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By-products as an amendment of a mine soil: effects on microbial biomass determined using phospholipid fatty acids

Enmienda de un suelo de mina con subproductos: efecto sobre la biomasa microbiana determinada mediante el uso de los ácidos grasos de los fosfolípidos

Correção de um solo de mina com subprodutos: efeito sobre a biomassa microbiana determinada através da utilização de ácidos gordos dos fosfolípidos

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ABSTRACT

In the present work, the effect of two by-products (pine bark and crushed mussel shell) on microbial biomass and community structure was studied in a soil from a mine tailing located in a copper mine. In a laboratory experiment, different doses (0, 12, 24, 48, 96 and 192 Mg ha^{-1}) of pine bark, crushed mussel shell or mixtures of both by-products were added to the soil. The amended soil samples were incubated for one year at 60% of water holding capacity, and then 33 phospholipid fatty acids (PLFAs) were extracted from these samples and quantified. The PLFAs concentrations were used for different microbial biomass estimations: total biomass, bacterial biomass, fungal biomass, gram-positive (G+) biomass and gram-negative (G-) biomass. The addition of crushed mussel had no significant effects on the total soil microbial biomass, either bacterial of fungal biomass. However, the addition of pine bark increased the total microbial biomass in the soil (up to 40%), mainly due to increases in the fungal biomass (it increased 1600%). No synergistic effects were observed when the soil was amended with both, pine bark and crushed mussel shell. The main community structure changes were due to the addition of pine bark to the soil, and were also due to modifications in fungal communities. Our results suggest that the microbial biomass was mainly limited in the mine soil by low organic matter concentrations, and therefore, practices increasing the amount of soil organic matter should be priorities for soil reclamation.

RESUMEN

En el presente trabajo se estudió el efecto de dos subproductos (corteza de pino y concha de mejillón triturada) sobre la biomasa y estructura microbiana de un suelo procedente de una escombrera localizada en una mina de cobre. En un experimento realizado en laboratorio fueron añadidas al suelo diferentes dosis (0, 12, 24, 48, 96 and 192 Mg ha^{-1}) de corteza de pino, concha de mejillón triturada y mezclas de ambos subproductos. Las muestras de suelo enmendado fueron incubadas durante un año al 60% de la capacidad de campo, y posteriormente 33 ácidos grasos de los fosfolípidos (PLFAs) fueron extraídos de estas muestras y cuantificados. La concentración de PLFAs fue utilizada para realizar distintas estimaciones de la biomasa microbiana: biomasa total, biomasa bacteriana, biomasa fúngica, biomasa de bacterias gram + y biomasa de bacterias gram –. La adición de concha de mejillón triturada no tuvo efectos significativos sobre la biomasa total ni sobre la biomasa bacteriana o fúngica. Sin embargo, la adición de corteza de pino al suelo incrementó la biomasa total del suelo (hasta un 40%), debido mayormente al incremento de la biomasa fúngica (se incrementó un 1600%). Tampoco se observaron efectos sinérgicos cuando el suelo fue enmendado con una mezcla de corteza de pino y concha de mejillón triturada. Los mayores cambios en la estructura de las comunidades microbianas fueron debidos a la adición de corteza de pino al suelo, y fueron además debidas

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a cambios en las comunidades fúngicas. Nuestros resultados sugieren que la biomasa microbiana del suelo de mina está mayormente limitada por la concentración de materia orgánica y, por tanto, deben ser priorizadas prácticas de manejo que contribuyan a incrementarla para la rehabilitación de este tipo de suelos.

RESUMO

Neste trabalho foi estudado o efeito de dois subprodutos (casca de pinheiro e concha de mexilhão triturada) na biomassa e estrutura microbiana de um solo procedente de uma escombreira localizada numa mina de cobre. Numa experiência de laboratório, doses diferentes (0, 12, 24, 48, 96 e 192 Mg ha^{-1}) de casca de pinheiro, concha de mexilhão triturada e misturas de ambos os subprodutos foram adicionados ao solo. Amostras de solo corrigido com os resíduos foram incubadas durante um ano a 60% da sua capacidade de retenção de água, e subsequentemente, 33 ácidos gordos dos fosfolípidos (PLFAs) foram extraídos a partir destas amostras e quantificados. A concentração de PLFAs foi usada para estimar vários tipos de biomassa microbiana: biomassa total, biomassa bacteriana, biomassa fúngica, biomassa de bactérias gram positivas e biomassa de bactérias gram negativas. A adição de concha de mexilhão triturada não teve nenhum efeito significativo na biomassa total ou na biomassa bacteriana ou fúngica. Porém, a adição de casca de pinheiro aumentou a biomassa microbiana do solo (até 40%), principalmente devido ao aumento da biomassa fúngica (a qual aumentou de 1600%). Não foi observado nenhum efeito de sinergismo quando o solo foi corrigido com uma mistura de casca de pinheiro e concha de mexilhão triturada. As maiores alterações na estrutura das comunidades microbianas foram produzidas pela adição de casca de pinheiro ao solo, as quais resultaram em alterações nas comunidades fúngicas. Os resultados sugerem que a biomassa microbiana do solo de mina é, maioritariamente limitada pela concentração de matéria orgânica. Assim, práticas que contribuam para o seu aumento devem ser prioritárias para a reabilitação deste tipo de solos.

KEY WORDS
Pine bark, crushed mussel shell, bacterial biomass, fungal biomass, soil degradation.

PALABRAS CLAVE
Corteza de pino, concha de mejillón triturada, biomasa bacteriana, biomasa fúngica, degradación del suelo.

PALAVRAS-CHAVE
Casca de pinheiro, concha de mexilhão triturada, biomassa bacteriana, biomassa fúngica, degradação do solo.

1. Introduction

Mining is an activity that causes an intense degradation of the land (Masto et al. 2015) with important impacts on ecosystems, causing soil, water and atmospheric pollution, as well as important landform modifications. Therefore, there is a growing need to develop management practices in order to reduce derived environmental impacts. Usually, mining activities generate large quantities of waste, generally accumulated in dumping sites. The soils developing on these dumps generally present acid pH values, metal(loid) concentrations and low organic matter contents (Álvarez et al. 2003; Perlatti et al. 2015). Under these conditions, microorganism development is very limited, presenting low activity (Fernández-Calviño et al. 2015; Zornoza et al. 2016) and low biomass (Zornoza et al. 2016) in mine soils. Thus, since soil microorganisms are the main agents responsible for long term sustainability of soil ecosystems (Nannipieri et al. 2003), management practices programmed to favor mine soil restoration must take into account the recovery of soil microbes as a main objective.

The controlled addition of organic and inorganic waste and by-products from different industries to degraded areas may be a good alternative for soil remediation, due to its low cost and the improvement of different soil properties (Fernández-Calviño et al. 2016; Abad-Valle et al. 2017). The addition of these low cost by-products to degraded soils may facilitate metal(loid) adsorption (Nguyen et al. 2013; Paradelo and Barral 2017), diminishing environmental and public health risks. Also, the addition of these materials may increase soil pH (Arias-Estévez et al. 2007) or soil organic matter concentration (Rodríguez-Salgado et al. 2016). Crushed mussel shells and pine bark are two by-products with high metal(loid) retention capacities (Cutillas-Barreiro et al. 2014; Farouq and Yousef 2015). Moreover, the addition of crushed mussel shell may increase pH in mine soils (Garrido-Rodríguez et al. 2013) and pine-bark amendments increase organic matter content since it is mainly composed of organic carbon (Cutillas-Barreiro et al. 2014). However, the effect on soil microbes due to the use of these two by-products in mine soils has been poorly studied. In this respect, Fernández-Calviño et al. (2015) determined the bacterial community growth in a Cu-polluted acid mine soil after amendment with different doses of crushed mussel shell, showing a positive effect on the bacterial growth for high doses of that by-product, but only for long incubation times (2 years).

The microbial biomass and community structure are important soil variables which can be studied using the phospholipid fatty acid (PLFA) analysis. This is a technique commonly used to analyze microorganisms in soils, which has been employed to detect changes in soil microorganisms biomass and structural patterns in soil microbial communities (Dangi et al. 2012; Nunes et al. 2016; Tian et al. 2016). Therefore, the determination of biomass and community structure responses to soil amendment with crushed mussel shells and pine bark may be useful for a better understanding of possible remediation strategies in acid mine soils polluted with metal(loid)s. We hypothesized that the use of these two by-products, individually or in combination, will increase the general microbial community biomass in acid Cu-polluted mine soils, contributing to soil reclamation, specifically regarding microbial communities.

The aim of this work is the evaluation of the effects of soil amendments with two by-products (crushed mussel shell and pine back) on microbial biomass and structure in a Cu-polluted acid mine soil. Both characteristics were assessed using the analysis of phospholipid fatty acids (PLFAs) extracted from amended soil samples after one year of incubation.

2. Materials and methods

2.1. Soil and by-products characteristics

The mine soil (42.87536°N, 8.35345°W) was sampled in the tailing of an abandoned Cu mine area located in Touro (Galicia, NW Spain). Plant species that have colonized the zone are mainly *Calluna vulgaris*, *Erica cinerea* and *Salix atrocinerea*. Main climatic conditions in the sampling area include average annual temperature 12.6 °C, with values 7.9-17.2 °C corresponding to the range of average minimum to maximum temperature; average annual rainfall 1886 mm, with maximum average monthly rainfall (281 mm) in December, and minimum average rainfall (39 mm) in July. The soil was collected at a 20 cm depth, air-dried and sieved through a 2 mm mesh. The pine bark was supplied by Geolia (Madrid, Spain), whereas the crushed mussel shell was provided by Abonomar S.L. (Illa de Arousa, Galicia, Spain). The soil was previously characterized by Fernandez-Pazos et al. (2013), the pine bark by Cutillas-Barreiro et al. (2014) and the crushed mussel shell by Garrido-Rodríguez et al. (2013). The mine soil was classified as a Spolic Technosol (Dystric, Arenic) according to the World Reference Base for Soil Resources 2014 (IUSS Working Group WRB 2015), had a sandy loam texture, was ultra-acidic (aqueous pH 3.0) and presented a very low organic carbon content (3 g kg^{-1}) compared with soils from the same area (Calvo de Anta et al. 2015). Also it presented a high total Cu concentration (773 mg kg^{-1}). The pine bark presented a strongly acid pH (4.5) and high carbon content (486 g kg^{-1}), mainly as organic carbon (≥ 99%). The crushed mussel shell presented a very strongly alkaline pH (9.4) and a total carbon concentration of 124 g kg^{-1}, mainly as inorganic carbon (≥ 99%).

2.2. Experimental design

The mine soil was amended with different concentrations of: a) pine bark (PB), b) crushed mussel shell (CMS), and c) a mixture (1:1, *m/m*) of pine bark and crushed mussel shell (MIX). The experiment was performed by adding separately 6, 12, 24, 48 and 96 g of each by-product per kg of soil, corresponding to 12, 24, 48, 96 and 192 Mg ha^{-1} considering an effective soil depth of 20 cm and a soil bulk density of 1 g cm^{-3}. Also, soil samples without any amendment were used as control. All mixtures and controls were duplicated, resulting in a total of 32 microcosms. Before amendment with the different by-products, the soil was rewetted up to 60% of water holding capacity and incubated at 22 ºC for one week. This period was considered enough for the recovery of soil microbial activity (Meisner et al. 2013). After one year of incubation, 2 g of each microcosm corresponding to the soil amendment with the different by-products, were sampled and frozen at -20 ºC for PLFA analysis.

2.3. Phospholipid fatty acids analysis

The phospholipid fatty acids (PLFAs) were extracted from the soil according to Frostegård et al. (1993) and quantified by Gas Chromatography. Briefly, lipids were extracted from 2 g of wet soil with a chloroform:methanol:citrate buffer mixture (1:2:0.8 *V/V/V*) and separated into neutral lipids, glycolipids and phospholipids using a pre-packed silica column. The phospholipids were subjected to a mild alkaline methanolysis and the fatty acid methyl esters were identified by gas chromatography (flame ionization detector) by the relative retention times of the fatty acids, using methyl nondecanoate (19:0) as internal standard. The PLFAs were designated in terms of total number of carbon atoms, double bonding and position of the double bonds. Prefixes ‘a’, ‘i’, ‘cy’ and ‘Me’ refer to anteiso, iso, cyclopropyl and methyl branching. Non-specific branching was designed by ‘br’ whereas cis and trans configurations were indicated by c and t, respectively. The total microbial biomass was estimated as the sum of all the determined PLFAs: i14:0, 14:0, i15:0, a15:0, 15:0, i16:0; 16:1ω7c, 16:1ω7t, 16:1ω5, 16:0, br17, 10Me16b, i17:0, a17:0, 17:1ω8, cy17:0, 17:0, br18, 10Me17, x3, 18:2ω6, 18:1ω9, 18:1ω7, 18:0, cy19 and 20:0. The bacterial biomass was estimated as the sum of the PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0), whereas the fungal biomass was estimated by using the PLFA 18:2ω6 (Frostegård and Bååth 1996). The sum of PLFAs i14:0, a15:0 and i16:0 were used for gram-positive (G+) bacterial biomass estimation, and the PLFAs cy17:0, cy19:0, 16:1ω7c and 18:1ω7 for gram-negative (G-) bacterial biomass (Zelles 1999).

2.4. Statistics

Significance of differences in the various types of microbial biomass, between the control and by-products spiked soils, were tested by one-way analysis of variance (ANOVA). Then Duncan's significant difference test, for significant differences to the un-amended control soil, was applied. Concentration of all the individual PLFAs data, expressed as relative values regarding total PLFAs, were subjected to principal component analysis (PCA), to elucidate the main differences in the PLFA patterns. The SPSS statistical programme was used for all calculations.

3. Results

The addition of CMS to the soil caused a significant increase in the pH values for all the CMS doses, up to 3.1 units for the highest dose applied (192 Mg ha^{-1}) (Fernández-Calviño et al. submitted), whereas the PB amendment was not associated with significant changes, and the addition of MIX caused significant pH increases for doses ≥ 24 Mg ha^{-1}, up to 1.3 units for the highest dose applied (192 Mg ha^{-1}). With respect to the organic carbon, calculations based on the PB and CMS percentage of organic carbon showed that the addition of CMS did not contribute to increases in this soil characteristic, while the addition of PB changed the organic carbon content from < 0.5 g kg^{-1} in the non-amended soil up to values around 47 g kg^{-1} in the soil sample amended with the highest dose (192 Mg ha^{-1}). In the case of soils amended with MIX, the organic carbon content increased up to 23 g kg^{-1} for the highest dose applied (192 Mg ha^{-1}).

After one year of incubation, the total amount of PLFAs extracted from the soil samples (an estimation of the total soil microbial biomass), was 13.3 ± 2.0 nmol g^{-1} in the non-amended mine soil. **Figure 1A** shows that the addition of CMS to the mine soil had no significant effects on the total microbial biomass for none of the doses applied during the same incubation period. However, when the soil was amended with PB, the microbial biomass showed a significant increase after one year of incubation for PB doses ≥ 96 Mg ha^{-1}, up to 40% for 192 Mg of PB ha^{-1} (**Figure 1B**). In the soil samples amended with MIX, the results were significantly higher than in the control only for the higher MIX dose (192 Mg ha^{-1}; **Figure 1C**).

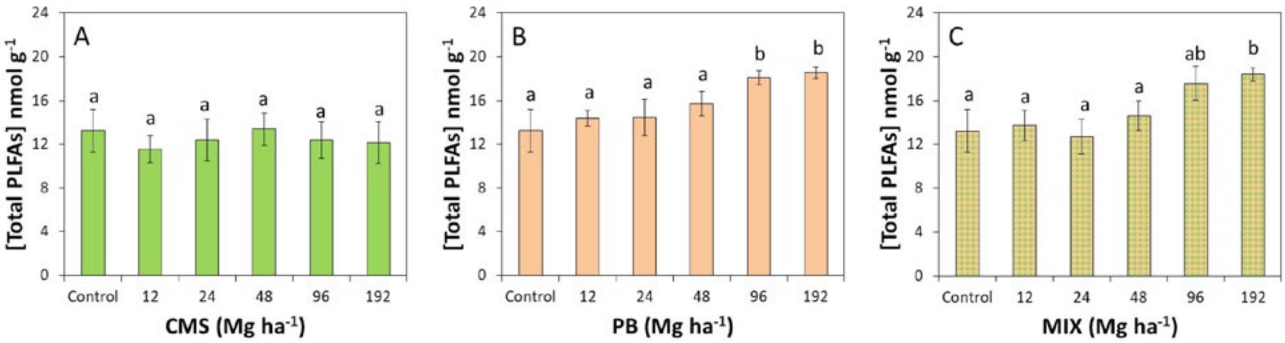

Figure 1. Total PLFAs concentration extracted from the soils amended with different amounts of crushed mussel shell (A), pine bark (B) and 1:1 crushed mussel shell: pine bark mixtures (C). The error bars show the standard deviation, and different letters indicate significant differences between doses (ANOVA, Duncan post-hoc test, $P < 0.05$).

The bacterial biomass was 5.4 ± 0.9 nmol g^{-1} in the non-amended mine soil after the same incubation period (one year). It was not significantly affected by the soil amendment with the by-products (CMS, PB and MIX) (**Figure 2**). The gram-positive bacterial biomass was 1.46 ± 0.14 nmol g^{-1} in the non-amended mine soil after the incubation period. As for total bacterial biomass, the addition of CMS or PB to the mine soil caused non-significant changes in the gram-positive biomass for any of the doses of amendments tested (**Figures 3A and 3B**). However, the addition of MIX to the mine soil increased the gram-positive bacterial biomass significantly (up to 50%) for the highest doses, 96 and 192 Mg ha^{-1} (**Figure 3C**). The bacterial communities can be depressed in the mine soil by low organic matter concentrations and extremely low pH values. The addition of MIX to the soil contributed to both increases in soil organic matter and pH, hence promoting better conditions for bacterial development. Among bacteria, the best adapted to extreme conditions are the gram-positive, and hence, they were capable of increasing biomass in response to environmental improvements. The gram-negative bacterial biomass was 2.7 ± 0.3 nmol g^{-1} in the non-amended mine soil after one year of incubation. None of the by-products added to the soil had significant effects on the gram-negative bacterial biomass for any of the doses tested (**Figure 4**).

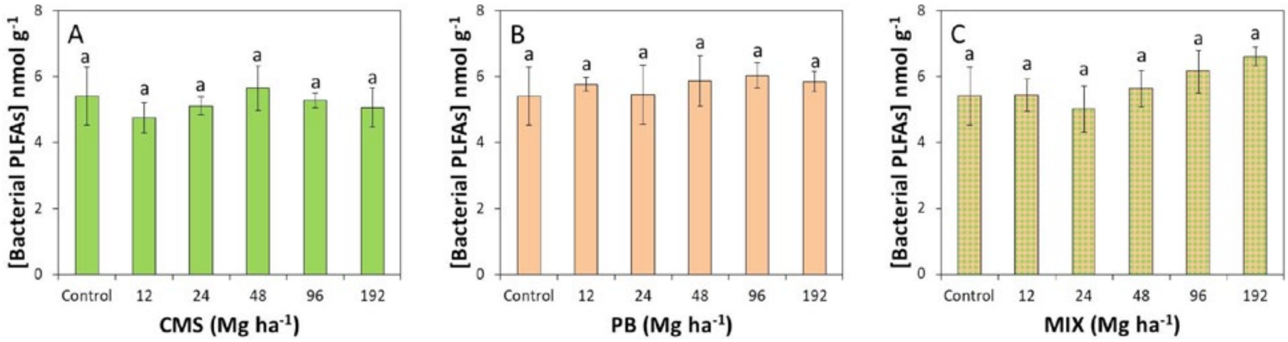

Figure 2. Bacterial PLFAs concentration extracted from the soils amended with different amounts of crushed mussel shell (A), pine bark (B) and 1:1 crushed mussel shell:pine bark mixtures (C). The error bars show the standard deviation, and different letters indicate significant differences between doses (ANOVA, Duncan post-hoc test, $P < 0.05$).

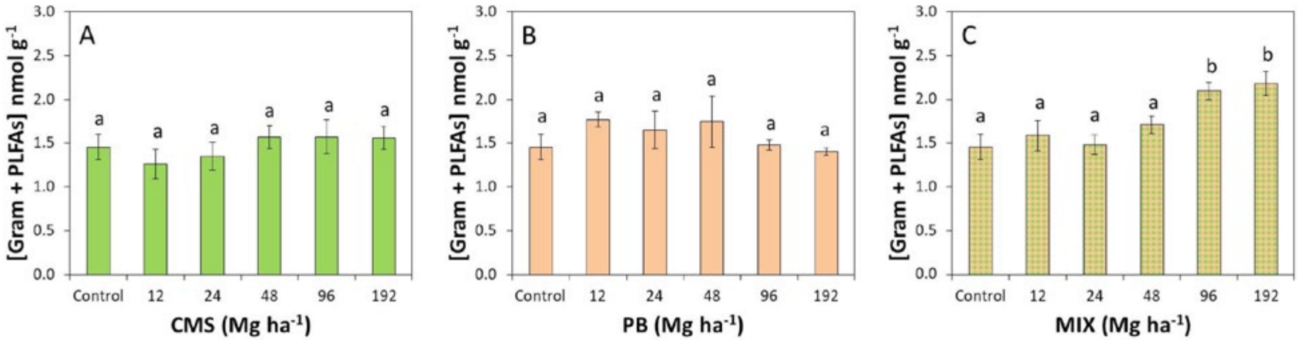

Figure 3. Gram-positive bacterial PLFAs concentration extracted from the soils amended with different amounts of crushed mussel shell (A), pine bark (B) and 1:1 crushed mussel shell:pine bark mixtures (C). The error bars show the standard deviation, and different letters indicate significant differences between doses (ANOVA, Duncan post-hoc test, P < 0.05).

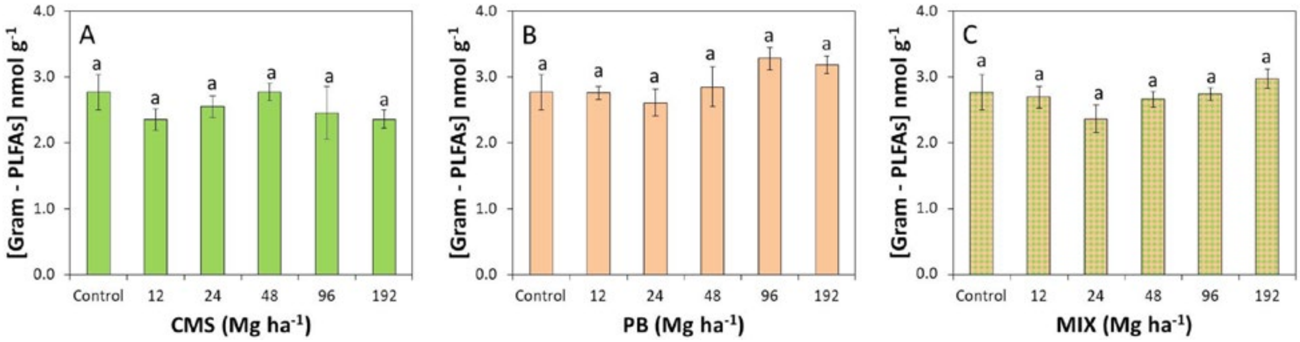

Figure 4. Gram-negative bacterial PLFAs concentration extracted from the soils amended with different amounts of crushed mussel shell (A), pine bark (B) and 1:1 crushed mussel shell:pine bark mixtures (C). The error bars show the standard deviation, and different letters indicate significant differences between doses (ANOVA, Duncan post-hoc test, P < 0.05).

The fungal biomass was 0.086 ± 0.014 nmol g^{-1} in the non-amended mine soil after one year of incubation. It was significantly affected by the by-products (CMS, PB and MIX) after the incubation period, but the magnitude of the induced changes was very different among them (**Figure 5**). The addition of CMS to the mine soil increased the fungal biomass (**Figure 5A**), reaching values significantly higher than the control (64%) only for the highest dose applied (192 Mg ha^{-1}). When the mine soil was amended with PB or MIX, the fungal biomass increased significantly for all the doses applied, reaching values 1600% higher than in the control when the soil was amended with 192 Mg ha^{-1} of PB (**Figures 5B and 5C**).

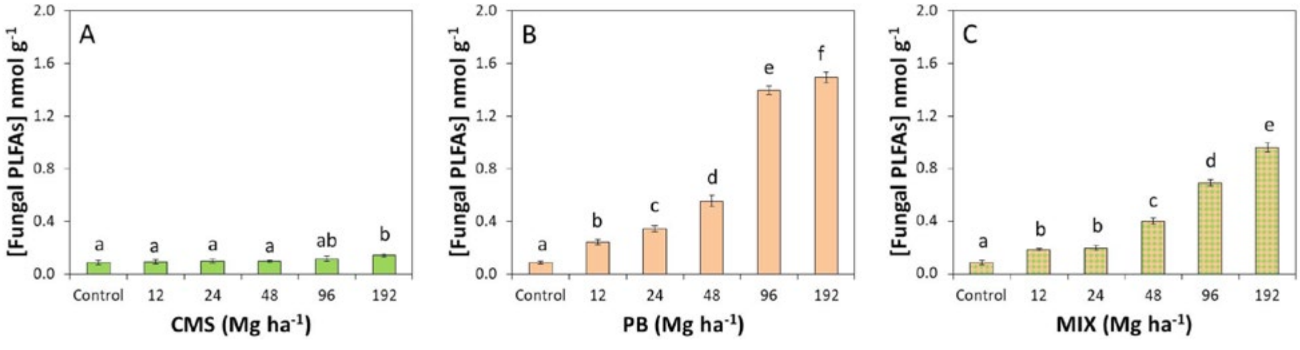

Figure 5. Fungal PLFAs concentration extracted from the soils amended with different amounts of crushed mussel shell (A), pine bark (B) and 1:1 crushed mussel shell:pine bark mixtures (C). The error bars show the standard deviation, and different letters indicate significant differences between doses (ANOVA, Duncan post-hoc test, P < 0.05).

Changes in the microbial community structure in response to by-product addition to the mine soil were analyzed using principal component analysis (PCA). The first PC explained 44.9% of the variance, whereas PC2 and PC3 explained 21.6% and 12.1%, respectively. The first PC (PC1) was significantly correlated (r=-0.691; P<0.01) with the pine bark concentration in the soil. This relation is quite clear (**Figure 6A**), but it seems that for PB concentrations ≥ 48 Mg ha^{-1} the changes in PC1 score are quite limited. The second PC (PC2) was also significantly correlated (r=-0.506; P<0.05) with the pine bark concentration in the soil but in this case the relation is not very clear (**Figure 6B**). The third PC (PC3) was significantly correlated (r = 0.824; P<0.01) with the crushed mussel shell concentration in the soil, and the PC scores were clearly higher for higher CMS concentrations (**Figure 6C**).

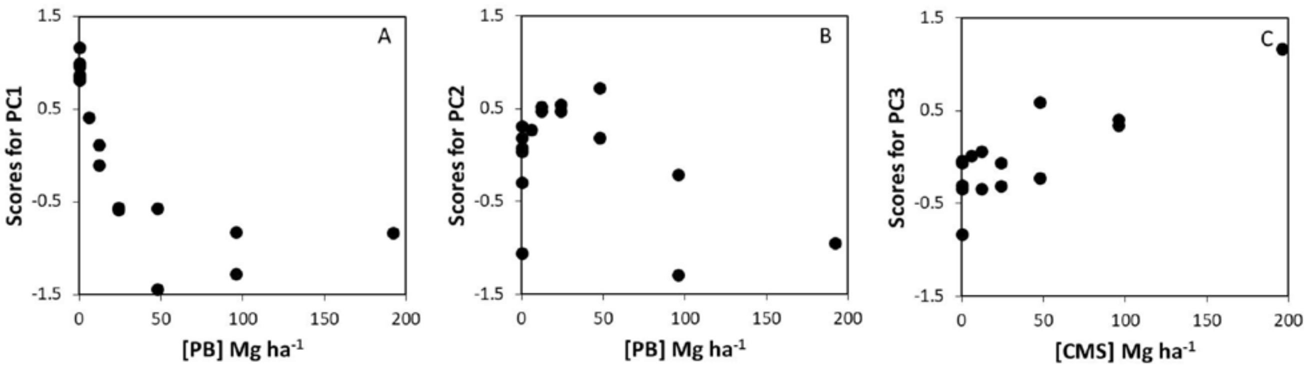

Figure 6. Relations between PB concentration and scores of soils along PC1 (A), between PB concentration and scores of soils along PC2 (B), and between CMS concentration and scores of soils along PC3 (C).

Figure 7 shows the loadings of the different PLFAs for PC1, PC2 and PC3, and the scores for the different soil samples along the first three principal components, which explain 78.6% of the microbial community structure variance. Loading values of different PLFAs indicated that there was a relatively high concentration of PLFAs 14:0, i15:0, a15:0; 16:1ω7c, 16:1ω7t, 16:1ω5, 16:0, i17:0, cy17:0, br17 and 18:0; and relatively low concentrations of PLFAs 10Me17, 18:2ω6, 18:1ω9 and 20:0 along PC1. Along PC2 there was a relatively high concentration of PLFAs i16:0, br17 and 10Me16b; and relatively low concentrations of PLFAs i14:0, 17:0, 18:2ω6, 18:1ω7, and 18:1ω9. The PC3 was mainly characterized by relatively high concentrations of the PLFAs i14:0, a15:0, 15:0, i16:0, a17:0 and x3; and relatively low concentrations of PLFA cy19:0.

4. Discussion

The total PLFAs concentration (an estimation of the microbial biomass) was quite low in the non-amended mine soil (13.3 ± 2.0 nmol g^{-1}) compared to the values found in agricultural soils (mean between 22 and 75 nmol g^{-1}; Fernández-Calviño et al. 2010) or forest soils (mean between 240 and 462 nmol g^{-1}; Barreiro et al. 2010) from the same area. Also, the biomass values of all the studied groups (bacteria, fungi, G+ and G-) were lower in the mine soil than in agricultural or forest soil. Therefore the use of restoration techniques helping to increase the microbial biomass is fully justified.

The application of crushed mussel shell (CMS) to mine soils contributed to the decrease in metal(loid) availability in this type of soil (Fernández-Calviño et al. 2016) and also to the increase in soil pH (Garrido-Rodríguez et al. 2013). The modification of both properties (decrease in metal(loid) availability and increase in soil pH) may increase the microbial

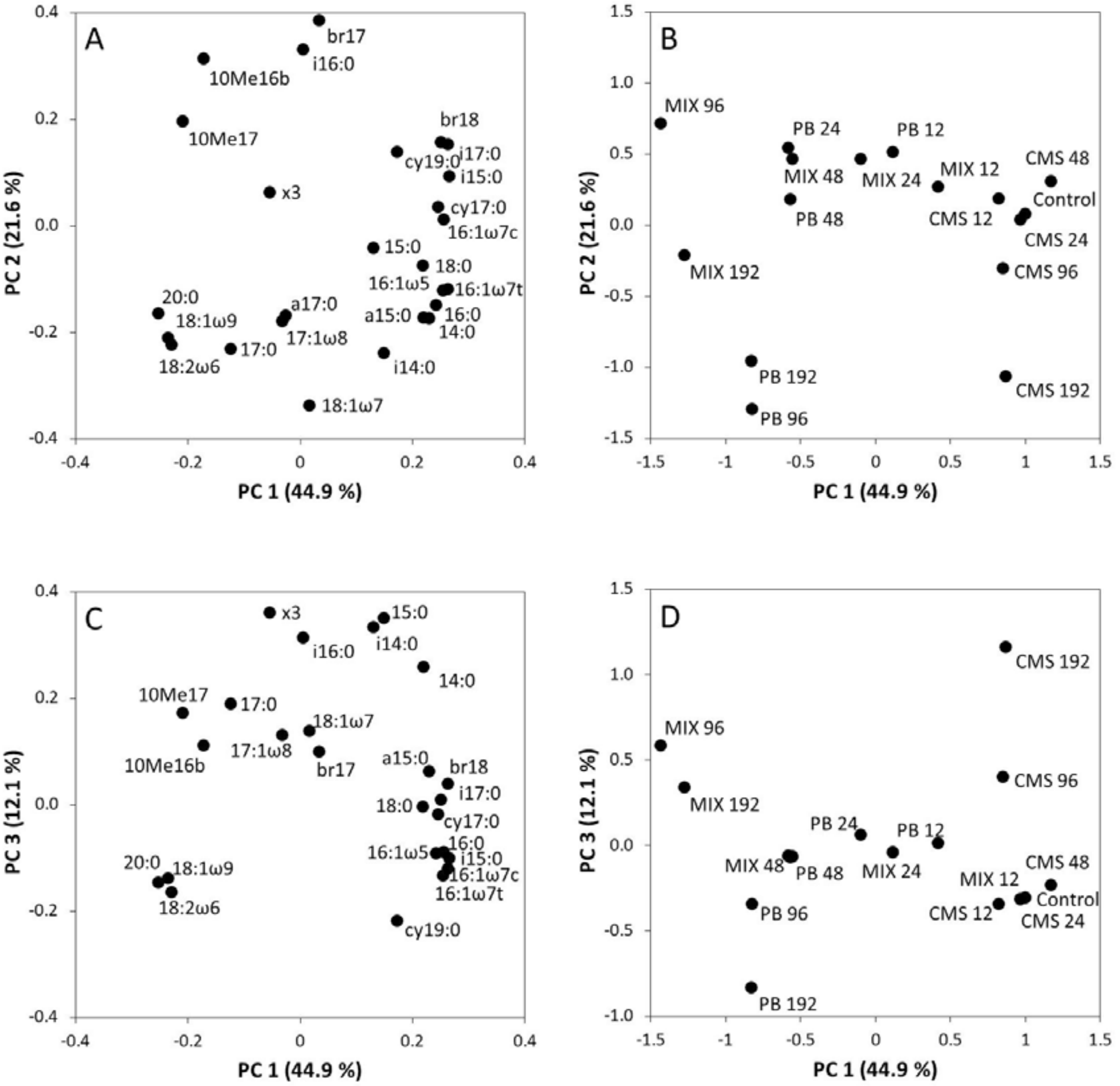

Figure 7. Principal component analysis of the PLFA pattern of mine soil samples (16): control and amended with different concentrations of CMS, PB and MIX (12, 24, 48, 96 and 192 Mg ha^{-1}. PC1, PC2 and PC3 accounted for 44.9%, 21.6% and 12.1% of the variance. (A) Loadings of the different PLFAs for PC1 and PC2; (B) Scores of the different soil samples for PC1 and PC2, (C) Loadings of the different PLFAs for PC1 and PC3; and (D) Scores of the different soil samples for PC1 and PC3.

development (Giller et al. 1998; Aciego-Pietri and Brookes 2009). However, the CMS amendment on the mine soil presented low effects on the microbial biomass, with only the fungal biomass increasing significantly (64%) in response to the highest CMS dose applied (192 Mg ha^{-1}). Therefore, metal(loid) pollution and low pH do not seem to be the principal factors for the low microbial biomass found in the studied mine soil. The increases in fungal biomass instead of bacterial biomass in response to CMS addition is surprising, since normally soil bacterial communities are favored by pH increases, whereas fungal communities decrease as the soil pH increases (Rousk et al. 2009). Decreases of fungal growth when the soil pH increases may be normally attributed to antagonistic effects between fungi and bacteria (Rousk et al. 2008), i.e. in long-term, soil pH increases stimulate bacterial growth, exerting a high competitive pressure on fungus, which concentration decreases (Rousk et al. 2009).

However, in the present work the bacterial biomass was not stimulated by the CMS addition and hence, there wasn´t competitive pressure on fungus. The application of crushed mussel shell (CMS) to mine soils had effects on the microbial community structure pattern via correlation with PC3 (**Figure 6C**). These effects may be due to the pH changes induced by the CMS application. The pH increase effects on PLFA pattern are normally associated to increases in 16:1ω5, 16:1ω7, and 18:1ω7, with a concomitant decrease in cy19:0 and i16:0 (Rousk et al. 2010). However, this behavior is not present in PC3 scores (**Figure 7**), and therefore the relation between PC3 and CMS dose cannot be directly attributed to changes in the soil pH.

The application of pine bark (PB) to mine soils may contribute to decrease the metal(loid)s availability in this type of soils (Fernández-Calviño et al. 2017), and also to increase the organic matter content, processes which would enhance the microbial biomass in the soils (Giller et al. 1998; Zornoza et al. 2015). In the present work, total microbial biomass increased significantly in response to PB doses ≥ 96 Mg ha^{-1}. These increases may be attributed mainly to growth in fungal biomass, since none of the bacterial biomasses here determined were affected by the PB amendment. Since PB is mainly organic matter (Cutillas-Barreiro et al. 2014), and the decrease of metal availability was marginal in promoting microbial biomass increases in the mine soil (see above discussion for CMS), the main factor in reclamation strategies in the mine soil, in order to increase the soil microbial biomass, should be the increase of soil organic matter. Similar results were found previously by Zornoza et al. (2016), although they found that the addition of pig slurry and pig manure significantly increased both bacterial and fungal biomass. The differences between these results and those found in the present work may be attributed to differences in the organic matter quality, since PB is composed mainly of lignin (47.9%) and glucan (18.6%) (Fernández-Calviño et al. 2017), and hence, the bacterial development may be limited in this type of substrate. However, other factors cannot be discarded, since the addition of amendments to mine soils normally increases the fungal:bacterial biomass ratios (Zornoza et al. 2015). The fact that fungi are more tolerant to metal(loid)s than bacteria (Khan and Scullion 2002) may have also some effect. However, the magnitude of the changes in fungal biomass in response to PB amendment was much higher than for CMS amendment (two orders of magnitude) and therefore this effect should be very limited. The results from crushed mussel shell and pine bark mixtures (MIX) addition were similar to those due to the pine bark amendment, but the magnitude of the induced changes was lower, i.e. synergistic effects between increases of soil organic matter concentration and soil pH were not found. Therefore, the results from MIX support that increases in the soil organic matter were most important than increases in soil pH (or than decreases in metal(loid) availability) for the recovery of microbial biomass in the Cu polluted acid mine soil. The first principal component from the PCA analysis explained 44.9% of the microbial community structure changes and it is correlated with the addition of pine bark to the soil, i.e. the main changes produced in the microbial community structure were due to the PB amendment. The 18:2ω6, a PLFA related with fungi, was one of the most important scores for PC1 (**Figure 7**), a result in agreement with the large increase found for fungal biomass.

5. Conclusions

The increase of total microbial biomass in the Cu polluted acid mine soil was not significant when amended with crushed mussel shell, after one year of incubation. However, using pine bark as soil amendment the total microbial biomass increased significantly for 96 and 192 Mg ha^{-1} doses. This increase was mainly due to growth in fungal biomass, while bacterial biomass did not change in response to pine bark amendment. Also, no synergistic effects were found between pine bark and crushed mussel shell when added as a 1:1 mixture to the soil. Also, the pine bark amendment was the main factor for community structure changes. The results of this research suggest that the addition of organic matter to the soil is the main factor contributing to microbial biomass reclamation in Cu polluted acid mine soils with initial low organic matter contents.

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