater abundance in pH 8.0 condition. In contrast,

in Mata area, where beside of pH acid condition there is also greater availability total organic carbon, the abundance this phylum was lower.

In different land uses in Brazilian Amazon researchers reported that abundance of many subgroups of the Acidobacteria phylum was higher in pasture with corrected pH than forest. In addition, the authors point these prokaryotes as good indicators of environmental changes (Navarrete et al., 2015). In general, Acidobacteria are quite versatile with carbon sources, from simple sugars to more complex substrates, such as hemicellulose and cellulose (Rawat et al., 2012), which justifies increase in abundance with application biochar our study.

Gram-negative bacteria, to the example of *Acidobacteria*, *Gemmatimonadetes*, *Bacteroidetes* and *Plactomycetes* tend to be more abundant at high pH conditions (Feng et al., 2010). *Firmicutes* and *Actinobacteria*, most of which are Gram-positive, showed sensitivity to pH reduction, with higher abundance in pH 6.0 and 4.3 conditions (Figure 3). The study conducted by Oka and Uchida (2018) also showed that abundance of *Firmicutes* was positively correlated with application of slag of pH ranging from 4.1 to 5.3, just like in present study. Besides, *Firmicutes* is among five most abundant phyla in areas contaminated with heavy metals (Liu et al., 2018).

The increase of diversity in affected area by iron ore tailings is related to increase in pH (Fierer and Jackson, 2006). We also attributed the reduction in abundance of some phyla to composition biochar used, since besides its potential to acidify soil, consists of a carbonic material very recalcitrant and with low nutrients availability in short term. The results observed by Sheng and Zhu (2018) also showed greater alpha diversity at alkaline pH and that addition of biochar increases abundance bacterial communities since material used was presented potential to alkalinize soil. However, in general, our study shows that sensitivity in abundance of the phyla is quite variable in to application of biochar.

The reduction richness and diversity when applied 5% of biochar reflects a imbalance caused by the quantity and quality of biochar in relation bacterial communities present area, requiring higher specificity metabolic. Additionally, we correlated this behavior with CO_2 emission observed this biochar rate and condition pH 8.0, because at 56 days incubation this treatment showed considerable increase CO_2 emission. We believe that higher rates of eucalyptus particulate biochar require greater adaptation by bacterial communities or even selection some groups, as was case in our study.

4. Conclusion

Application of biochar affected the composition of the soil bacterial communities, mainly of the phyla Acidobacteria, Actinobacteria, e Proteobacteria. The reduction of pH promoved changes in CO2 emission, showing increased in rates at 1% and 2% of biochar. The alpha diversity was, in general, higher in the pH 8.0 condition, in the 1% and 2% of biochar rate, indicating adaptation by bacterial communities to new edaphic conditions.

Thus, we conclude that application of biochar may be an interesting technology during rehabilitation of areas affected by the iron ore tailings, since, depending on the material used, it can reduce soil pH and improve many ecosystem functions. And, although it is able of altering bacterial populations, biochar does not reduce alpha diversity, which ecologically positive. However, this study was only a pilot for future investigations.

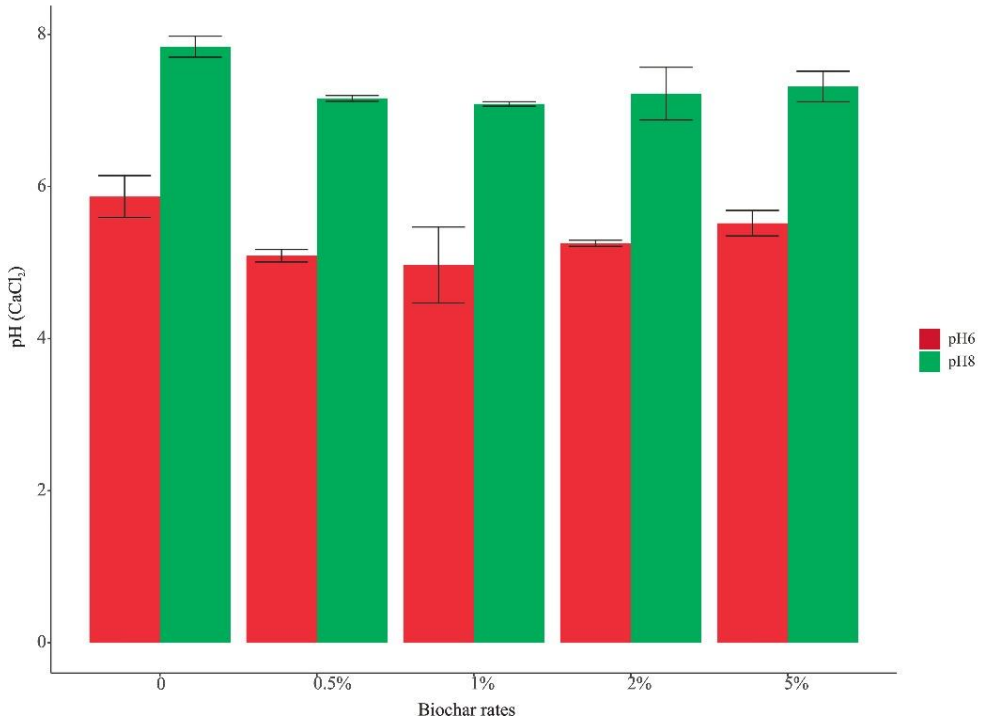

Figure 1. Effect of biochar on two pH conditions of samples of mining tailings, after 90 days of incubation. Error bar demonstrates values of standard deviation.

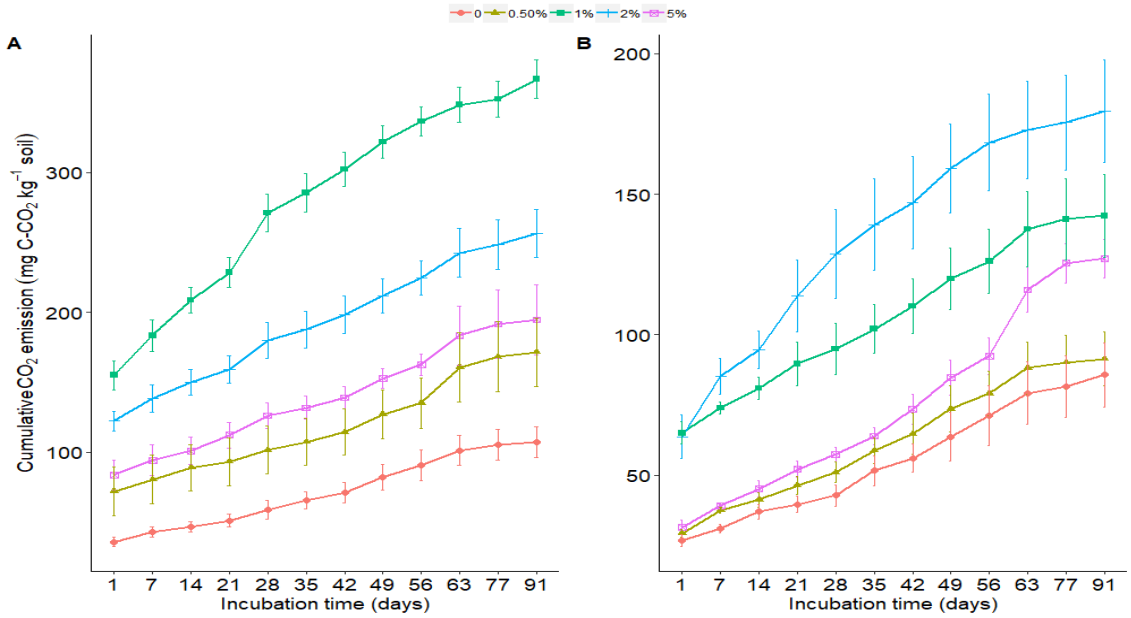

Figure 2. Cumulative CO_2 emission under the effect of different rates of biochar under conditions of pH 6.0 (a) and pH 8.0 (b) in soil samples. Error bar demonstrates values of standard deviation.

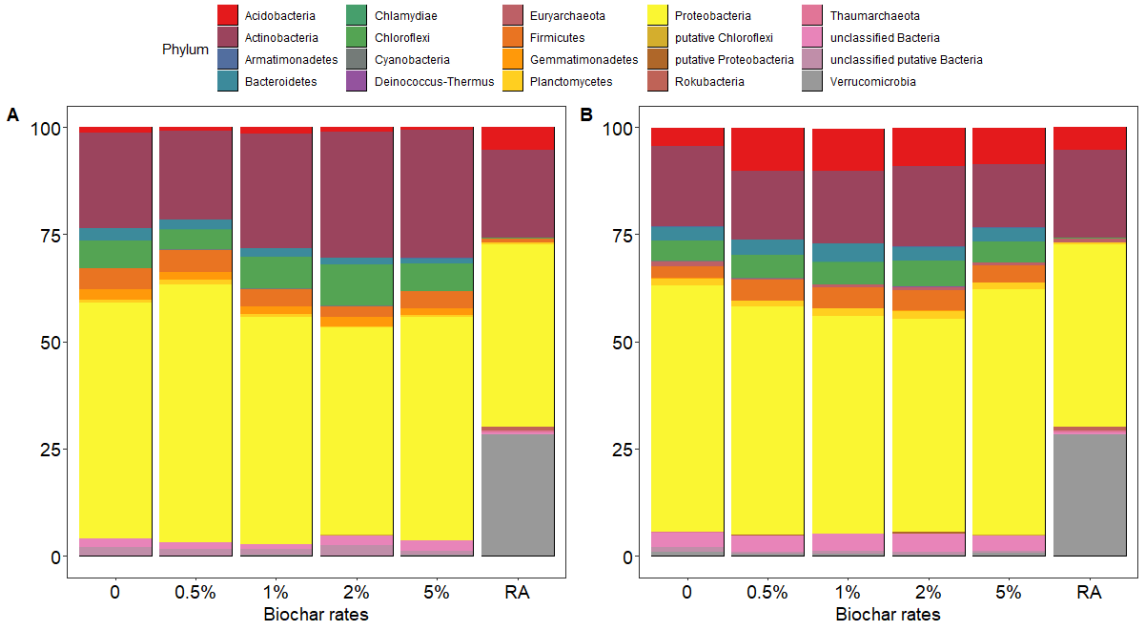

Figure 3. Taxonomic composition of bacterial communities, level of phylum, after application of bichar in soil samples with pH 6.0 (A) and pH (B) of area affected by the iron ore tailings in Mariana, Brazil. Only the 20 most abundant taxa are shown. * RA - reference area.

Table 1 – Selected properties of the soil collected on the banks of the Gualaxo do Norte River, in impacted areas (I) with deposition of the tailings and RA - reference area.

Area		I	RA
pH		8.04	5.15
P disponível	mg kg^{-1}	6.68	1.54
K disponível	$cmol_c$ dm^{-3}	0.03	0.06
TOC	g kg^{-1}	4.56	16.52
CEC	$cmol_c$ dm^{-3}	2.72	8.53

pH determined in water; Available P value were extracted with Mehlich 1; CEC - cation exchange capacity at pH = 7 determined indirectly by the sum of the bases; TOC: organic carbon determined by the oxidation of potassium dichromate.

Table 2. Comparison of a-diversity indices, read numbers and rhiqueza, under different condition pH and rates biochar, at a genetic distance of 3% (RA: reference area).

Treatments	Read Numbers	OTUs	Chao1	Shannon
		pH 8.0		
0	30,078	446	453	5.05
0.5%	20,489	357	374	5,19
1%	22,687	412	423	5.31
2%	22,457	386	402	5.25
5%	19,271	316	328	5.06
		pH 6.0		
0	26,492	194	197	4.37
0.5%	30,341	215	218	4.29
1%	33,798	248	249	4.47
2%	25,992	207	210	4.42
5%	23,461	172	174	4.07
*RA	7,848	79	80	3.88

* RA - reference area.

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