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INFLUENCE OF SAPROPHYTIC FUNGI AND INORGANIC MATERIALS ON WHEAT STRAW COMPOSTING: THEIR EFFECTS ON CO₂ EMISSIONS AND COPPER DISTRIBUTION IN MINING CONTAMINATED SOILS

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INFLUENCE OF SAPROPHYTIC FUNGI AND INORGANIC MATERIALS ON WHEAT STRAW COMPOSTING: THEIR EFFECTS ON CO_2 EMISSION AND COPPER DISTRIBUTION IN MINING CONTAMINATED SOILS

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"Nuestra mayor gloria no está en no haber caído nunca, sino en levantarnos cada vez que caemos"

Oliver Goldsmith.

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Thesis outline

Mining activities may produce a negative impact on natural and agricultural ecosystems, with important effects in soils including, pollution, loss of organic matter (OM), loss of soil quality and soil erosion, which may result in a loss of vegetation cover. Therefore, it is necessary to develop technologies that allow minimizing the negative impact produced and technologies capable to enhance soil quality, reduce contaminant availability and maintain a better vegetation cover in contaminated ecosystems. For this purpose, the use of organic amendments appears to be an interesting practice for the amelioration of contaminated soils.

On the other hand, high amounts of crop residues are annually produced and some of them, like wheat straw (WS) are predominantly burned in fields. Thereby, some treatments and practices are necessary in order to decrease the high amounts of these organic residues and reusing them. Under this context, their utilization as soil amendments is a widespread practice for agricultural systems, ecological restoration and remediation of contaminated soils. Nevertheless, in order to avoid some negative effects in soils due to their application as fresh material, to stabilize them in previous stages is an essential procedure. In this sense, composting is an economic method to manage and stabilize these residues which has shown a high effectiveness on the transformation of organic materials.

Composts of some agricultural residues may have a relatively high rate of decomposition under uncompleted stabilization process. Then, a significant part of carbon (C) source resulting from the composting process eventually becomes quickly mineralized in soil. Some co-composting practices has shown a high effectiveness in compost stabilization enhancing its quality and some physicochemical properties as cation exchange capacity, nevertheless, there are still many unknowns about these practices. Thus, studies aimed to enhance the stabilization of composted residues and promote a higher permanence and residence time of them when applied to soils are still necessary in order to increase the beneficial effects in contaminated soils.

Thus, this Thesis study aimed to promote the stabilization of WS residues in an aerobic composting system by using inorganic materials such as metallic oxides (co-composting) and saprophytic fungi in order to generate a potentially resistant to mineralization and

highly reactive soil amendments for being used in the immobilization of copper in metal contaminated soils.

Firstly, in chapter two are reviewed novel (bio) technologies that can be used to manage agricultural residues for land application and remediation of contaminated and eroded soils. This information provided the theoretical background.

In chapter three, information concerning to composting process, OM transformations and the final compost characterization are shown (see specific objective 1). In this chapter, the effects of different fungal strains and inorganic materials in aerobic composting systems were analyzed. A complete characterization of the final products is provided. In a first experiment, WS was inoculated with 4 different fungal strains (*Coriolopsis rigida; Pleurotus ostrautus, Trichoderma harzianum* and *Trametes versicolor*) and co-composted with inorganic materials such as Fe/Al-oxides and soil clay minerals (Soil AC) (B horizon from an allophane enriched-soil) and enzymes related to the substrate decomposition were evaluated. In addition, humification and OM stabilization by physcicochemical interactions were determined. For this purpose, different chemical, biochemical, physicochemical, microscopic and spectroscopic methods such as Fourier transformed infrared (FTIR) and Cross polarization magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS^{13}CNMR) were used. Part of these spectroscopic and microscopic analyses were performed in Agriculture and Agri-Food, Canada and Instituto de Recursos Naturales y Agrobiología de Sevila (IRNAS-CSIC).

Differences between treatments in terms of enzymatic activities, humification levels, functional groups content and composition were observed. *Trametes versicolor* inoculated WS in combination or co-composted with Fe-oxide (Tv+Fe-oxide treatment) showed higher enzymatic activities (~300 U L^{-1} of LAC and 0.350 U L^{-1} of MnP) and humification levels (lower E_4/E_6 ratios; < 7) than the other treatments. However, the evaluated enzymatic activities were low and differ with the reported literature in similar experimental conditions. On the other hand, partial stabilization of these treatments was confirmed and complemented by FTIR and solid state ^{13}C NMR analyses, which provided information about chemical structure, aromaticity and acidic functional groups. Microscopic analyses allowed characterizing the humic fraction and understanding some properties of materials as consequence of the stabilization process during the aerobic

composting process. Images showed metal-OM interactions between humic like-substances (HS) from compost and iron at scales of 1-to-200 nm and the aggregation phenomena. Results indicated that under our experimental conditions there was differences between the application of inorganic materials and fungal strains in wheat straw composting. In this sense, the combination of inorganic materials and fungi could promote an effective treatment during 126 days of aerobic composting and may produce a partial stabilization of this agricultural residue by potentially similar mechanisms to soil OM stabilization.

In Chapter four, the effects of compost application in a Cu contaminated soil were evaluated.. For this, wheat straw based-composts (WSC) obtained produced on stage 1 (compost production; chapter 3) were incubated with a metal contaminated soils (Cu basal=330 ppm and Cu spiked soil= 830 ppm). Treatments inoculated with *T. versicolor* (Tv; with no inorganic material addition) and co-composted with Fe-Al oxides and Soil AC (Tv+Fe-oxide, Tv+Al-oxide, Tv+Soil AC) were selected and mixed with the contaminated soils. The microbial activity was assayed by the CO_2 release (Respiration experiment). In addition, the enzymatic activities dehydrogenase (DHA) and β-glucosidase were measured. Copper sequential extraction was conducted in order to analyze the effects of composts in Cu availability. Finally, OM transformations in soils were analyzed by CPMAS[13]CNMR. Compost application markedly increased the CO_2 release in comparison with control incubations without compost application. However, compost treated with metallic oxides and clay minerals showed a lower respiration (CO_2 release) than Tv compost (with no inorganic material addition). Under our experimental conditions, a significant decrease of the enzymatic activities (DHA) was observed in spiked soils –compost incubations, suggesting a possible limiting step of its determination as consequence of the high level of Cu, which may act as reducing agent of the enzymatic products. On the other hand, compost application reduced the easily exchangeable fraction (~30 to 15%) and increased the fraction of Cu associated to OM (~10 to 15 %) in compost amended soils, especially in those with a basal contamination, what suggest a potential Cu immobilization by effect of the organic amendments. Finally, OM in soils changed its chemical structural composition after 1 year of soil-compost incubation. Under this context, CPMAS[13]CNMR showed an increase in aromatic and O-alkyl structures associated to lignin-like products and carbohydrates compounds from composts.

In conclusion, our results indicated that the inorganic materials (metallic oxides and clay minerals) addition and fungi inoculation positively affected the WS composting enhancing the physicochemical properties of the produced composts. Moreover, compost application increased the microbial activity in contaminated soils. However, the application of inorganic materials into the wheat straw composting could promote the OM protection and decrease the compost decomposition in soils under the studied conditions. Therefore, the application of the partial stabilized composts to the mining contaminated soils promoted the Cu immobilization as effect of the incorporation of OM which was present even one year after the compost application.

In summary, we concluded that inorganic materials application in combination with ligninolytic fungi inoculation may produce partially stabilized composts, which are able to be applied in Cu contaminated soils and promote the Cu immobilization in short-terms studies.

The scheme, main purposes and stages of this Doctoral Thesis are summarized in Figure I.
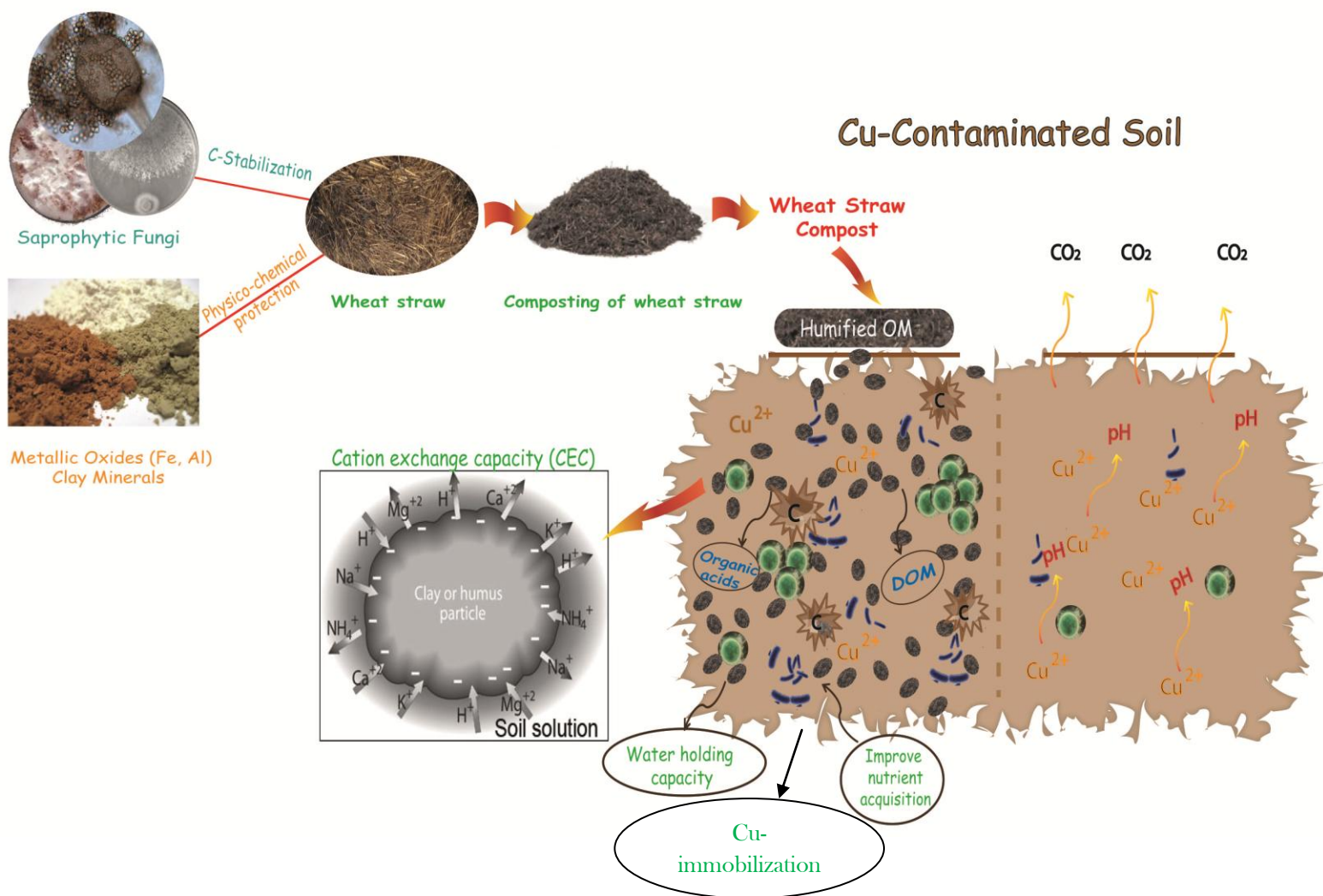
Figure I Overview of this Doctoral Thesis, which was developed with the aim to stabilize wheat straw in an aerobic composting system using and combining inorganic materials and saprophytic fungi. In the graphical abstract the main effects of compost in Cu-contaminated soil are summarized as well.

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Chapter I

General Introduction

1 General introduction

Industrial activities are associated with important contamination processes in soils (Lottermoser et al., 2003). Several anthropogenic activities have caused important increases in soil copper levels in urban areas as well as in wild and agricultural lands. In developing countries, mining is a major economic activity, being responsible for generating a greater amount of contaminants, hence significantly contributing to erosion and contamination of soils by toxic elements (TE). The discharge of metal-enriched solid and effluent wastes into the air, water, and soils generates a deleterious effect in ecosystems, particularly soils, where these events produce, for example, the loss of organic matter (OM), soil physical degradation and contaminant incorporation into the soil matrix (Park et al., 2011). Based on this scenario to include technologies that increase the accumulation and stabilization of OM and decrease the availability of contaminants like TE have became necessary in soils affected by pollution episodes especially if the risk of human or ecological health damage is deemed high enough.

Several environmental remediation systems using physical, chemical, or biological treatments have been developed in recent decades (Meier et al., 2012; Uqab et al., 2016), highlighting the immobilization techniques which are focused on reducing the availability and activity of TE (Lomonte et al., 2011; Smolinska, 2015). The aim of the immobilization techniques is to accelerate natural processes such as sorption, complexation and precipitation reactions using amendments for decreasing mobility and availability of the contaminants (*e.g*., metal(oid)s) in soils (Bolan et al., 2003; Park et al., 2011; Bolan et al., 2014). Different types of amendments have been used for this purpose including inorganic materials such as iron oxides, phosphate, calcium and nanomaterials among others (Castaldi et al., 2005; Derome, 2009; Stuckey et al., 2009; Nielsen et al., 2011: Rabbani et al., 2016) and organic materials like composts and the combinations of them (Tandy et al., 2009; Neaman et al., 2012; Scotti et al., 2015). Organic amendments like compost have been widely used due to its composition support the plant growth, increase the OM content in soils, promote the microbial activities and reduce the availability and mobility of TE through its physicochemical properties promoting different immobilization processes and reactions (Chen et al., 2006; Park et al., 2011; Medina et al., 2015).

Sorption reactions of metal(oid)s to OM is attributed to an increase in surface charge (Chen et al., 2006; Clark et al., 2007) and the presence of metal/metalloid binding compounds (Gondar and Bernal, 2009). A complex of a metal ion and organic molecule arises when water molecules surrounding the metal ion are replaced by other molecules or ions, with the formation of a coordination compound. The organic molecule that combines with the metal ion is commonly referred to as the ligand (Stevenson, 1994: Chen et al., 2006). In this sense, several studies of compost application found that the incorporation of OM increased TE immobilization in contaminated soils, which is mainly attributed to increases in pH and the significant surface charge and binding properties of composts (Bolan et al., 2003; Park et al., 2011; Jones et al., 2016; Madejón et al., 2016). Moreover, the application of mature organic amendments has positive effects on soils improving soil physical characteristics such as particle size distribution, cracking pattern and porosity (Park et al., 2011).

On the other hand, crop residues are an important source of nutrients and high value chemicals (Azam, 1990; Habets et al., 2013; Schnitzer et al., 2014), which can positively influence the biological, chemical and physical properties of soils. In Chile, huge amounts of these residues are annually produced. Despite to be a rich source of nutrients, stubble burning is the most common management approach and it is estimated that 80 to 90% of wheat stubble surface in La Araucanía and Bío Bío regions is burned (Taladriz and Schwember, 2012). The incorporation of organic residues increases the soil microbial biomass and activity, promoting among other carbon (C) and nitrogen (N) mineralization (Singh, 1995; Cassman et al., 1996). Several studies have shown that the re-incorporation of crop residues into the soil improved tilth, reduced water and wind erosion processes and prevented nutrient losses by leaching (Lal, 1980; Lal et al., 1980; Maurya and Lal, 1981; Bukert et al., 2000; Shah et al., 2003; Andrews, 2006; Shafi et al., 2007). As a consequence of the crop residues incorporation the detrimental or erosive processes in soil are decreased due to the high amount of OM incorporated. This is a very important aspect to be taken into account in remediation and amelioration of soils, since degraded and contaminated soils usually have lower OM contents (Barea et al., 1999; Lal, 2003). Thus, to increase soil OM contents by incorporation of organic materials is considered a fundamental practice in soil remediation (Senesi and Plaza, 2007; Ben Achiba et al., 2009; Scotti et al., 2015).

Nevertheless, some recent researches indicate that excessive incorporation of organic fresh materials (non-stabilized) may have adverse impact in soils, environment and even in crop yield (Jiang et al., 2012) associated to the fact that fundamental processes in soils required stabilized OM (Medina et al., 2015). For example, under aerobic and anaerobic conditions the incorporation of non-stabilized organic materials may promote the biotic and abiotic N immobilization by soil minerals and soil microorganisms (Olk et al., 2002; Schmidt-Rohr et al., 2004; Senesi and Plaza, 2007; Hubbe et al., 2010). Moreover, other adverse effects in soil and environment include, among others: (i) an increase of the mineralization rate of native soil organic C, (ii) the induction of anaerobic conditions by mineralization of large amounts of non-stabilized organic C, (iii) the associated extended O_2-consumption, (iv) the alteration of soil pH (Senesi and Plaza, 2007) and (v) the stimulation of green house gas (GHG) emissions (Luo et al., 1999; Yan et al., 2005; Plaza an Senesi, 2009; López-Cano et al., 2016) and losses of nutrients by leaching. Also, some authors have reported lower crop yields in systems amended with high amount of fresh crop residues as consequence of increased diseases, poor seed germination and presence of phytotoxic compounds like polyphenols (Benlboukht et al., 2016).

Thereby, it is well known that stabilization of organic materials like crop residues before to their application into soils may help to reduce their oxidation to CO_2 in soils. In this sense, composting is one of the most used technologies for organic waste management and an important way for C stabilization involving the partial humification of the OM (Senesi and Plaza, 2007; Scotti et al., 2015). In these processes humic-like substances (HS) are generated which have a higher residence time providing numerous essential functions once applied to soils and minerals previously contained in residues (Singh and Sharma, 2002). This technology reduces the waste volume and weight about 50% resulting in a stable product that can be used to enhance chemical, biochemical and physical properties of soils (Sánchez-Monedero et al., 2002). Aerobic composting is a microbiological-mediated process which carried out in presence of O_2 process (Sharma et al., 1999; Li et al., 2013; Mekki et al., 2013; Anand and Apul, 2014; Han et al., 2014; Cavagnaro, 2015). The resulting product is a useful material for agricultural and contaminated soils which have positive effects in soil characteristics such as structure and porosity, nutrient levels (de Bertoldi, 1983) and microbial activity (Liu et al 2007). Moreover, as we mentioned above,

stabilized-compost have been described as an effective alternative in the amelioration and remediation of degraded and contaminated soils enhancing C stock (Bustamante et al., 2010; Park et al., 2011), microbial activity (Alguacil et al., 2011), vegetation cover (Tandy et al., 2011) and improving some physical properties such as bulk density, aggregate stability and mean comparable weight diameter (Srinivasan et al., 2012). The stabilized-compost may decrease the availability of TE through different physicochemical reactions between OM and metals (Park et al., 2011; Jones et al., 2016; Kästner and Miltner, 2016).

However, some compost may have a relatively fast rate of degradation (Bolan et al., 2012; Pan and Sen, 2013) into soils (*e.g*., 139 days for poultry manures compost) when the organic materials have not achieve a complete stabilization and maturity. Under this context, up to now the influence of compost application on C sequestration due to compost management is limited in terms of C use efficiency and long-term C preservation even combined with organic farming and no till management (Fischer and Glaser, 2013). Some studies have shown that a part of the C source resulting from the composting process eventually becomes quickly mineralized in soils and returns to the atmosphere as CO_2 form (Chang et al., 2009; Godbout et al. 2010). Then, the beneficial effects of these materials in soils could be temporary and the applications should be frequently performed. Long-term observations of the effect of biannual compost application on soils showed that frequent amendments are able to increase soil C sequestration (Lashermes et al., 2009; Paetsch et al., 2016), although a large part of the organic matter applied may be subject to decomposition thereby increasing GHG emissions. In this framework, alternative methods to stabilize C and promote the recalcitrance of composted organic wastes in order to enhance nutrient dynamics reducing their losses should be considered in agricultural and contaminated systems.

Therefore, alternative methods to stabilize C in composted agricultural residues has been developed highlighting the use of microorganisms and inorganic materials such as metallic oxides and clay minerals (Brunetti et al., 2008; Bolan et al., 2012; Chan et al., 2016) which may catalyse and promote their stabilization, humification and the physicochemical protection. For example, some studies have shown that the application of metallic oxides such as iron (FeOx), aluminium (AlOx) and manganese oxides (MnOx) have positive effects on the transformation of organic materials (Qi et al., 2012) and

agricultural residues (*e.g.,* olive residues) promoting the humification process of OM into composting systems (Brunetti et al., 2008). Also, some authors noticed the use of clays minerals (*e.g.,* allophane) as compost protectors through the formation of organo-mineral complexes, preventing its microbial decomposition in soils (Bolan et al., 2012). Moreover, Jolanun and Towprayoon (2010) observed that addition of clay residues to organic waste composting was advantageous for aeration, moisture, and absorption enhancing N dynamics and losses.

In addition, one of the constituents of crops residues like wheat straw forming a macromolecular complex with cellulose and hemicellulose correspond to lignin, which avoid to microbes access to simple C sources (Tuomela et al., 2000; Huang et al., 2006). Lignin is a randomly constructed polymer of phenylpropanoid substructures, and the decomposition of this polymer requires a non-specific enzymatic system (Eriksson et al., 1990; Hatakka, 2001; Hofrichter, 2002; Madhavi and Lele, 2009; Hubbe et al., 2010). This complex structure inhibits or limits the lignocellulose transformation; thereby, lignin decomposition could be considered as an important rate-limiting step during the lignocellulosic residues-composting (Lopez et al., 2006). Some basydiomycetes are able to decompose and/or even mineralize lignin. These microorganisms posse a ligninolytic machinery of extracellular enzymes that includes phenol oxidases, peroxidases and hydrogen peroxide generating enzymes among others, which allows to these kind of fungi be proficient lignin degraders (Eriksson et al., 1990; Hatakka, 1994; Hofrichter, 2002; Grinhut et al., 2007).

The problem to address

Although several composting studies carried out during the last decades have enhanced our understanding about the composting process, contributing significantly to the compost science knowledge, there are still many unknows. For example, how the combination of microorganisms and inorganic materials (*e.g*., clay minerals) affect the OM stabilization in composting systems, and how these final products modify the chemical, biochemical and physicochemical properties of mining contaminated soils are still largely unknown. Thereby, complementary studies related to biotic and abiotic stabilization of crop residues

(wheat straw) in aerobic composting systems, and their effects in mining contaminated soils are still necessary. Considering this, here we proposed to conduct a Doctoral Thesis to study key aspects of composting with the following hypotheses and objectives:

1.1- Hypothesis and research objectives:

- The combination of saprophytic fungi and inorganic materials (metallic oxides and clay minerals) will promote the organic matter (OM) stabilization of aerobic composted wheat straw.

- The incorporation of wheat straw based-compost (WSC) will modified the chemical, biochemical and physicochemical properties of Copper (Cu)-contaminated soils, decreasing the availabilty of Cu through different interactions with the OM.

1.2- General objective:

To promote the OM stabilization of wheat straw in aerobic composting systems by using inorganic materials (metallic oxides) and saprophytic fungi in order to generate suitable soil amendments to be used in copper contaminated soils.

1.3- Specific objectives:

1) To determine the effects of saprophytic fungi, inorganic materials, addition times and their possible combinations in terms of OM stabilization in aerobic composted wheat straw systems.

2) To analyze the mineralization and OM stabilization of wheat straw composts in Cu-contaminated soils.

3) To evaluate the effects of wheat straw composts on physico-chemical, microbiological and biochemical properties of Cu-contaminated soils and analyze the availability of copper after compost application.

Chapter II

State of the art

Crop Residue Stabilization and Application to Agricultural and Degraded Soils: A review.

Abstract

Agricultural activities produce vast amounts of organic residues including straw, unmarketable or culled fruit and vegetables, post-harvest or post-processing wastes, clippings and residuals from forestry or pruning operations, and animal manure. Improper disposal of these materials may produce undesirable environmental (e.g. odors or insect refuges) and health impacts. On the other hand, agricultural residues are of interest to various industries and sectors of the economy due to their energy content (i.e., for combustion), their potential use as feedstock to produce biofuels and/or fine chemicals, or as a soil amendments for polluted or degraded soils when composted. Our objective is review new biotechnologies that could be used to manage these residues for land application and remediation of contaminated and eroded soils. Bibliographic information is complemented through a comprehensive review of the physico-chemical fundamental mechanisms involved in the transformation and stabilization of organic matter by biotic and abiotic soil components.

2.1 Introduction

Organic residues remaining after crop or wood harvesting in agriculture and forest industries can be transformed into energy, biofuels, high-value chemicals, forage or soil amendments (Liu et al, 2008; Schnitzer et al., 2014). Agricultural wastes are classified into two categories, crop residues (i.e., plant residues such as roots, leaves, straw, stubbles, among others), and residues from agro-industrial activities which are by-products of post-harvest and food processing, among others. Crop residues are one of the major organic wastes, with an annual production of about 74 Tg of dry crop residues produced worldwide. The global annual production of rice and wheat crop residues is about 45 Tg (Kim and Dale, 2004) whereby they may become a potential environmental problem or useful feedstock for new byproducts.

Crop residues are an important source of nutrients (Azam, 1990), which can positively influence the biological, chemical and physical properties of soil. The incorporation of crop residues increases nitrogen mineralization (Singh, 1995) as well as organic carbon (C) and total nitrogen (N) in soil (Cassman et al., 1996). In this context, several studies have shown that the incorporation of crop residues into soil improves tilth, reduces erosion processes, prevents nutrient losses by run-off and leaching, and increases microbial biomass (Lal, 1980; Lal et al., 1980; Maurya and Lal, 1981; Bukert et al., 2000; Shah et al., 2003; Shafi et al., 2007). Consequently, when organic residues are incorporated to the soil, detrimental or erosive processes decrease due to the high amount of organic matter (OM) that they supply. This is a very important aspect to take into account in both soil remediation and amelioration practices, since degraded and polluted soils usually have lower organic matter content (Lal, 2003).

Mining is an industrial activity, which may have a deleterious effect on soils, agro ecosystems and wildlife. Extensive mining produces a large amount of metal-enriched solid and liquid effluents, which can pollute air, water, and soils (Meier et al., 2012). The productivity of contaminated soils by mining activities requires subsequent expensive reclamation practices. Current and future reclamation technologies are designed to minimize such deleterious impacts through the use of socially friendly technologies, such as organic amendments from crop residues, which can even cause a prolonged sequestration of potentially toxic elements (PTE) such as metals (Pb, Cu, Zn, Cd, Fe) and metalloids (As, Se) into the organic matrix of soils.

Recent research indicates that incorporation of excessive residues as a soil amendment may have an adverse impacts on soil, environment and crop yield (Jiang et al., 2012) because many soil functions require mature and stable organic matter (Senesi, 1989; Senesi and Brunetti, 1996; Senesi and Plaza, 2007; Brunetti et al, 2008; Plaza and Senesi, 2009; Guenet et al., 2012). For these reasons, further studies concerning transformation and stabilization processes of organic wastes, prior to soil application are imperative. Ensuring a high supply and availability of nutrients for crops, and the long-term storage of atmospheric C in soils, will be favored if organic residues are well stabilized before their application to soil. Composting is one of the most used technologies for agricultural residues treatment. This technology reduces volume and weight by about 50% resulting in a

stable product that can be used to enhance chemical and physical properties of soils for agricultural purposes (Sánchez-Monedero et al., 2002). Alternative composting methods to stabilize C in composts are being developed. These methods enhance their quality and the physical, chemical and biological fertility of soils (Bolan et al., 2012).

Composting as technology has been widely described and summarized in several contributions as review articles and books chapters since decades. These articles describe different aspects of composting such as composts feedstock, production methods and properties of composts, composting parameters, methodologies for the characterization of the final product, biochemical transformations of organic matter, microorganisms involved in the process and the effects of compost in different types of soils (e.g. Agricultural, polluted or degraded soils), soils properties, plant growth and pathogen protection (de Bertoldi et al., 1983; Senesi, 1989; Schaub and Leonard, 1996; Senesi and Brunetti, 1996; Senesi et al., 1996; Scheuerell and Mahaffee, 2002; Chen, 2003; Mason, 2006; Larney and Hao, 2007; Bernal et al., 2009; Plaza and Senesi, 2009; Park et al., 2011; Mohammad et al., 2012; Singh and Kalambdha, 2012; Li et al., 2013; Mekki et al., 2013; Anand and Apul, 2014; Han et al., 2014; Cavagnaro, 2015) among others, which contribute significantly to the compost science knowledge. However, we think is necessary to summarize new findings and novel approaches described in current scientific articles due to the relevance of this topic for agriculture and environment.

Thus, our objective is to review the most relevant and current information regarding compost stabilization, use and application of composted agricultural residue, with emphasis on organic-metal complexes and metal-OM interactions. We hope that in the near future such knowledge may be useful for developing novel approaches, processes and technologies for the production and application of biotical and chemically stabilized composted organic amendments in polluted and/or degraded soils. Furthermore, we hope that these new composting approaches and technologies may be also used as tools for C sequestration in soils based on scientific evidence.

2.2 Crop residues production

As a consequence of the increase in world population, there has been a rise in the demand for food and food production. This has generated huge amounts of agricultural residues. As stated earlier in this article, agricultural residues can be classified conventionally into two categories: crop residues and agro-industrial residues (Tripathi et al., 1998). Crop residues refer to plant remains staying in the field after crop harvest. Crop residues include wheat straw, shafts and shoots, among others. On the other hand, agro-industrial residues are byproducts of the postharvest processes generated from cleaning, sieving, milling (Lim et al., 2012) or residues left by the food processing industry. Worldwide, straw, roots, shaft and other tissues of corn, wheat and rice are the main crop residues annually produced, which account for 40.6%, 24.2% and 15.7% respectively (Jiang et al., 2012, Schnitzer et al., 2014). For example, it has been estimated that the total production of crop residues only in China during 2009 was about 807 million tons, and the average yield was 716 million tons in the past 10 years (Jiang et al., 2012). Total yields and annual production of crop residues and the major producer countries and regions are summarized in Tables 2.1 and 2.2.

Kim and Dale (2004) estimated annual production rates of main crops produced around the world. For instance, they estimated that the maize annual production is about 520 Tg of dry matter. They also reported that major production regions are North America (42%), Asia (26%), Europe (12%) and South America (9%). Regarding maize, the highest grain yields occurs in North America, in which U.S. average grain yield is 7.2 Mg/ha.. About 5% of corn in the world is wasted, generating about 203.6 Tg of corn stover annually (Kim and Dale, 2004). On the other hand, the annual global production of dry rice grain is about 526 Tg. In this sense, Asia is the leading production region of rice with over 90% of global production and the largest yield occur in Australia. About 4.8% of rice annually production is lost as waste (Kim and Dale, 2004) and about 22 Tg of these loses residues occur in Asia. Finally, the annual global production of wheat dry grain is about 529 Tg, with Asia (43%) and Europe (32%) representing the main regions. North America is the third largest production region with 15% of global wheat production (Kim and Dale, 2004). The latter authors noticed that grain yield of wheat ranges from 1.7 to 4.1 dry Mg ha^{-1}.

About 20 Tg of dry wheat residues (4% of global production) is lost as waste. About 10 Tg of dry wheat in Asia ends up in the waste stream. Although wheat straw is being used as an animal feed, the scope of paddy straw as cattle feed is limited due to its high silica content (>12%) (Gaind and Nain, 2010).

Table 2.1

Annual production of the wheat straw and crop residues in the main producers' countries.

References	Country	Residues	Annual production (10^6 Mg y^{-1})
Li et al. (2012)	Canada	Wheat straw	37.4
		Total crop residues	82.4
Scarlat et al. (2010)	European Union	Wheat straw	108.6
		Total crop residues	258. 0
Liu et al. (2008)	China	Wheat straw	126.6
Jiang et al. (2012)		Total crop residues	630 .0
Cala-Rodríguez et al. (2003)	Spain	Wheat Straw	10 .0
		Total crop residues	38 .0
Schomberg et al. (1994); Lal (2005)	United Stated	Wheat Straw	74.0
		Total Crop residues	421.6

Table 2.2

Annual production of rice residues in the world, modified from Lim et al. (2012)

Regions	Residues	Annual production (10^6 Mg y^{-1})
Africa	Rice straw	24.5
	Rice husk	4.9
Asia	Rice straw	618.0
	Rice husk	123.65
Europe	Rice straw	4.10
	Rice husk	0.82
Oceania	Rice straw	0.29
	Rice husk	0.06
America	Rice straw	38.10
	Rice husk	7.62
World	Rice straw	685.24
	Rice husk	137.05

2.2.1 Potential environmental issues and economic benefits

Production of large amounts of lignocellulosic materials such as straw and shafts play a useful role in dealing with potential environmental problems. For example, the full harvest of crop residues and soil tillage may give rise to soil erosion and decrease soil OM content (Mann et al., 2002). Under anaerobic conditions N immobilization by soil OM can occur (Olk et al., 2002; Schmidt-Rohr et al., 2004), due to the binding of proteinaceous compounds to the lignin-derived phenolic component (Olk et al., 2006). Moreover, some studies have shown that when organic waste, such as crop residues, are used as a soil amendment the application of these fresh materials may cause several adverse effects on soils due to the lack of mature and stable OM (Senesi and Brunetti, 1996; Brunetti et al, 2008). These effects include, among others: i) an increase of the mineralization rate of native soil organic C through extended microbial oxidation, ii) the induction of anaerobic conditions by mineralization of large amounts of non-stabilized organic C, iii) the associated extended O_2-consumption, and iv) the alteration of soil pH (Senesi and Brunetti, 1996). In addition, these fresh residues may be phytotoxic due to the production of ammonia, ethylene oxide and organic acids (Tam and Tiquia, 1994). Otherwise, in some places their combustion have a negative impact on air quality (Madariaga, 2003). For this reason, environmental friendly treatments of agricultural residues are required.

2.3 Transformation and stabilization of agricultural residues

Crop residues treatment, their safe use and disposal become important issues for human society. With the increase in agricultural wastes, landfill space has become the limiting factor for disposal (Amir et al., 2003). Thus, residue processing and recycling become interesting alternatives for their treatment. The application of agricultural residues (e.g. crop residues, animal manures) to soil have become since decades interesting environmental and agricultural practice for maintaining and enhancing soil OM, being known that this remediates degraded soils and supplies crop nutrients. Thereby, it is well known that stabilization of these residues before their application to soil may help increase

their residence time by reducing their oxidation to CO_2 in soil agro-ecosystems. Some recent methods of stabilizing agricultural wastes are reviewed in the article.

2.3.1 Composting

Composting is one of the most important and common technologies for the treatment of agricultural residues. Composting reduces volume and weight about 50% and results in a stable product that can be used to enhance chemical and physical properties of soils for agricultural purposes (Senesi, 1989). In general, aerobic composting is defined as a microbiological process that converts waste into organic manure rich in plant nutrients and humus (Bewick, 1980; de Bertoldi et al., 1983; Senesi, 1989; Schaub and Leonard, 1996; Senesi and Brunetti, 1996; Sharma et al., 1999; Senesi et al., 2007). Composting is a technology for waste treatment and an important way for stabilization of organic residues. Different phases of composting process have been recognized and defined. The composting process, especially when it is carried out in batch mode, can be divided into phases characterized by temperature. The phases are conventionally defined in terms of the kinds of bacterial and fungal populations that thrive in different temperature ranges, i.e., psychrophilic (optimum ≤ 13 °C), mesophilic (21-48 °C), and thermophilic (45-68 °C) (de Bertoldi et al., 1983; Chen and Inbar, 1993; Haug, 1993; Mason, 2006; Hubbe et al., 2010; Li et al., 2013).

During composting, bio-macromolecules such as lignin, cellulose, hemicellulose, starch, among others, are partially transformed or decomposed by microorganisms into more simple molecules. The main microorganisms involved in the different phases of composting process include some groups of mesophilic such as *Escherichia*, *Streptococcus*, *Bacillus* and *Serratia*. At thermophilic and cooling down phases fungi such as Aspergillus sp., *Fusarium* sp. and *Penicillium* sp. appear to predominate (Table 2.3) (de Bertoldi et al., 1983; Hubbe et al., 2010; Mohammad et al., 2012).

During composting the biodegradable organic compounds are mineralized, whereas part of the remaining organic materials become precursors of humic substances (HSs) (de Bertoldi et al., 1983; Senesi, 1989; Schaub and Leonard, 1996; Senesi and Brunetti, 1996; Senesi and Plaza, 2007). During the decay and transformation processes of biomolecules

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(from plants tissues and other dead organism) HSs are formed by secondary biotic synthesis reactions or re-synthesis (Stevenson, 1994; Schnitzer and Monreal, 2011). The HSs are considered as the final product of OM stabilization by composting. The HSs have a crucial role in the main environmental reactions such as reductive and oxidative reactions, sorption and complexation of pollulants and soil C sequestration (Senesi, 1992 a; Bradley et al., 1998; Coates et al., 1998; Plaza and Senesi, 2009; Park et al., 2011; Singh and Kalamdhad, 2012).

Table 2.3
Type of Microorganisms involved in the different stage of composting. Modified from Hubbe et al. (2010).

References	Composting stage	Involved organism in the composting stage	Type of (micro)organism
Steel et al. (2010)	Thermophilic the beginning of composting process, (inmediatily after of heat peak)	*Rhabditidae sp.* *Panagrolaimidae sp.* *Diplogasteridae sp.* *Cephalobidae sp.*	Nematode Nematode Nematode Nematode
	At the cooling and maturation of the composting procedure	*Mononchoides sp.* *Dytilenchus filimus*	Nematode Nematode
Silva et al. (2009)	Mesophilic (composting before mixing the pile)	*Bacillus sp.* *Paenibacillus sp.* *Actinomycetes* *Aspergillus fumigatus*	Bacteria Bacteria Filamentous bacteria Fungi
Días et al. (2009)	At the end of composting procedure (samples in different points)	*Aspergillus fumigatus* *Emericella sp.* *Aspergillus ochraceus* *Aspergillus terraus* *Penicillium oxalicum*	Fungi Fungi Fungi Fungi Fungi

Chang et al. (2009); Tuomela et al. (2000)	At the cooling and maturation	*Thermoactinomyces sp.*	Yeast
Rebollido et al. (2008)	Mesophilic	*Pseudomonas sp.*	Bacteria
		Azotobacter sp.	Bacteria
		Azospirillium sp.	Bacteria
		Bacillus sp.	Bacteria
Salar and Aneja (2007)	Mesophilic and thermophilic	*Chaetomium sp.*	Fungi
		Thermophile sp.	Fungi
		Malbranchea sulfurea	Fungi
		Thermomyces lanuginosus	
		Torula thermophila	
Taiwo and Oso (2004)	Mesophilic	*Feacal coliformes*	Bacteria
		Streptococcus sp.	Bacteria
		Proteus sp.	Bacteria
		Serratia sp.	Bacteria
		Bacillus sp.	Bacteria
	Thermophilic	*Aspergillus sp.*	Fungi
		Fusarium sp.	Fungi
		Penicillium sp.	Fungi
		Humicola sp.	Fungi
		Mycothypa sp.	Fungi
		Scopurialopsis sp.	Fungi
		Cephalosporium sp.	Fungi
		Trichothesium sp	Fungi
	Cooling down stage	*Aspergillus sp.*	Fungi
		Fusarium sp.	Fungi
		Penicillium sp.	Fungi
		Cladosporium sp.	Fungi
		Mycothypa sp.	Fungi
		Scopulariopsis sp.	Fungi
		Coprinus sp.	Fungi
		Cephalosporium sp.	Fungi
		Trichothecium sp.	Fungi

Hassen et al. (2001)	At the cooling of composting procedure	*Escherichia coli*	Bacteria
	Mesophilic and thermophilic	*Streptococcus sp.*	Bacteria
	At the remainder of composting cycle	*Bacillus sp.*	Bacteria
De Bertoldi et al., (1983)	Thermophilic	*Absidia ramosa* *Allescheria terrestris* *Mucor pusillus* *Chaetomium thermophilum* *Talaromyces thermophilis* *Aspergillus fumigatus* *Humicola insolens* *Humicola lanuginosa* *Lenzites sp.* *Penicillium duponti* *Scytalidium thermophilum* *Sporotrichum thermophile* *Thermoascus sp.*	Fungi
	Meshophilic	*Allescheria terrestris* *Chaetomium sp.* *Dasyscipha sp.* *Emericella nidulans* *Mollisia sp.* *Thermoascus aurantiacus*	Ascomycota
		Armillaria mellea *Clitopilus insitus* *Coprinus cinereus* *Fomes sp.* *Lentinus lepiakus* *Lenzites trabea* *Pleurotus ostreatus* *Polyporus versicolor*	Basidiomycota
		Alternaria tenuis *Artrobotrys oligospora*	Deuteromycota

Aspergillus amstelodami
Aspergillus sp.
Botryotrichum piluliferum
Cephaliophora tropica
Cephalosporiopsis alpina
Cephalosporium sp.
Cephalosporium sp.
Cephalosporium sp.
Cephalosporium sp.
Cladosporium herbarum
Dorathomyces sp.
Geotrtchum candidum
Gliocladium sp.
Gliomastox murorum
Graphium sp.
Harpographium sp.
Leptographium lundbergii
Paecilomyces sp.
Penicillium sp.
Penicillium sp.
Phlyctaena sp.
Rhinocladiella artrovirens
Scopulariopsis brevicaulis
Scopulariopsis sp.
Sporotrichum thermophile
Stachybotrys sp.
Trichoderma viride

Humification of simple organic molecules produces changes in the chemical characteristics of the OM constituents, which are usually considered as quality indices for the end product of composting. These indices are usually based on the ratios between different extractable alkali fractions of OM such as humic acids (HA), fulvic acids (FA), total extractable carbon and absorbance of acetone extract (Iglesias and Pérez, 1989; Senesi et al., 1989; Senesi, 1992b; Romero et al., 2007; Khan et al., 2009). Different types of raw materials are used for composting and generate various types of HSs with different chemical compositions (de Bertoldi, 1983; Senesi, 1989; Senesi et al., 2002; Larney and Hao, 2007; Senesi and Plaza, 2007; Bernal et al., 2009; Mohammad et al., 2012; Li et al., 2013). For example, Brunetti et al. (2007) found differences among HS compositions in different organic amendments and native soils, highlighting that HA like-substances from the organic amendments exhibit higher H, N, S and phenolic OH group contents, and lower

C/N and C/H ratios, O, carboxylic, total acidity and organic free radical contents than native soil. Thus, Brunetti et al. (2008) reported that aliphatic and polysaccharide structures and S- and N-containing groups of HA-like fractions of organic amendments are partially incorporated into native soil HAs, hence, determining modifications to various extents of their composition, structure and reactivity. In the soil environment, chemical components of HA such as polyketides and humin, are extremely resistant to biodegradation (Schnitzer and Monreal, 2011). Their half-decay time ($t_{1/2}$) reach thousands years. For example, ^{14}C dating has shown that soil OM in whole and aggregate size fraction in soils from the Great Plains and Eastern regions of North America, or from volcanic environments is in the order of hundreds-to-thousands of years old (Monreal et al., 1997; Grinhut et al., 2007).

The resulting product of the composting process is used frequently for agricultural purposes such as amelioration of soil characteristics (as structure and porosity) and enhancing the nutrient supply for crop growth (Bewick, 1980; de Bertoldi et al., 1983; Senesi, 1989; Plaza and Senesi, 2009; Mekki et al., 2013; Cavagnaro, 2015). For example, Tejada and Gonzales (2003) found that the application of crushed cotton gin residues compost was of great agricultural interest due mainly to its high OM content, which produced an increase in soil microbial activity, structural stability and porosity. Carbon sequestration is another effect of composting process which is currently receiving an increased interest (Pipatti and Wihersaari, 1998; Lou and Nair, 2009). Lynch et al., (2006) found that some compost sources, such as corn residues, had a high recalcitrance in soils after being applied. The above authors noticed that one year after application, 75−95% of the applied compost C remained in soil, and hence composts increase OM recalcitrance with prolonged storage in non-mineral soil fractions. Moreover, Bustamante et al., (2010) found that applying compost could be a great contribution to the reduction of atmospheric CO_2, because it increases C storages in soils after compost application. However, authors as Godbout et al. (2010) has shown that a part of the C source resulting from the composting process eventually becomes mineralized in the soil and returned to the atmosphere as CO_2 (Senesi, 1989).

New approaches have been developed for enhancing the residence time of HS produced by composting in soil before being decomposed (Godbout et al., 2010). Decreasing the large amount of energy needed for producing the optimal conditions of

biological composting at optimum moisture and temperature, and decreasing the long composting time needed for OM humification (90-365 days) has been studied by Alfano et al. (2008). Some novel composting technologies which aim to promote the stabilization of the composted organic amendment by physico-chemical interactions has been analyzed by Bolan et al. (2012) and Qi et al. (2012a, b), and are reviewed in the following sections.

2.3.2 Biotransformation of crop residues by saprophytic fungi

Lignin, hemicelluloses and celluloses are major components of crop tissues. Lignin is a complex aromatic macromolecule that provides strength and rigidity to cell walls and tissues of all vascular plants by acting as glue between the polysaccharide filaments and fibres (Monties and Fukushima, 2001). Lignin is a chemically heterogeneous molecule which is linked by several covalent bonds (Brunow, 2001). These bonds cannot be cleaved by hydrolytic enzymes as in most other natural polymers (cellulose, starch, proteins, etc) (Hofrichter, 2002). Lignin tends to be less abundant than the cellulose or hemicellulose components of biomass during the composting processes (Tuomela et al., 2000; Eiland et al., 2001a,b; Jouraiphy et al., 2005; Huang et al. 2006; Alburquerque et al., 2009; Spaccini et al., 2009). In the future, the addition of organisms such as saprophytic fungi that can act over the chemical bonds in lignin could be a way to accelerate the transformation and humification of residues with high lignin content.

It is well known that saprophytic fungi act as primary, secondary and tertiary decomposers in natural ecosystems (Grinhut et al., 2007). Moreover, fungi are the most efficient degraders of macromolecules such as lignin, hemicelluloses and celluloses and humic substances (HS). Fungi are considered more efficient than bacteria because most of bacteria cannot degrade various of these substances (Esham et al., 2000; Machnikowska et al., 2002; Filip and Tesarova, 2004). However, recent studies showed that certain bacterial isolates have an enhanced capability for lignin breakdown, where apparently extracellular enzymes as peroxidases and laccase appear to be involved (Vargas-Garcia et al., 2007; Bugg et al., 2011).

Saprophytic fungi secrete non-specific oxidoreductases extracellular enzymes, such as manganese peroxidase (MnP), laccase (LAC) and lignin peroxidase (LiP). Apparently,

MnP and LiP production is only limited to certain basidiomycetous fungi (Grinhut et al., 2007). In this context, two ecophysiological groups have been described as able to secrete these enzymes: white-rot and litter decomposers (WRF and LDF) (Dix and Webster, 1995), which secrete MnP in multiple forms into their surrounding microenvironment. When these enzymes are secreted by saprophytic fungi, decomposition of lignin (making up the so-called ligninolytic system) lead to the formation of unstable compounds (e.g. phenoxy radicals), which can undergo subsequent condensation and polymerization (humification) (Stevenson, 1994; Chefetz et al., 1998; Grinhut et al., 2007). The pathway followed by each enzymatic product (degradation or polymerization) is probably dependent not only on the enzymes and substrates involved, but also on reaction conditions, such as pH, humidity, percent oxygen and electrical conductivity (Grinhut et al., 2007). In addition, other enzymes such as cellulases, hemicellulases and esterases which depolymerize cellulose, hemicellulose and glucosides are also considered to be extremely important in the degradation process of lignocellulosic biomass (Tabka et al., 2006).

About 8,500 species of basidiomycetes have been described, which are lignocellulose-degrading saprothops and half of them occur in soils, wood and fallen plant litter (Lynch and Thorn, 2006). Moreover, these groups of fungi are able to degrade recalcitrant molecules as well as HS. Based on their large size and stability, HS are not likely-metabolized intracellularly by microbial cells, instead they appear to be initially degraded by extracellular non-specific enzymes (Kastner and Hofrichter 2001). It has been observed that white-rot fungi and litter decomposer fungi can degrade and transform macromolecules only in presence of other easily degradable C sources. For a review about this topic see Grinhut et al. (2007).

As a consequence of their extracellular enzymatic activity, WRF and LDF have been widely used for degrading lignocellulosic compounds of agricultural residues (crop residues), and thus, reducing the time-course of the stabilization into HS. For example WRF are well known for biodelignification of wood chips (Eriksson and Kirk, 1985), wheat straw (Tsang et al., 1987; Arora, 1995) and bamboo sticks (Reid, 1989).

2.4 Stabilization of OM by composting of crop residues

2.4.1 Biotic stabilization

Two main mechanisms are responsible for OM stabilization during composting: biochemical alteration and physicochemical protection. Biochemical alteration occurs when simple organic compounds are transformed by biotic (microorganism) processes into more resistant and larger molecular size forms of OM. These transformations are usually both referred to as "humification processes" and altered or transformed products as "humus" material (Senesi and Plaza, 2007). Humification is believed to be the primary process by which simple OM components are stabilized under the form of HSs (Stevenson, 1994). Under this context, the aim of any treatment for organic residues, such as composting, is to promote OM humification by increasing its biostability and maturity, thus improving its potential as an organic fertilizer and avoiding adverse environmental effects that may occur in soil (Senesi et al., 1989; Senesi and Brunetti, 1996; Plaza et al., 2002). Recently, Schnitzer and Monreal (2011) indicated that a microbial and biochemical humification path proceeds via synthesis of polyketides. In the latter process, intracellular and complex multi-enzyme systems cooperate to catalyze processes of condensation, aliphatic chain elongation, polyaromatic ring formation (aromatases) and a few other reactions. As a result of the latter biotic process, a large stable and chemically diverse alkyloaromatic structures are formed that serve as the backbone of HAs.

2.4.2 Abiotic stabilization

2.4.2.1 Stabilization by metal oxides

Recent studies have shown that abiotic oxidation of OM by oxides such as iron oxides (FeOx), aluminum oxides (AlOx) and manganese oxides (MnOx) have a positive effect on organic residues, increasing the residence time and accelerating the humification process of organic matter in soils (Brunetti et al, 2008; Qi et al., 2012a, b; Saidy et al., 2012). Humification by abiotic agents has been described for several agricultural wastes. In

particular, MnOx have shown to be more reactive catalyzers than other soil mineral constituents such as AlOx and FeOx in the polymerization of phenolic compounds based on their high oxidation potential and high specific surface reactivity (Shindo and Huang, 1984; Wang and Huang, 2000). In this context, Brunetti et al. (2008) found that manganese (II) oxide (MnO_2) was able to catalyze efficiently the humification of oil mill wastewater promoting the chemical OM alteration, possibly acting in some reactions such as condensation, oxidative polymerization, ring cleavage, decarboxilization, and dealkylation reactions (Jokic et al., 2004).

The use of abiotic humification process by inorganic oxidants has shown various advantages. This method has a fast processing time, and no selectivity for different OM sources. Qi et al. (2012a, b) used "steel slag", which is characterized by a high content of calcium, iron, manganese and silicon, all of which are present mainly as oxides that are tightly bound to the slag matrix. Qi et al. (2012b) reported that Fe(III) and Mn(IV)-oxides in steel slag can act as effective oxidants and substantially accelerate poly-condensation of model humic precursors (glycine, glucose and catechol) to form FA, which are subsequently transformed into HA. Moreover, Qi et al. (2012a, b) found that humification reactions can inhibit the release of metals from steel slag under acidic conditions, which presents environmental advantages. Xu et al. (2012) found similar results in stabilized sewage sludge using fly ash as catalyst. For these reasons, this method of abiotic composting has attracted particular attention, and currently a series of novelty materials are being used for humification of organic amendment (Table 2.4).

2.4.2.2 Stabilization by clay colloids

The application of clay materials as stabilizing agents (goethite, gibbsite, allophane, among others) aims at decreasing the decomposition rate of HS. For example, it has been described that allophane spherules, a non-crystalline or 'short-range order' aluminosilicate, have an important role in soil OM stabilization. Calabi-Floody et al. (2011) found that in volcanic soils a significant amount (11.8%) of carbon is so strongly held by nanoclay surfaces as to resist repeated treatments with hydrogen peroxide. About 50% of this carbon is identifiable as black carbon-derived compounds, suggesting that allophanic nanoclays in volcanic soils

play an important role in carbon stabilization. The role of some clay minerals in the stabilization of compost (poultry and cow manures) has been evaluated in a recent study (Bolan et al., 2012). Results showed that the addition of clay materials to compost decreased the rate of decomposition, thereby increasing the stabilization and half- life of C. Half-life value of poultry compost manure increased from 139 days to 620, 806 and 474 days with the addition of goethite, gibbsite and allophane, respectively (Bolan et al., 2012). In this case, the decrease in decomposition rate may be attributed to the interaction between C of OM and Al and Fe oxides mainly through the formation of stable organo-mineral complexes. In addition, Schnitzer and Monreal (2011) indicated that polymerization and cross-linking of alkylaromatic moieties of HAs by surfaces of clay colloids result in stabilizing HA macromolecules and soil organic matter.

Table 2.4

Abiotic additives used for organic compounds and compost stabilization.

References	Amendment or organic substrate	Abiotic additives	Mechanism
Qi et al. (2012)a; Qi et al. (2012)b; Nishimoto et al.(2013).	Glycine, glucose Catechol	Steel slag (Fe(III) and Mn(IV) oxides)	Polycondensation reactions of humic precursors as quinones, amino acids and saccharides
Brunetti et al. (2008).	Oil mill wastewater	Manganese oxide	Condensation, oxidative polymerization, ring cleavage, decarboxilization, and dealkylation reactions
Bolan et al., (2012).	Poultry manure and cow manure compost, green Waste biochar	Goethite, Gibbsite, Allophane.	Physicochemical protection of OM by complexation, reduced Bacterial activity,

			low pH and poor availability of nutrients.
Xu et al. (2012).	Sewage sludge	Fly ashes.	Promoting humification process.
Himanen and Hänninen (2009).	Peat mixture	Sulphates and oxides of iron, magnesium, manganese, and zinc mixed with clay; calcium hydroxide, peroxide, and oxide.	pH alteration, decrease water-soluble ammonia among others.

Bolan et al. (2012) and others suggests that OM preservation from stabilized compost occurs in a similar way as that in soils, basically influenced by a number of processes including: i) physicochemical protection of OM by complexation with iron, aluminum and allophane in these cases (Eusterhues et al., 2003; Mikkuta et al., 2006; Matus et al., 2008; Calabi-Floody et al., 2011; Garrido and Matus, 2012) ii) reduced bacterial activity that results from the presence of free iron and aluminum; and iii) low pH and poor availability of nutrients, especially phosphorus to soil microorganisms involved in OM degradation (Parfitt, 2009).

The physicochemical protection is referred to as organo-mineral interactions between HSs and minerals, which occur at the molecular level from namometer to micrometer scales. As a result of these interactions, the microbial attack of OM (decomposition) is prevented. Some physicochemical ways for OM stabilization are the complexation with soil minerals and occlusion within soil aggregates, among others, which produce highly inaccessible OM to decomposers and extracellular enzymes (Monreal et al., 1997; Jastrow et al., 2007). Therefore, the physicochemical protection can be defined as the spatial inaccessibility for biotic decomposers due to several mechanisms such as intercalation, occlusion and interaction with mineral surfaces and metal ions. Mineral

surface interaction (Fe-, Al-, Mn-oxides, phyllosilicates) with OM is one of the most important mechanisms for OM stabilization that protects it against the decomposers. Sorption occurs by diverse organo-mineral associations, such as polyvalent cation bridges, hydrogen bonding, van der Waals forces, and interactions with hydrous oxides and aluminosilicates (Jastrow et al., 2007).

2.4.3 Properties of Stable OM in Compost

It has been well established that poorly-ordered Fe, Mn and Al-Fe Oxides are the most reactive components that facilitate humification from precursors such as amino acids, polyphenols, and reducing sugars (Wang and Huang, 2000; Chen et al. 2010), this being concomitant with the organo-metal complex formation, among other physicochemical interactions. Currently, these inorganic colloidal agents are an alternative to agricultural residues treatment and stabilization processes which are applied to several agricultural residues such as olive oil mill wastes, poultry manure, cattle manure, among others. Noteworthy, there is no sufficient information about using these inorganic compounds in the humification of organic materials, or in composting processes of lignocellulosic materials. The reader is directed to an article published by Mtui (2009) dealing with recent technologies that have been used for the treatment and pretreatment of lignocellulosic biomass and production of value-added products which include mechanical, physical and biological systems.

2.5 Biotechnological applications of stabilized agricultural wastes

2.5.1 Restoration of eroded and degraded soils

Stabilized agricultural residues are usually used as soil amendments for enhancing soil properties (i.e., structure, nutrients, OM content). These amendments have been used for decades in amelioration and remediation of polluted, degraded and eroded soils. Soil erosion affects physically and chemically to soils and degrades its structure, altering soil fertility among other properties (Lal, 2003). This destructive process causes physical loss of

topsoil with its constituent nutrients and soil OM, exposing at the surface less fertile subsoil, which is usually characterized by a low structural stability and productivity. This type of erosion adversely affects soil quality and ecosystem functions (Srinivasan et al., 2012). Annually, the global rate of erosion is about 75 billion Mg (Pimentel et al., 1995) and 100 Mg ha^{-1} for severely eroded soils (Lal, 2003). As a consequence of soil OM losses by erosion, the content of soil organic C is often < 1-2 %, resulting thus necessary to increase soil OM content, by intensification of crops or by incorporation of organic amendments.

Currently, the use of compost for greenhouse gases (GHG) mitigation has drawn increased attention. Compost application is considered an important tool, because it recycles crop nutrients and decreases the requirements of synthetic fertilizers and pesticides; thereby reducing GHG emissions due to use of fossil fuel associated with their industrial production and application (Cogger, 2005; Favoino and Hogg, 2008). Use of composted OM also increases and accelerates the growth in plants, thereby increasing CO_2 uptake and C storage within the plant and C sequestration in soil (Mondini et al., 2007; Favoino and Hogg, 2008; Arriagada et al., 2014). Composts may also improve tillage and workability of soil, thereby reducing emissions (Favoino and Hogg, 2008; Lou and Nair, 2009).

2.5.2 Ecological restoration

Soil amendments produced from agricultural residues are widely used for soil and ecological restoration to enhance the health of degraded systems. After environmental degradation, restoration and reclamation efforts can be hampered by poor physic-chemical soil characteristics and reduced diversity in the community of soil microorganisms (Ohsowsky et al., 2012). In general, soil amendment with composts enhances the nutritional characteristics (Allievi et al., 1993), soil properties (Table 2.5) and microbial activity (Liu et al., 2007). For this reason, compost application in ecological restoration has a positive effect on the vegetation cover. Tandy et al. (2011) reported that in a short-time experiment, compost applied to a nutrient-poor alkaline soil increased OM, nutrient content, and water holding capacity, whilst lowering soil pH. These changes were linked to

an increase in vegetation cover. In an eroded soil from the Sonoran desert (USA), however, compost and inoculant (bacteria and arbuscular mycorrhizal fungi) application together with native plants (*Prosopis articulata*, *Parkinsonia mycrophilla* and *Parkinsonia florida*), had no long-term beneficial response on vegetation cover in this degraded ecosystem (Bashan et al., 2012). In the latter study and in the short-term (< 3 months), plants had a positive response on growth (height, number of branches and diameter of the main stem) until three months after the application of compost. The short-term benefits can be explained by the quick mineralization of some carbon sources from compost (Godbout et al., 2010).

Table 2. 5
Effects of stabilized agricultural wastes applied as soil amendment.

References	Effects in soils
Crecchio et al. (2004); Adani et al. (2006,2007) Bustamante et al. (2010); Bolan et al. (2012)	Enhancing the C stock and increasing the colloidal humified organic matter
Spark et al. (2008); Olivier et al. (2012)	Increasing the activity of microbial communities and VOC emissions by plants.
Alguacil et al. (2011)	Promoting the activity of arbuscular mycorrhiza
Stucky and Hudak (2001)	Plant growth promoting
Gaind et al. (2010); Srinivasan et al. (2012)	Improving chemical and physical properties of soil
Eriksen et al. (1999); He et al. (2000); Wortman and Walters (2007)	Mineralization of nitrogen and phosphorus content in soil
Tandy et al. (2011); Bashan et al. (2012)	Ecological restoration of eroded soils: Enhances WHC, increases soil OM, promote bacteria and AMF activities
Bolan et al. (2003); Park et al. (2011), Nielsen et al. (2011); González et al. (2012)	Remediation of metal-polluted soils: increasing metal sorption, changes in pH, increase of microbial activities, reduction of metals.

2.5.3 Remediation of polluted soils

As a consequence of some industrial activities, there are significant levels of pollutants in soils, and other natural systems, which alter the functioning and equilibrium of ecosystems. Industrial activities, such as mining, are associated with important pollution problems in soils (Lottermoser, 2003). The discharge of metal-enriched solid and liquid effluents into the air, water and soils generate a deleterious effect on the ecosystems particularly in soils (Meier et al., 2012). In developing countries, mining is an important economic activity, being responsible for generating a great amount of pollutants. Solid mine wastes contain a large amount of metals in particulate matter, and also a high volume of wastewater is generated during the mining and metal extraction processes (Gray et al., 2003).

When heavy metals and metalloids are incorporated into the soil, they undergo different reactions. Their adsorption and complexation at the interface between the soil solution and solid phases result in their accumulation. Adsorption reactions reduce the formation of surface complexes which can be either physical attachment or bonding of metal ions and molecules onto the surface of another component. Metal adsorption is highly dependent of soil pH; e.g. the sorption of cationic metals increases when increasing pH while that of anionic species decreases when increasing pH (Adriano et al., 2004), and on the presence of other soil organic and inorganic components, redox potential and cation exchange capacity (Naidu et al., 2008). For instance, soil OM has a high affinity for metal cations due to the presence of ligands or functional groups (Senesi, 1992 c; Harter and Naidu, 1995). When soil pH increases, H^+ dissociates from functional groups such as carboxyl, phenolic and hydroxyl functional groups, thereby increasing the affinity for metal cations (Park et al., 2011).

Heavy metal and metalloids can form different complexes with soil inorganic surfaces due to various hydroxyl groups with different levels of reactivity. These complexes can be inner-spheres complexes, when metal(loid) ion is directly bound to functional groups of soil particles or outer-sphere complexes when water molecules are interposed in between functional groups of soil particles and metal(loid) ions (Park et al., 2011). In the same sense, organic complexes between metal(loid) and organic acids such as HA and FA, can be affected by different factors such as temperature, soil pH, ionic

strength, dominant cations and soil type (texture), being soil pH the main factor on organic-metal complexes formation (Senesi, 1992c; Bolan et al., 2003; Luo et al., 2010). Another important reaction experienced by metals in soils is the precipitation, which is defined as the process of metal(loid) immobilization in presence of inorganic anions such as sulfate, carbonate, hydroxide and phosphate when the soil pH and the metal(loid) concentration are high (Adriano, 2001). Because phosphate are able to precipitate metals, they are the most common compounds used to effectively precipitate heavy metals and metal(loid)s in contaminated soils or water. According to the degree of stability of precipitates between phosphate and metals, Pb forms the most stable precipitate and Zn the lowest one (Bolan et al., 2003). Other important metal stabilizing reactions in the environment are: a) oxidation/reduction reactions by microorganisms; e.g. reduction of Se(VI) to Se(0) by bacteria; b) methylation/demethylation, which is considered to be the major process of volatilization of As, Hg and Se in soils, resulting in the release of toxic methyl gases (Cernansky et al., 2009); and c) the biological modification of local soil environment as a consequence of some microbial processes which can enhance metal(loid) solubility, thereby increasing their bioavailability and potential toxicity (Park et al., 2011).

The use of stable composts may help remediate soils contaminated by mining activities especially if the concentration of one or more metals exceeds the specified threshold level in soil profile. A number of environmental remediation systems using physical, chemical, or biological treatments have been developed in recent decades (Meier et al., 2012), highlighting the immobilization techniques, which are focused on reducing the availability and activity of trace elements, but not on removing the pollutant from the soil. The purpose of immobilization processes is to accelerate natural ones such as sorption, complexation and precipitation reactions using amendment with the aim of decreasing the mobility and bioavailability of pollutants (heavy metals among others) in soils (Bolan et al., 2003).

Inorganic materials (i.e iron oxides, phosphate and calcium) and/or organic materials) have been used for increasing the stability and complexation of contaminants in polluted soils (Castaldi et al., 2005; Derome, 2009; Nielsen et al., 2011). González et al. (2012) found in a metal-As polluted soil that the combination between an agricultural residue and marble sludge (8% marble sludge with 2% compost) was most effective

increasing the immobilization of Cd, Cu, Pb and Zn. None of these treatments, however, proved to be effective in reducing the soluble concentrations of all trace elements involved. Actually, amendment was effective at immobilizing one pollutant, but may increase the mobility of others (Hartley et al., 2004).

Organic amendments usually enhance bioremediation of heavy metal(oid)s through various processes that include immobilization, reduction, volatilization and rhizosphere modification (Park et al., 2011). The immobilization is referred to adsorption reactions, where the organic amendments induced retention of metal(loid)s which is attributed to an increase in surface charge (Clark et al., 2007) and the presence of metal(loid) binding compounds (Gondar and Bernal, 2009). Bolan et al. (2003) found that the incorporation of compost increased the surface charge of the amended soils, which is attributed to the higher pH and surface charge of the composted biosolid. Other organic amendments have been effective in decreasing metal bioavailability by immobilization. For example, Cu is immobilized in soil because of increased the Cu-organic complexes formation, and Cr by reduction from Cr(VI) to Cr(III), and subsequent precipitation as chromic hydroxide (Park et al., 2011). Generally, organic amendment application has shown to have a positive effect in soil improving soil physical characteristics such as particle size distribution, cracking pattern and porosity. Improved soil structural characteristics contribute to prevent the dispersion of metal contaminated particles by formation of water stable soil aggregates (Park et al., 2011).

Further, metal(loid)s are subject to redox reactions which can have a biotic or abiotic origin, being the reduction reaction the most important in some metalloids such as Arsenic. It has been noticed that organic amendments such as compost from crops residues among other, have a positive effect enhancing the Cr and Se reduction (Bolan et al., 2003; Chiu et al., 2009; Hsu et al., 2009), where Cr(IV) is reduced to Cr(III) which is less available to plants. Under anaerobic conditions and increased OM content, As(VI) can be biotically reduced to As(III) resulting in higher toxicity and mobility in the soil environment (Wakao et al., 1988). The amendment application in polluted soil enhances the dissolved organic carbon (DOC) content, and the easily oxidable fraction of DOC providing the energy source for soil microorganisms involved in the reduction processes of metal(loid) (Chiu et al., 2009). Moreover, DOC and natural OM may be associated with Hg

altering its speciation and bioavailability in ecosystems; this form strongly bonds with humic substances and appears to stabilize Hg(II) in soil environment (Park et al., 2011). Another important effect of organic amendment such as crop residues compost application is the modification of rhizospheric environment. As a consequence of these changes (pH, organic acids, soil solution composition, microorganism activity) the metal chemistry can influence transformation, mobility and availability of metal(loid)s in soils (Akhtar and Malik, 2000: Caravaca et al., 2005; Perez-de-Mora et al., 2006). The complexation of heavy metals with soluble organic compounds increases their mobility (Neal and Sposito, 1986). In comparison, organic ligands of stable soil macromolecules (i.e., with aliphatic components) in humic substances may reduce metal mobility and associated environmental toxicities (Monreal et al., 1998). Thus, increasing the content of long-chain components as lipids and alkanes in humified organic materials will help stabilize heavy metals in soils preventing their transfer into environment and food chain.

Bioremediation, such as phytoremediation is an important technique used for remediation of heavy metal polluted soils (Brunetti et al., 2012: Ghanem et al., 2013). Plant-mediated decontamination/detoxification processes are commonly referred to as "phytoremediation" (Rajkumar et al., 2012). Phytoremediation is generally considered an inexpensive and environment friendly technique; and it may involve various processes: phytoextraction, phytoinmobilization, phytotransformation, phytodegradation, phytostimulation, phytovolatilization, and rhizofiltration (Ashraf et al., 2010). The main disadvantage of phytoremediation is, however, a slow process that requires several years or decades to reduce metal concentrations in soil at levels that are harmless to humans and other organisms (McGrath and Zhao, 2003). This slowness is due to the limited growth and biomass production of hyperaccumulator plants (Peuke and Rennenberg, 2005). For this reason, enhancing the soil conditions for metal hyperaccumulator plants may be a priority in phytoremediation research. Hajishah et al. (2010) evaluated the phytoextraction of Ni, Cd and Pb using different amendments (compost and EDTA among others) and found that the effectiveness of sugarcane compost to stimulate the accumulation of Cd, Pb in plant shoots were 2.8 and 2.9 times higher than the control respectively. Further, they found that all treatments (compost and EDTA) were higher in terms of solubilizing at different levels soil Pb, Cd and Ni for root uptake and translocation into canola shoots. These results

suggested that the amendment used could be an alternative in the remediation and phytoremediation of heavy metal polluted soils. Finally, several microorganisms such as bacteria and mycorrhizal fungi have been described as enhancers of phytorremedation process in heavy metal polluted soils (Arriagada et al., 2009a; Brunetti et al., 2012; Meier et al., 2012).

Actually, soil microorganisms as arbuscular mycorrhizal fungi (AMF) are involved in diverse biochemical processes and nutrient cycling. These processes enhance or accelerate the re-establishment of a plant cover, thereby increasing the stability of polluted ecosystems (Reynolds et al., 1999; Moynahan et al., 2002; Arriagada et al., 2007). In this context, soil microorganisms have developed several mechanisms to resist or tolerate the toxic effects of metals in polluted soils. Particularly, in the case of soil fungi, the tolerance/resistance mechanisms developed include; (a) adsorption of metals to the cell wall surface (biosorption), (b) transportation and cellular incorporation (bioaccumulation), and (c) transformation of metals through redox or methylation reactions (Gadd, 1986, 1993).

The ability of AMF to confer resistance to plants against metal and metalloids has been reported in several studies (Janousková et al., 2005; Hildebrant et al., 2007; Arriagada et al., 2009b; Aguilera et al., 2011; Meier et al., 2011; 2015 Barea et al., 2013; Cornejo et al., 2013; Seguel et al., 2013). These observations and the positive effect of mycorrhizal symbiosis on the phytoremediation of metal-polluted soils are of great biotechnological interest, as mycorrhizal plants are as effective in extracting metals (e.g., Cu, Cd, Pb, Zn) as non mycorrhizal-hyperaccumulator plants (Huang and Cunningham, 1996; Ebbs and Kochian, 1998; Arriagada et al., 2010). Mycorrhizal plants also improve phytostabilization because metals (Zn, Cd, and Cu) are bound to hyphae and roots without translocating these elements to shoots (Joner and Leyval, 1997; 2001). As such, metals remain in the soil but because they are less bioavailable, toxicity to other organisms is reduced (Leyval et al., 2002). The production of extracellular polymeric substances (EPS), mucopolysaccarides and proteins by plant-associated microbes can also play an important role in complexing toxic metals and in decreasing their mobility in the soils (Rajkumar et al., 2012). However, production and accumulation in soils of glomalin, a glycoprotein produced by AMF, is an important mechanism to enhance phytorremediation process, since glomalin can

immobilize in a stable form several toxic elements, such as Pb, Cu, Zn, Al (González-Chávez et al., 2004; Cornejo et al., 2008; Vodnik et al., 2008; Aguilera et al., 2011; Seguel et al., 2015). Therefore, the manipulation and use of AMF as a tool for polluted soils begins to be considered in phytoremediation programs using mycorrhizal plants (Meier et al., 2012). Further research is required to understand the long-term effects on metal sequestration plant-AMF association and the combined effects of organic amendments application.

Several studies showed that the application of organic amendments as compost derived from agricultural residues have a positive effect on microbial activity and plant growth (Table 3) suggesting their possible use and also the use of phytoremediation plans to enhance the immobilization of metals and promote soil microbial activity. Alguacil et al. (2011) found that the addition of sugar beet waste amendment was a good strategy for the remediation and/or phytostabilization of mine tailing sites. Alguacil et al. (2011) and Meier et al. (2011) have also found that the application of sugar beet waste (treated with temperature) was very efficient with regard to increasing AMF communities in the majority of the host plant species assayed and decreasing the concentration of metals in shoot tissues.

Within this context, Medina et al. (2010) found a positive interaction between a dry olive cake (compost) with a AMF strain (isolated from a Cd-contaminated soil). Such interaction resulted in a highest growth of *Trifolium repens*, which may be associated to enhanced nutrient acquisition and the immobilization of Cd in the compost amended soil. Despite the latter results, the long-term potential of organic amendment from agricultural residues, for metal(loid) immobilizing material, however, is still unknown. Little is known about the nutrient mineralization rates of different amendment materials in soils, especially the long-term effects. Current studies have reported that different organic amendments have high decomposition rates (Godbout et al., 2010). Thus, the positive effects of plant-AMF associations on metal(loid) immobilization in polluted soil could be functioning in a short-term (<1 year), being necessary frequent compost applications. Therefore, the development of improved and stabilized amendments whit an increased residence time in soils is still necessary in order to enhance the waste management and promote the remediation of degraded and polluted soils.

2.6 Conclusions and future prospects

The application of composted organic amendments derived from different crop residues has a positive impact on the physical, chemical and biological properties of soils. The application of organic amendments also helps to immobilize metals and some metalloids through adsorption and complexation reactions, reducing their bioavailability and limiting their further transfer through plant uptake and leaching. Moreover, their joint use (microorganisms, plants, organic residues and inorganic materials) could be a useful tool for enhancing phytostabilization processes in environmental conditions. In this sense, the novel technologies of OM stabilization here reviewed appears as an important tool for the production of improved amendments finalized to agriculture and remediation of polluted and physically degraded soils. Finally, the new strategies for improved compost production based on agricultural residues may stimulate farmers to keep those materials revaluing and reusing them in scenarios as above described.

Chapter III

Compost production and organic matter stabilization

Associated to specific objective 1

Manuscripts associated to this chapter:

- Use of saprophytic fungi and inorganic materials for enhancing the aerobic composting of wheat straw. I (paper to be submitted to Waste Management).

- Supramolecular structure of humic substances extracted from a compost-amended mining soil: Main features and their potential effects on Cu-immobilization.
(Paper under peer review process, Environmental Science and Pollution Research).

Abstract

Composting transforms highly resistant organic materials through microbial activities into a stable and complex organic matter (OM). Some saprophytic fungi decompose lignocellulosic biomass through their enzymatic systems. Moreover, the addition of metallic oxides and clays to composts may enhance carbon (C) stabilization via changes of physical, chemical and biochemical properties of OM. In order to promote compost stabilization of wheat straw (WS), the effects of saprophytic fungi (*Coriolopsis rigida*, *Pleurotus ostreatus*, *Trichoderma harzianum* and *Trametes versicolor*) inoculation and the supply of inorganic materials (allophanic clays and Fe-Al oxides) were studied. Laccase (LAC), manganese peroxidase (MnP), and β-glucosidase activities were measured. The chemistry of compost and humic like-substances (HS) extracted from composted WS was characterized by its elemental composition, E_4/E_6 ratios, and changes in functional groups of OM, determined by Fourier transform infrarred (FTIR), and cross polarization magic angle spinning ^{13}C nuclear magnetic resonance (CP/MAS $^{13}CNMR$) spectroscopy. In addition, its structure and shape were observed by electron microscopy (TEM/SEM). Wheat straw inoculated with *T. versicolor* showed highest enzymatic activities for LAC (~3.0 U L^{-1}), MnP (~0.350 U L^{-1}) and β-glucosidase (~450 µmol pNP g^{-1} h^{-1}) relative to those shown in composted WS treated with the other fungal species. At the end of composting, the E_4/E_6 ratio was lower in the Fe-oxide treatments than the untreated ones indicating that extracted HS presented a high molecular weight and poly-condensation of aromatic rings. Information from the FTIR and microscopic observations indicates that Fe in compost may be bound to acidic functional groups promoting the HS aggregation. Finally, $^{13}CNMR$ spectra showed that WS composts (WSC) inoculated with *T. versicolor* and supplied with Fe-oxide achieves the highest relative aromaticity at the end of process, indicating a partial stabilized and mature compost. These results suggest that WS inoculation with saprophytic fungi in combination with metal oxides can helps to accelerate composting and produce a stable end-product in ~120 days of process.

Keywords: Compost maturity, enzymatic activities, humic like-substances, lignocellulosic materials.

3.1 Introduction

Agricultural residues are one of the major organic materials generated worldwide every year, which usually are burnt in fields causing an environmental problem (Ruiz, 2011) and missing out their energetic potential and their suitable use as biomass for different purposes like high-value chemicals extraction (Habets et al., 2013; Schnitzer et al., 2014) and soil-amendments, since this residue can positively influence the biological, chemical and physical properties of soils and crop yields (Andrews, 2006; Shafi et al., 2007; Zhang et al., 2015).

Nevertheless, recent research indicates that incorporation of excessive fresh residues as soil amendments may have adverse impacts in soil environment and crop yield (Jiang et al., 2012) because many soil functions require mature and stable organic matter (OM) (Plaza and Senesi, 2009; Guenet et al., 2012). The application of non-stabilized materials to soil may generate a negative impact with some unwanted effects, highlighting among others: *i)* an increase of the mineralization rate of native soil organic carbon (OC) through extended microbial oxidation, *ii)* the induction of anaerobic conditions by mineralization of large amounts of non-stabilized OC, *iii)* associated extended O_2-consumption, *iv)* the alteration of soil pH (Senesi and Brunetti, 1996; Senesi and Plaza, 2007) and the stimulation of green house gases (GHG) emissions through increasing C availability for methanogenics in paddy soils (Yan et al., 2005) and denitrifiers in dry-land soils (Luo et al.,1999). Moreover, many studies found that additional N-fertilizer is needed when residues are left on soils to avoid N uptake (immobilization) from soil or allow for soil C accumulation (e.g. Clapp et al., 2000).

Composting is considered an important process for C stabilization involving partial OM humification. The resulting humified-OM is a potentially suitable amendment for agricultural (Castaldi et al., 2005; Scotti et al., 2015), degraded and/or contaminated soils (Park et al., 2011; Medina et al., 2015). Composting of organic materials through successive microbial activities results in more stable and complex OM, which chemically and biologically resembles humic substances named humic like-substances (HS) (Dinel et al., 2004). These materials are refractory organic compounds; dark colored and heterogeneous produced by humification process and contribute with essential functions to global soil fertility and health (Senesi and Plaza, 2007). However, incompletely

stabilization of composts from agricultural residues result in relatively high rate of OM degradation (Bolan et al., 2012). Some practices are used to enhance the quality and stabilization of composted organic materials. Some of them include the use of inorganic materials such as allophane, and Fe-Al-Mn oxides, lime and zeolite (Brunetti et al., 2008; Bolan et al., 2012; Qi et al., 2012; Chan et al., 2016; Wang et al., 2016). These materials interact with OC catalyzing the humification processes, changing chemical properties, and/or through inter-molecular interactions (*e.g*., complex formation), thus preventing the microbial degradation and increasing C stock in soils.

Lignin, which is one of the main constituents of WS, forms a macromolecular complex with cellulose and hemicellulose preventing microbes from accessing simple organic substrates (Tuomela et al., 2000; Huang et al., 2006). Lignin is a randomly constructed polymer of phenylpropanoid substructures, and the decomposition of this polymer requires a non-specific enzymatic system (Hofrichter, 2002; Madhavi and Lele, 2009; Zeng et al., 2014). Lignin decomposition is considered as an important rate-limiting step during the lignocellulosic residues-composting (Lopez et al., 2006). Some basydiomycetes are able to decompose and/or even mineralize lignin. These fungi have a ligninolytic machinery of extracellular enzymes that includes phenol oxidases, peroxidases making them proficient lignin degraders (Hofrichter, 2002; Grinhut et al., 2007; Sahadevan et al., 2016). White rot fungi contain specific enzymes Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), Laccase (LAC), and hydrogen peroxide-generating enzymes. Along with these enzymes, Reactive oxygen species (ROSs) is also considered to be an important agent for wood decay by fungi. Various combinations of these enzymes are produced by different fungi which suggest different mechanisms of lignin degradation (Sahadevan et al., 2013).

Based on the above, we hypothesized that the inoculation of saprophytic fungi in combination with inorganic materials should achieve apropiate stabilization in aerobic composting systems. Thus, the main objective of this study was to promote the stabilization of lignocellulosic residues (wheat straw) through aerobic composting by using saprophytic fungi as lignin-biotransformers in combination with inorganic materials (allophanic clays and metallic oxides) as humification-catalyst and physico-chemical protectors of transformed OM.

3.2 Material and methods

3.2.1 Wheat straw, saprobe fungi and inorganic materials

Wheat (*Triticum aestivum* L. var. Kumpa) straw based-composts (WSC) were produced at pilot-scale under controlled conditions of temperature (25°C) and moisture (50%) during 126 days. The initial WS material was collected after crop harvest from plots located at Maquehue experimental station (Universidad de La Frontera) in La Araucanía region, Chile. The compost units, with an initial weight of about 1.5 kg each, were composted in piles into autoclave bags as individual containers. Before, the initial WS was chopped into 1-2 cm pieces with a mill. Then, the material was immersed in hot water at 60-80 °C for 2 hours in plastic containers as pretreatment, in order to reduce the contamination, enhance the fungi colonization, and decrease the population of other microorganisms (Colavolpe et al., 2014). Bags were kept open; moisture was measured and maintained by applying distilled water. Wheat straw piles were initially inoculated with different fungal strains corresponding to *Coriolopsis rigida* (Berk. Et Mont.) Murrill, *Trametes versicolor* (L.:Fr.) Pillat, *Trichoderma harzianum* Rifai and *Pleurotus ostreatus* (Jacq.) P. Kumm.*,* which were obtained from the fungal collection at the Laboratorio de Biorremediación, Universidad de La Frontera, Temuco, Chile. The inocula were prepared on Malt Extract Agar (MEA) plates growing for 15 days at 28°C. Afterwards, an aqueous suspension of each fungal strain was obtained by means of the disruption of culture media (plates) in 400 mL of sterilized deionized H_2O, and a similar quantity of fresh mycelia was added to the WS experimental units, being thoroughly homogenized. Iron oxide (Fe_2O_3; Sigma Aldrich®), Aluminum oxide (Al_2O_3; Sigma Aldrich®) and an allophane-enriched soil horizon (Soil AC) (Pemehue soil series, 25 % of allophane content in B horizon, Pitrufquén locality, Araucanía Region; 39º05`25``, 72º31`47``, 140 m a.s.l.) were added to different composts as potential catalysts of humification process and compost protectors. These catalysts were added to composting units at 2 % w/w of total compost mixture (Gadepalle et al., 2008).

3.2.2 Composting process and experimental design

The inorganic materials: Fe_2O_3, Al_2O_3 and Soil AC, were incorporated to the experimental units at the following addition times: *i)* 2 weeks (at day 14) after fungal inoculation (T0); *ii)* 8 weeks (at day 56) after fungal inoculation (T1); and *iii)* 16 weeks (at day 112) after fungal inoculation (T2). Moisture was adjusted to initial 65-70% and kept at 50% in later stages of composting (Khan et al., 2009; Pan and Sen, 2013) and the process was maintained for 16 weeks (126 days). Aerobic composting was kept under greenhouse conditions, and sampling was made every two weeks. Three replicate samples were taken from the different experimental units in each pile for the subsequent biochemical and physicochemical analyses. The experimental design was a completely randomized factorial experiment, with three replicates for each fungal strain (4 fungal species), inorganic materials (addition of 3 inorganic materials and 1 control), and time of inorganic material application (3 application times), for a total of 48 composting treatments.

3.2.3 Enzyme assays

Enzymatic extracts from composting treatments were obtained every 2 weeks by the following procedure: 2 g of compost material were added with 25 mL of sodium acetate, shaken at 100 rpm for 2 h at 4 °C and centrifuged at 5000 rpm for 20 min. The supernatant was filtered through Whatman filter paper no. 3 and kept at -20 °C until their use. The following enzymatic activities; laccase (LAC) and manganese peroxidase (MnP) (Castillo et al., 2000) for ligninollityc activities (phenoloxidase and peroxidase enzymes), and β-Glucosidase (Tan et al., 1987) for global hydrolytic activity, were determined. Laccase enzyme was determined by the oxidation of 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The reaction mixture contained 300 μL of buffer sodium acetate (50mM; pH: 5.0), 100 μL ABTS (20mM), and 1000 μL of enzymatic extracts. Reactions were carried out by 5 minutes and absorbance was obtained at 420 nm. Manganese peroxidase (MnP) was assayed through the oxidation of 2, 6-Dimethoxyphenol (DMP). The reaction mixture contained 200μL of buffer sodium malonate (250 mM; pH 4.5), 50μL of DMP (20 mM), 100 μL of H_2O_2 (4 mM), 50 μL $MnSO_4$ (20mM), and 600 μL of enzymatic

extract. Determination was followed by reactions of 5 minutes and measured at 469nm. The activity of β-glucosidase was carried out by the determination of *p*-nitrophenol (pNP) released by the catalytic action of the enzyme using *p*-nitrophenil-β-D-glucopyranosyde (pNPG) as substrate. Reaction mixture contained 400 µL of pNPG in 50 mM of sodium citrate buffer (pH: 4.8) and 100 µL of enzymatic extract. Each tube was incubated at 50°C during 30 minutes. At the end of the incubation 1 mL of 2 M Na_2CO_3 (pH 11.0) was added in order to stop the reaction.The amount of *p*-nitrophenol released was determined by measuring absorbance at 410 nm and the enzyme activity was calculated according to standard curves.

3.2.4 Composting stabilization tests and humic like-substances extraction

For analyzing the evolution of composting and compost stabilization, bulk samples were collected from all treatments at different times during the process. At the end of composting process, humic like-substances (HS) were extracted for chemical characterization. Briefly, 10 g of air-dried compost sample were placed into a 150-mL propylene flask and 100 ml of NaOH 0.5 M were added under N_2 flux. Flasks were shaken for 24 h at room temperature and the dark colored supernatant was recovered by centrifugation (4500 rpm for 10 min). The humic acid fractions were coagulated by acidifying the alkaline extract to pH 2.0 adding HCl 2 M, and separated by centrifugation (Schnitzer, 1982). Finally, in order to to clean and minimize the ash content of HS, a purification of HS by shaking for 24h with a HF-HCl solution (5 mL 52% HF + 5 mL conc. HCl + 990 mL H_2O) was carried out and repeated twice (Schnitzer, 1982; Zbytniewski and Buszewski, 2005). The HS were also washed to completely remove Cl^-.

The pH was measured on water-extracts (1:10 w/v) and compost temperature was determined in the middle of the pile around 20 cm in depth every week. In order to determine a humification index of composting which express the condensation grade of the different treatments E_4/E_6 ratios were assayed. For this, 1g of material was extracted using 50 mL of NaOH 0.5 M, shaken overnight at room temperature, centrifuged at 4500 rpm for 25 min and the absorbance was measured at 472 nm (E_{472}) and 664 nm (E_{664}) (Zbytniewski and Buszewski, 2005; Khan et al., 2009).

3.2.5 Spectroscopic analysis

At the end of WS composting (after 16 weeks) the chemical characterization of compost and HS including, the elemental composition (C, N, H, S, and O), content of functional groups and the transformation of C types in OM were performed. Considering the results obtained previously as criteria (see section 3.2.7), these analyses were conducted for the following treatments: WS inoculated with *T. harzianum*, *T.versicolor* and *P.ostreatus* fungal strains; no co-composted (Control) and co-composted with Fe_2O_3 and Soil AC (B horizon from an andisol) incorporated at T1 addition time (56 days after the inoculation).

3.2.5.1 Elemental analysis

Elemental analysis was performed using an elemental analyzer Vario EL III Elemental Analyzer G.G. (Hatch Stable Isotope Laboratory, Ottawa University, Canada). Details for the elemental analysis method used please refer to http://www.isotope.uottawa.ca/welcome.html

3.2.5.2 Functional groups of OM by FTIR spectroscopy

Functional groups of OM were characterized by Fourier-Transform Infrared (FTIR) spectroscopy. For this, bulk compost samples and HS from composts were prepared as described by Dinel et al. (2004). Briefly, HS were ground and sieved at 450 nm, dried overnight at 70°C, prepared by mixing 400 mg KBr with 2 mg of the materials and then compressing the mixture into pellets. Spectra were obtained by using Varian Scimitar 1000 FTIR. The analysis was performed at the Department of Chemistry, Carleton University, Ottawa, Canada.

3.2.5.3 Cross Polarization/Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (CP/MAS ^{13}C NMR)

Structural changes of C-types in OM of bulk samples and HS from compost were determined using CP/MAS ^{13}C NMR. The ^{13}C solid-state NMR spectra were obtained with a Bruker Ascend^{tm} 400 MHz wideboard spectrometer operating at a ^{13}C resonance frequency of 150.93 MHz. The cross polarization magic angle spinning (CPMAS) technique was applied with a spinning speed of 14 kHz. The ^{13}C chemical shifts were calibrated relative to tetramethylsilane (=0 ppm) with glycine (176.04 ppm). Approximately 10000 single scans with pulse delays between 300 and 500 ms were accumulated for each spectrum (de la Rosa et al., 2014). Spectra regions were divided as follow: alkyl-C (0-45 ppm), N-alkyl/methoxyl C (45-60 ppm), O-alkyl C (60-110 ppm), aryl C (110-160 ppm), carboxyl/amide C (160-185 ppm) and aldehyde and ketones C (185-245 ppm).

3.2.6 Content of functional groups

The following chemical methods were used to determine the content of functional groups of OM in bulk compost and extracted HS samples. For the active hydrogen and total acidity (sum of carboxylic and phenolic), both compost and HS were treated with an excess of Ba (OH)$_2$ solution under N$_2$ for 24 h. The Ba (OH)$_2$ remaining after reaction was then back-titrated with standard acid (Wright and Schnitzer, 1959). Whereas, the carboxylic group content was estimated with an excess of Ca (OAc)$_2$ solution. For this, samples were shaken for 24 h with Ca (OAc)$_2$ and the acetic acid liberated was then titrated with NaOH solution (Wright and Schnitzer, 1959). Phenolic OH groups were determined as follows: total acidity (meq g^{-1} of humic material)-Carboxylic groups (meq g^{-1} of humic material)=Phenolic OH groups (meq g^{-1} of humic material). Finally, the carbonyl groups were measured by a quantitative oximation with hydroxylamonium salts (Fritz et al., 1959).

3.2.7 Humic like-substances shape and aggregation

For determinations of shape, HS-metal interactions and macromolecular structures by direct observation (size, degree of aggregation), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques were used. Presence and distribution of

elements such as, metals ions into the organic matrix of HS from compost were supported by the X-ray Energy Dispersive spectroscopy (X-EDS) (see next section). For obtaining TEM images, HS, samples were diluted (1/100) in Milli-Q® quality H_2O, applied with a nebulizer on carbon-coated copper grids and then were air-dried for 24 hrs. Formvar Carbon Films on 400 Mesh Copper Grids (Electron Microscopy Sciences, Cat # FCF400-Cu) were used and samples were observed with a Hitachi H-7000 (Hitachi, Tokyo, Japan) microscope. Images were collected with a Gatan ORIUS^{TM} SC 200 CCD camera (Gatan, Inc., Pleasanton, CA, USA) and the observations were performed in the Laboratory of Microscopy at Agricultural and Agri-Food, Canada. In addition, for analyzing the presence of crystalline structures on the organic matrix of HS, the electron diffraction patterns (ED) were performed at 60 cm from the focus.

For SEM analysis, HS from compost were dissolved in a NaOH 0.1 M solution at 100 mg HS L^{-1} which was used as stock. This stock solution was used to prepare dilutions at 1/100, adjusted to pH 4.0 and 8.0, and one drop of each one was placed on 400 Mesh Copper Grids (EMS, cat # FSF400-Cu), and dried at room temperature for 24h (Chen et al., 2007). Then, samples were coated with gold and the observations were performed with a Quanta 600 FEI (FEI Inc., Hillsboro, OR, USA) at 20 kV under high vacuum conditions in the Laboratory of Microscopy at Agriculture and Agri-Food, Canada.

3.2.8 X ray Energy Dispersive(X-EDS) analysis

In this study, was possible to identify spectroscopically some elements like N, O, Na, Mg, Al, Si, Cl, K, Ca, Fe, Cu, Zn, in HS extracted from wheat straw based-composts (Tv+Fe-oxide treatment). For this characterization, HS samples were coated with a C film and observed by the electronic microprobe JEOL JXA8600M, using EDS Bruker detector; model XFLASH 5010. Images and spectra were analyzed by QUANTAX200 (Bruker) software. Samples were worked at 15 kV and 10 nA as experimental conditions. X ray Energy dispersive analyses were performed in the Laboratory of Electronic Microprobe, associated to the Institute of Geology (GEA) from Universidad de Concepción, Chile.

3.2.7 Statistics

All data sets were subjected to three-way analysis of variance (ANOVA) after the corroboration of normality and homocedasticity to determine the main effects of fungal strain, inorganic materials addition, application time and all the possible interactions among the factors, for each sample stage. In some cases, data sets did not meeting the assumptions for ANOVA and were transformed by means of Ln, but the results are shown in the original scale of measurement. Repeated measures analyses were performed to analyze the global effect of the above mentioned factors. Moreover, the global means were subjected to a factorial analysis including the extraction of principal components (PC) to obtain different groups of experimental individuals with a global homogeneous behavior. The correlation among the different variables and the obtained PC were analyzed using the Pearson correlation coefficient. A non-hierarchical cluster analyses using the farthest-neighbor as agglomerative method was performed to the obtained PC for clustering the different treatments and select some of them (in each cluster) for the spectroscopic procedures as previously mentioned. Significance level was established at $p < 0.05$. Statistical analyses were performed with the SPSS software v.15.0 (SPSS, Inc., Chicago, Il.).

3.3 Results

3.3.1 Enzymatic activities

3.3.1.1 Treatment effects on temporal changes

Enzyme activities changed during the composting process according to the effects caused by various treatments. Laccase (LAC) activity increased markedly 14 days after fungal inoculation and reached its highest level on day ~112 of composting in the treatment *P.ostreatus*+Al-oxide applied at T0. Moreover, when Fe_2O_3 was applied to the *T. versicolor* inoculated compost treatment (*T.versicolor*+Fe-oxide) LAC activity reached its maximum at day 42 of composting when the inorganic material was incorporated 14 days after fungi inoculation (T0) and at day 98 of composting when the metallic oxide was incorporated 56

day after fungi inoculation (T1) ($\sim$4 U L^{-1}) (SD 1; annex 1). Addition of Al$_2$O$_3$ and Soil AC 14 days after fungi inoculation also increased the LAC activity in the *T. versicolor* inoculated treatments at days 70 and 42 respectively of composting. When *T. versicolor* inoculated compost were supplied with Al$_2$O$_3$ and Soil AC 56 days (T1) after fungi inoculation there was an increase of LAC activity for Al$_2$O$_3$ and a decrease for Soil AC treatment. Besides, in *T.versicolor* treatment, the application of Fe$_2$O$_3$ at day 56 decrease the activity at the day 70 with the metallic oxide addition, but then increased LAC activity up to the end of composting. Contrariwise, the addition of Fe$_2$O$_3$ to *C. rigida* inoculated treatment at day 112 (T2) drastically decreased this enzymatic activity up to the end of composting. At the end of composting (126 days), the WSC inoculated with *T. versicolor* showed the highest LAC activity (a mean value of 3.0 U L^{-1}) relative to those shown in composted WS treated with other fungal species (Fig. 3.1). *Trichoderma harzianum* showed the lower values for this enzymatic activity ($\sim$0.35 U L^{-1}). The addition of Fe$_2$O$_3$ showed an increase for LAC activity, especially when it was applied on T1 of composting process. Control treatments for each inoculated treatment (with no inorganic material addition) did not show a clear tendency of the evolution of LAC activity during the process.

The activity of MnP in WS inoculated with both, *T. versicolor* and *C. rigida*, and co-composted with the metallic oxides showed an unclear tendency and irregular evolution during the composting process (SD2; annex 2), especially when Al$_2$O$_3$ was incorporated 14 days after fungal inoculation. On the other hand, the addition of inorganic materials (Al$_2$O$_3$) to composting treatments inoculated with *P. ostreatus* decreased MnP activity at day 84. Additionally, composted WS inoculated with *T. versicolor* also showed the highest level of MnP activity (0.517 U L^{-1}) whereas *T. harzianum* showed the lower value for MnP activity (0.049 U L^{-1}) (Fig. 3.2). Similarly, addition of Fe$_2$O$_3$ and Soil AC increased the MnP activity in *T. versicolor* inoculated treatments, especially when the inorganic materials were incorporated at day 56 and 112 (T1 and T2). Composted WS inoculated with *C. rigida* showed a high enzymatic activity as well, highlighting the higher MnP activity at day 28 for Al$_2$O$_3$ added 14 days after compost inoculation (Al-oxide T0 treatment) and at day 112 for Fe$_2$O$_3$ treatment (SD2; annex 2). Controls from different inoculated treatments (with no inorganic materials addition) showed an irregular evolution during the process as well and -
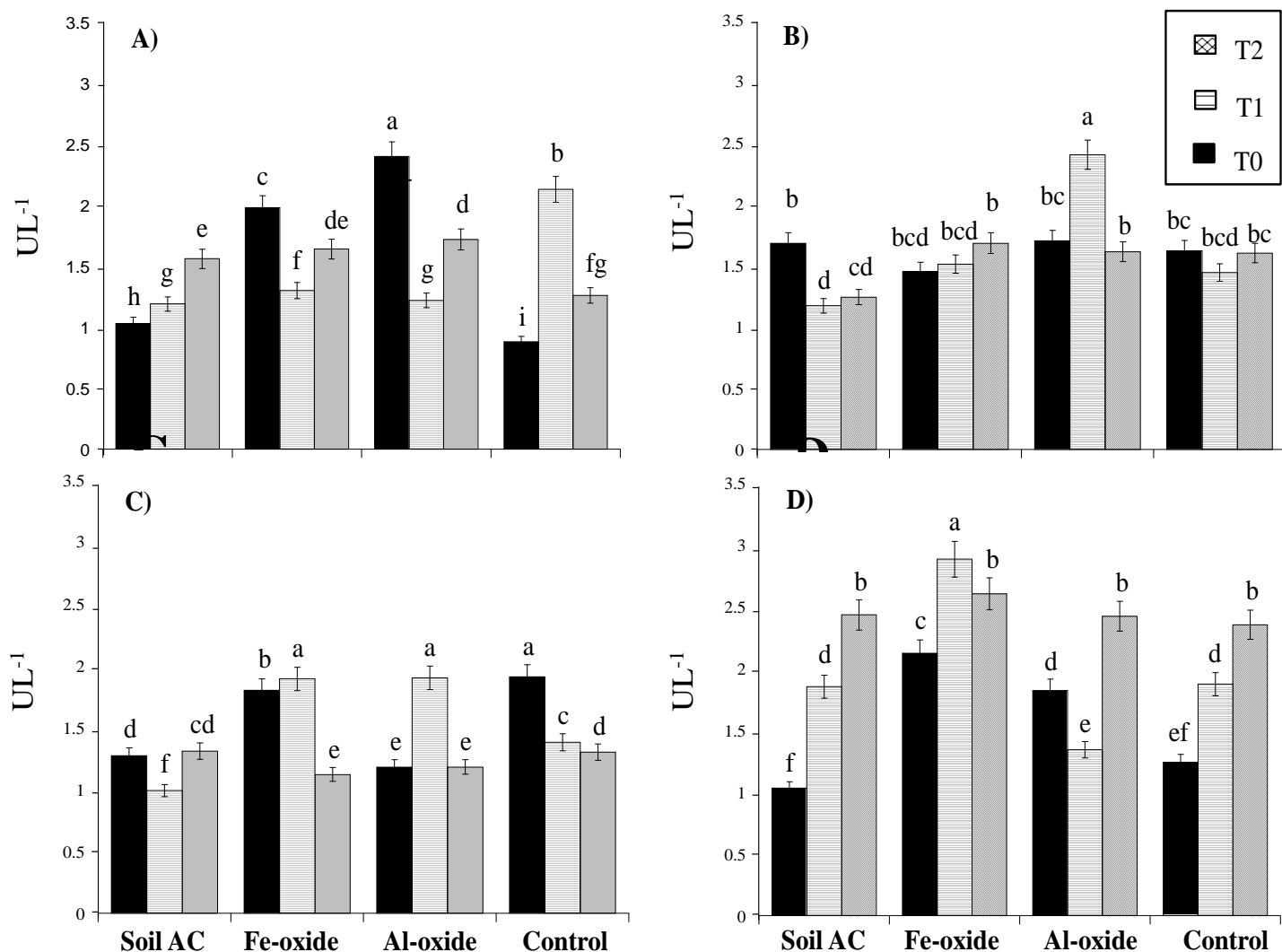
Figure 3.1 Mean activity of laccase (LAC) enzyme (U L^{-1}) in composted wheat straw systems inoculated with A) *Coriolopsis rigida*; B) *Pleurotus ostreatus*; C) *Trichoderma harzianum* and D) *Trametes versicolor* strains, no co-composted (Control) and co-composted with the b horizon of an Andisol (Pemehue soil series) (Soil AC); Iron oxide (Fe-oxide) and Aluminum oxide (Al-Oxide). The inorganic materials were applied at different times of application as follow, T0: applied 14 days after fungi inoculation (black bars); T1: applied 56 days after fungi inoculation (hachured bars); and T2: applied 112 days after fungi inoculation (reticulate bars). Letters represents significant differences (*P<0.05*).
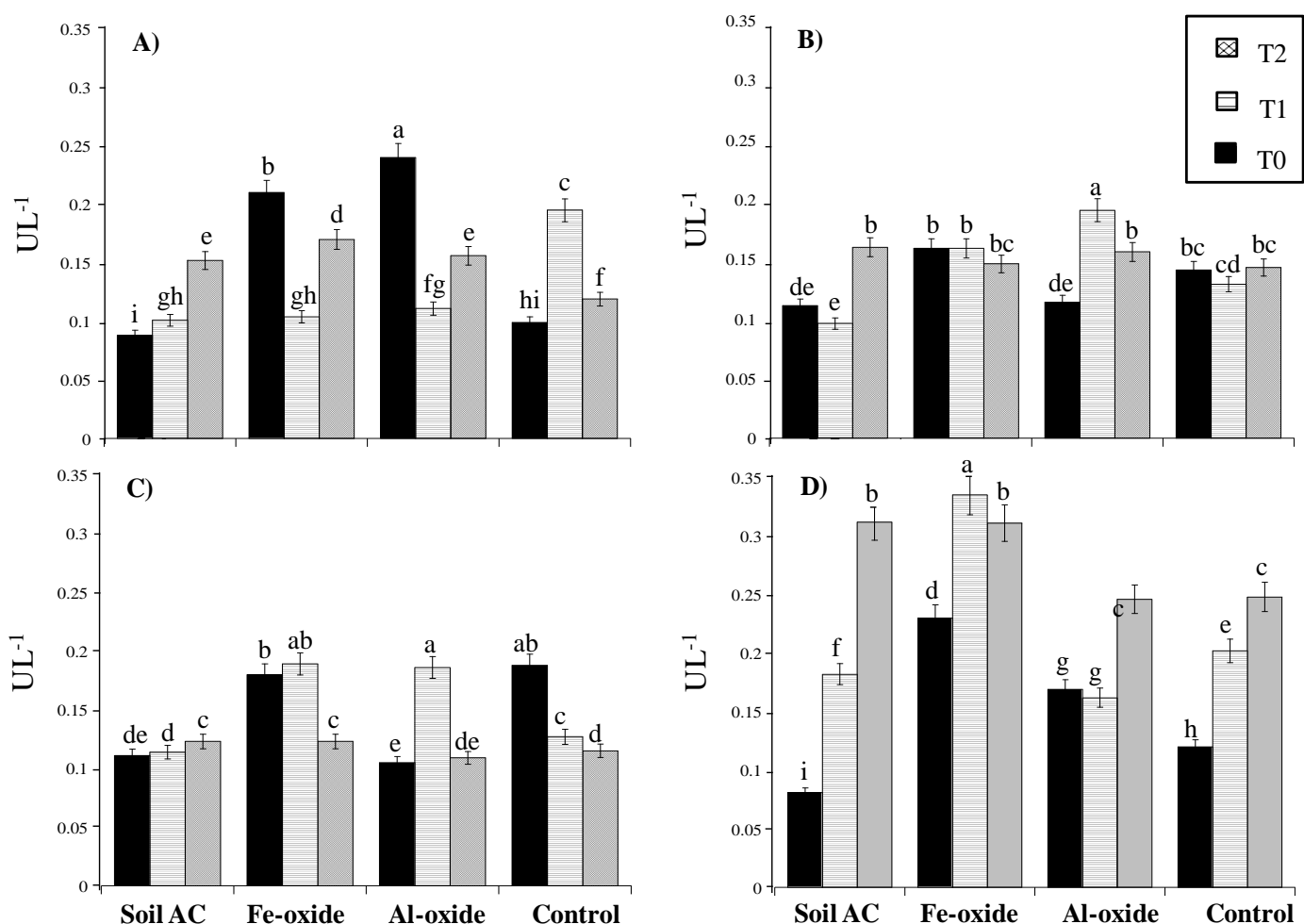
Figure 3.2 Mean activity of Manganese peroxidase (MnP) enzyme (U L^{-1}) in composted wheat straw systems inoculated with A) *Coriolopsis rigida*; B) *Pleurotus ostreatus*; C) *Trichoderma harzianum* and D) *Trametes versicolor* strains, no co-composted (Control) and co-composted with the b horizon of an Andisol (Pemehue soil series) (Soil AC); Iron oxide (Fe-oxide) and Aluminum oxide (Al-Oxide). The inorganic materials were applied at different times of application as follow, T0: applied 14 days after fungi inoculation (black bars); T1: applied 56 days after fungi inoculation (hachured bars); and T2: applied 112 days after fungi inoculation (reticulate bars). Letters represents significant differences (P<0.05).

-highest activity was observed in *T. versicolor* treatments with a mean activity of ~0.28 U L^{-1}.

There was not a clear tendency in the evolution of the β-glucosidase activity during the composting process. In this sense, WS inoculated with *P. ostreatus* showed a high initial activity that decreased as composting progressed. Whereas, compost samples inoculated with *T. harzianum* showed a high enzyme activity at the beginning of composting period mainly in the first 50 days, increased and decreased at days 14 and 52, respectively (SD3; annex 3). In comparison with the other control treatments (no inorganic materials addition), WSC inoculated with *T. versicolor* showed the highest activity for β-glucosidase (~600 µmol pNP g^{-1} h^{-1}) (Fig. 3.3). Moreover, the addition of Al_2O_3 induced the highest overall β-glucosidase activity in the compost inoculated with *T. versicolor* at the end of 126 days of aerobic composting (Fig. 3.3). The addition of Al_2O_3 to WS inoculated with *T. versicolor* at days 14 and 112 (T0 and T2), and that of Soil AC at day 112—resulted in the higher enzymatic activities (*p<0.05*) than the other inoculated treatments. WS inoculated with *C. rigida* showed a higher β-glucosidase activity when Al_2O_3 and Fe_2O_3 were incorporated at day 14 (T0).

3.3.2 Composting maturity test

Compost pH, E_4/E_6 and C/N ratio of composting material and HS were used as index of compost maturity. In addition, changes in compost temperature were analyzed to stop the process of composting. The pH in all compost treatments increased as the composting process progressed; it increased from pH ~ 4.0 at the beginning of composting up to pH ~9.0 at the end of the process (126 days)(Fig. 3.4). Wheat straw inoculated with *C. rigida* and co-composted with Al_2O_3 at day 112 (Al-oxide T2) showed the extreme values for pH (3.42 at beginning and 8.53 at the end of composting). On the other hand, *P. ostreatus* inoculated treatments showed the highest pH at the end of composting (9.14). *Trametes versicolor* and *T.harzianum* showed a similar evolution of pH with values below 4.0 at the first stages since days 1 to 42 of composting and up to 8.0 at the final stage of composting (Fig. 3.4).
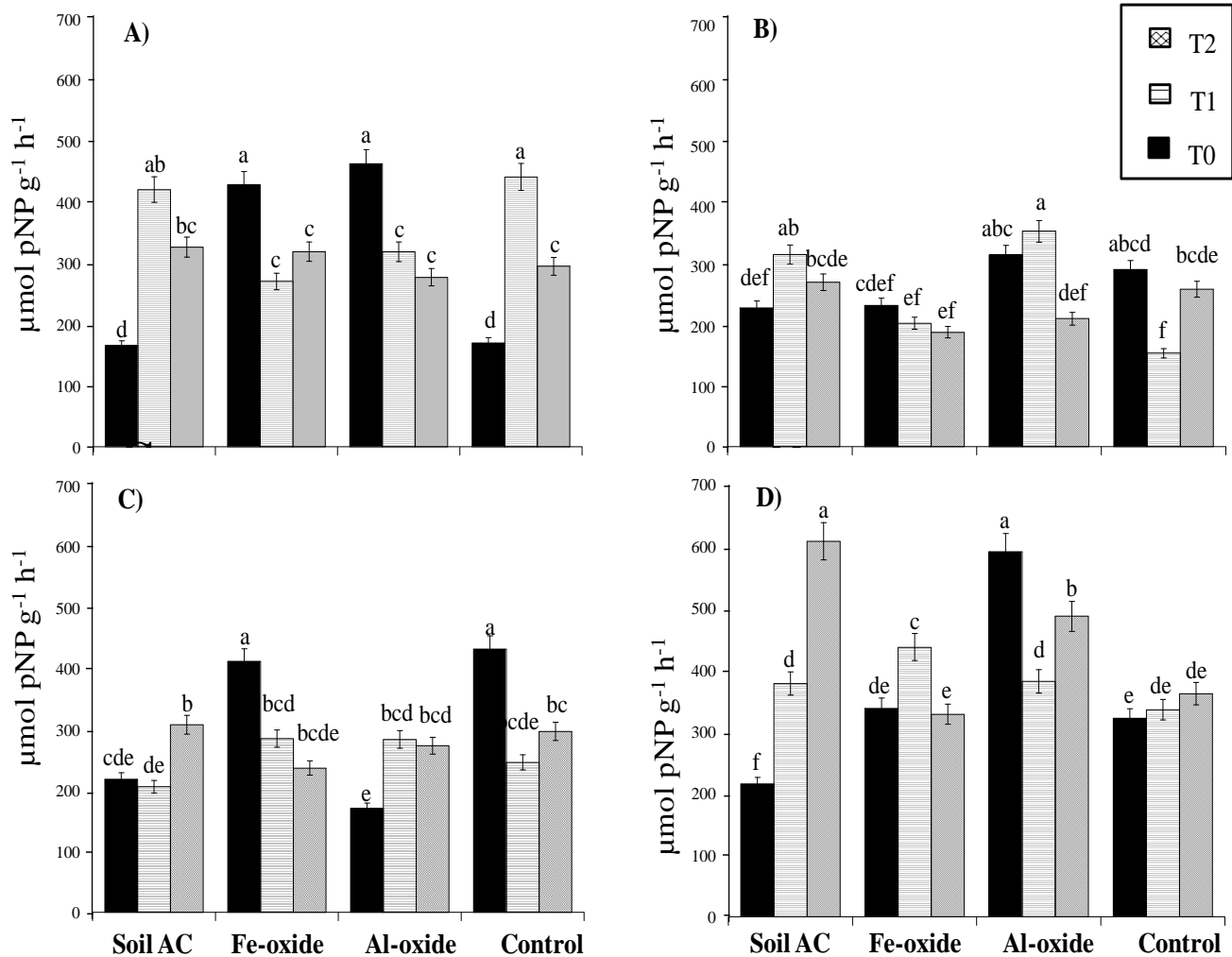
Figure 3.3 Mean activity of β-glucosidase (μmol pNP $g^{-1}h^{-1}$) in composted wheat straw systems inoculated with A) *Coriolopsis rigida*; B) *Pleurotus ostreatus*; C) *Trichoderma harzianum* and D) *Trametes versicolor* strains, no co-composted (Control) and co-composted with the b horizon of an Andisol (Pemehue soil series) (Soil AC); Iron oxide (Fe-oxide) and Aluminum oxide (Al-Oxide). The inorganic materials were applied at different times of application as follow, T0: applied 14 days after fungi inoculation (black bars); T1: applied 56 days after fungi inoculation (hachured bars); and T2: applied 112 days after fungi inoculation (reticulate bars). Letters represents significant differences (P<0.05).
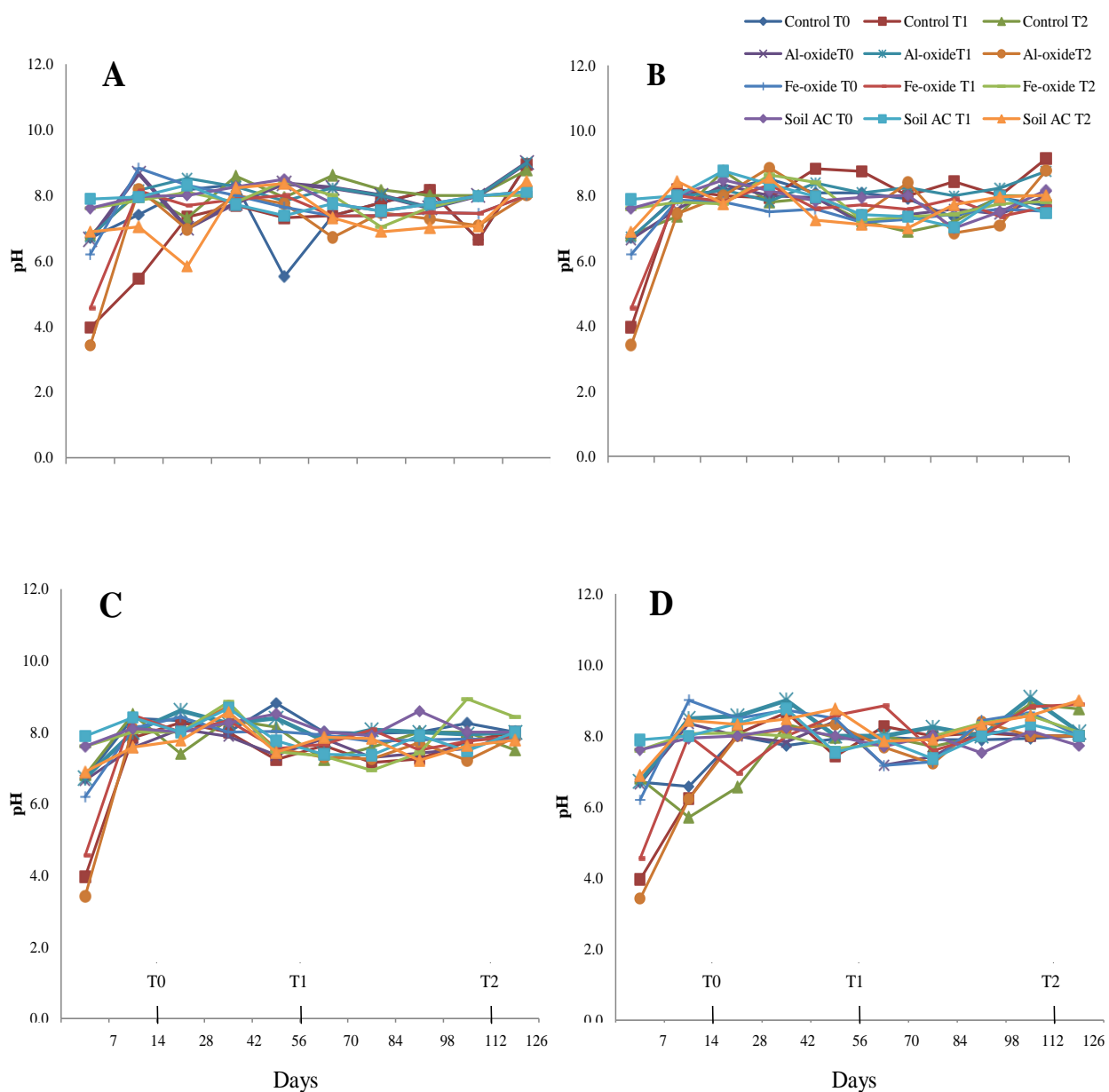
Figure 3.4 pH evolution of composted wheat straw during 126 of composting. A) *Coriolopsis rigida* inoculated wheat straw B) *Pleorotus ostreatus* inoculated wheat straw C) *Trichoderma harzianum* inoculated wheat straw and D) *Trametes versicolor* inoculated wheat straw. Treatments were co-composted with the b horizon of an Andisol (Pemehue soil series) (Soil AC); Iron oxide (Fe-oxide) and Aluminum oxide (Al-Oxide). The inorganic materials were added at different times of application: 14 days after fungi inoculation (T0); 56 days after fungi inoculation (T1); 112 days after fungi inoculation (T2). X axis shows days of composting.

In our study, the E_4/E_6 ratio decreased over the first weeks of composting and roughly stabilized thereafter being lower than 6 (~4.5) at the end of composting (Fig. 3.5). In this sense, *T. versicolor* and *T. harzianum* inoculated composts showed the lower mean E_4/E_6 ratios in Al and Fe-oxide treatments (~7.9 and 7.6 respectively) applied at days 14 and 56 (T0 and T1) for Al_2O_3 (Al-oxide T0, T1treatments) and at days 56 and 112 (T1 and T2) for Fe_2O_3 in *T.versicolor* (Fe-oxide T1, T2 treatments) at the end of composting. Conversely, higher mean E_4/E_6 ratios were observed in *C.rigida* +Fe-oxide treatment (~12.5) (Fig. 3.5). Control treatments with no inorganic addition were lower in *T. harzianum* inoculated treatments (~7.5).

3.3.3 Multivariate relationships of composting process

Based on the overall average value for the experimental parameters reported in section 3.2, highly significant correlations ($p < 0.001$) were observed between the ligninolytic enzymes LAC and MnP (r=0.945) and between β-glucosidase and ligninolytic enzymes (r=0.642, for LAC; r=0.624, for MnP). Conversely, pH and E_4/E_6 ratio were independent and did not have significant correlations with the enzyme activities. These high correlations are evident after the PC analysis (Fig. 3.6) wherein variables LAC, MnP and β-glucosidase are clustered in the same quadrant; whereas, pH and E_4/E_6 appear to be independently distributed. In this sense, treatments as *T.versicolor* inoculated WS and treated with metallic oxides and soil as well as *P.ostreatus* appeared mainly located at second quadrant where the variables pH and E_4/E_6 are clustered. According to our results, and after clustering all treatments, 9 selected treatments were chosen covering the majority of established groups (SD4; annex 4). In general, application time of the inorganic material at day 56 (T1) was chosen for analyzing the OM transformations by chemical and spectroscopic analysis, because the highest values for pH and lower E_4/E_6 considered in this study as stabilization parameters were observed at this time. The cluster tree (SD4, annex 4) showed that the group involving the *T. versicolor* x Fe_2O_3 combination is the treatment with highest enzymatic activity and lowest E_4/E_6 ratio values, indicating the most stabilized treatment at the end of composting.
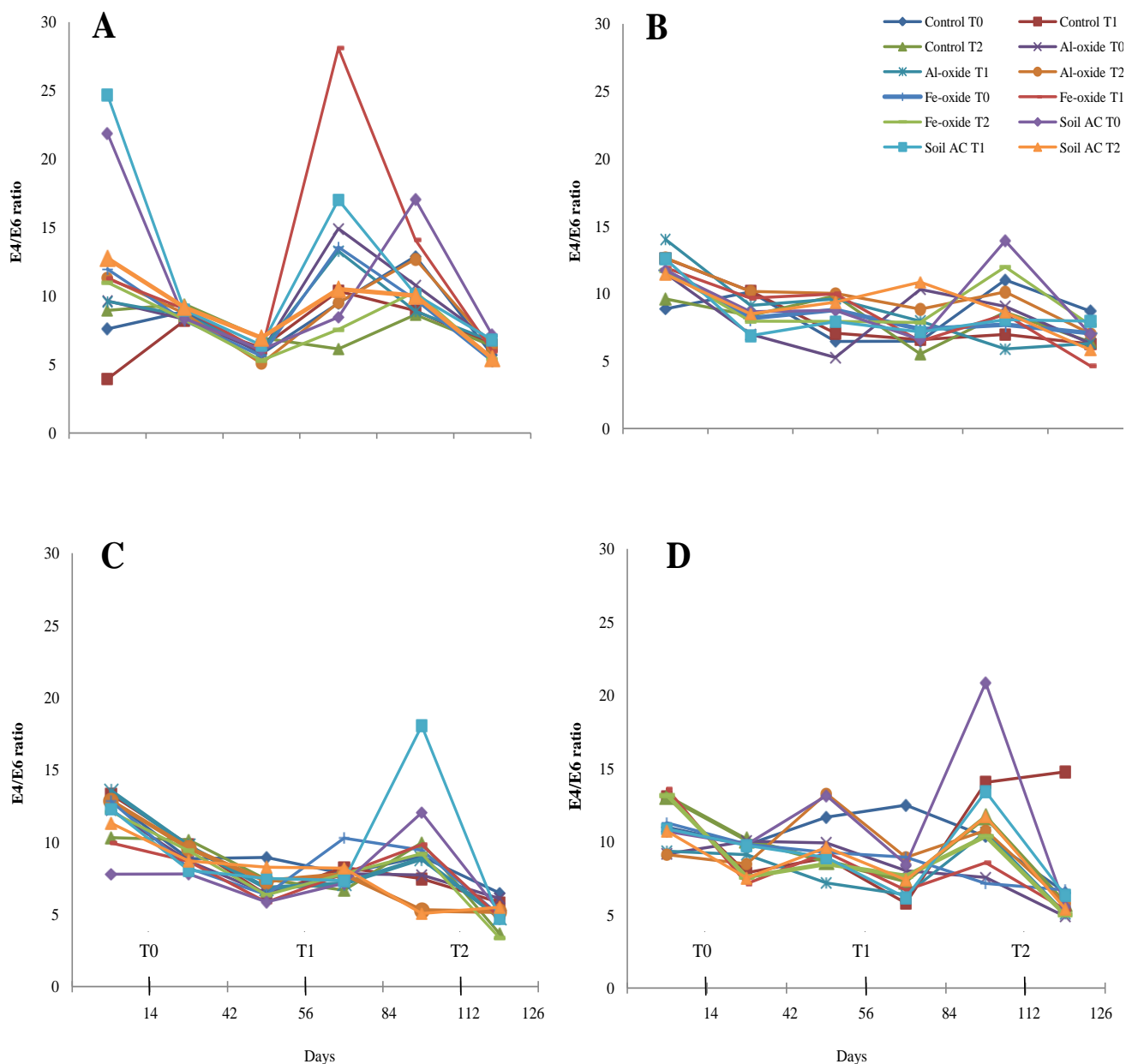
Figure 3.5 E$_4$/E$_6$ evolution of composted wheat straw during 126 of composting. A) *Coriolopsis rigida* inoculated wheat straw B) *Pleorotus ostreatus* inoculated wheat straw C) *Trichoderma harzianum* inoculated wheat straw and D) *Trametes versicolor* inoculated wheat straw. Treatments were co-composted with the b horizon of an Andisol (Pemehue soil series) (Soil AC); Iron oxide (Fe-oxide) and Aluminum oxide (Al-Oxide). The inorganic materials were added at different times of application: 14 days after fungi inoculation (T0); 56 days after fungi inoculation (T1); and 112 days after fungi inoculation (T2).X axis shows days of composting.
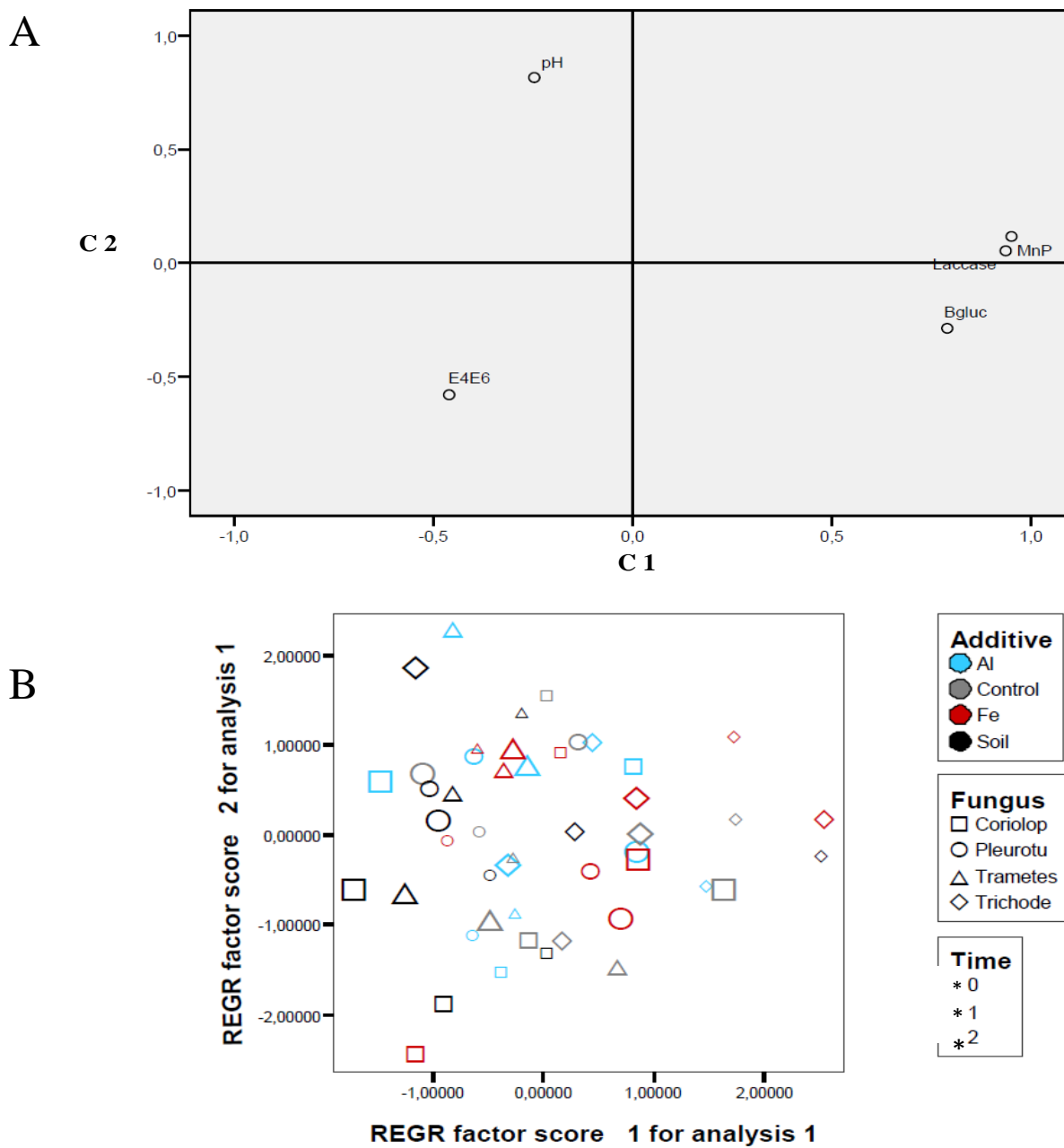
Figure 3.6 A) Principal component (PC) analysis of pH, enzymatic activities (Laccase, MnP and β-glucosidase) and humification index (E_4/E_6) and B) potential locations and interactions between variables and factors from the studied composting systems.

3.3.4 Elemental and spectroscopic characterization of composts and extracted HS

3.3.4.1 Elemental composition

At the end of the composting period , the C content among the treatments ranged from ~42 to ~44 % (wt/wt) for bulk composted samples and ~57 to ~60 % for HS (Table 3.1). Nitrogen content ranged from ~0.3 to ~1.5 % in composted samples and ~0.8 to ~2 % in HS. Oxygen content varied between ~48 to 50% in bulk compost samples whereas it ranged from ~32 to 39% for HS. There was similar content of H ranged from ~5.4 to 5.7% and ~5.0 and 5.7% for bulk compost samples and HS respectively. The range of S content was from ~0.25 to 0.55% for compost and ~0.40 to 0.70 for HS samples extracted from compost.

3.3.4.2 Fourier Transformed Infrared analysis (FTIR)

Figure 3.7 shows the FTIR spectra for bulk composted samples. The main absorbances observed were a deep band at 3650 cm^{-1} (H-bonded OH groups), a distinct peak at 2920-2925 cm^{-1} (aliphatic CH$_3$ stretch), weak peak at 2849 cm^{-1} (CH$_2$ aliphatic C), pronounced peaks in the 1630-1640 cm^{-1} region (C-O stretch of COO^{-1}), a peak at 1580 cm^{-1} region corresponding to the stretching vibrations of conjugated C=O bonds in aromatic rings, small peaks at 1450-1465 cm^{-1} (-CH$_3$, -CH$_2$, -asymetric bending), 1420-1425 cm^{-1} (C-OH deformation of COOH and COO^{-} synnetric stretch), 1375-1385 cm^{-1} (C-CH$_3$, C-CH$_2$ deformations), and at 1235 cm^{-1} (C-O stretch or OH-deformations of COOH). Finally, a very strong peak could be detected near to 1050-1070 cm^{-1} due to C-O stretching in cellulose (Fig. 3.7). For the HS spectra the main absorbances are similar than the bulk compost spectra; however, signals corresponding to shoulders at 1700- 1720 cm^{-1} (C-O stretch of C=O and COOH of uronic acids), strong signals between 1500-1515 (C=C aromatic rings of lignins), peaks at 1360-1365 cm^{-1} (CH$_2$OH aliphatic OH), 1265-1270 cm^{-1} (C-O stretching and OH deformations of alcohols and phenols) weak peaks at 1125 cm^{-1} (alcoholic OH), and strong peaks at 1035-1070 cm^{-1} due to C-O stretching of cellulose were found in the HS samples spectra (spectra not showed).

Table 3.1 Elemental analysis, C/N, H/C and final E_4/E_6 ratio of bulk compost samples and humic like-substances samples from the selected treatments.

Treatment	%N	%C	%H	%S	O%	C/N	H/C	Final E_4/E_6
Compost Samples								
ThC	0.45	44.43	5.76	0.51	48.85	98.48	0.129	5.8
Th+Fe oxide	0.59	42.54	5.42	0.55	50.9	71.53	0.127	4.8
Th+Soil Ac	0.31	43.46	5.63	0.39	50.2	138.72	0.129	4.7
TvC	1.35	43.19	5.45	0.54	49.47	32.05	0.126	14.7
Tv+Fe-oxide	0.6	44.42	5.58	0.36	49.04	73.93	0.125	5.3
Tv+Soil AC	0.56	43.24	5.61	0.52	50.07	76.77	0.129	6.3
PoC	0.69	43.53	5.48	0.4	49.9	63.33	0.125	6.2
Po+Fe-oxide	0.5	43.09	5.44	0.43	50.53	86.35	0.126	4.6
Po+Soil AC	0.27	43.18	5.57	0.23	50.76	160.41	0.128	7.9
Humic like-substances								
ThC	0.94	60.36	5.52	0.71	32.48	64.34	0.091	-
Th+Fe-oxide	1.52	57.96	5.53	0.41	34.58	38.21	0.095	-
Th+Soil AC	1.63	58.13	5.56	0.4	34.28	35.69	0.095	-
TvC	2.07	57.89	5.62	0.47	33.95	27.95	0.097	-
Tv+Fe-oxide	1.81	53.04	5.01	0.43	39.71	29.32	0.094	-
Tv+Soil AC	1.96	58.93	5.5	0.59	33.02	30.03	0.093	-
PoC	1.75	59.12	5.56	0.47	33.09	33.7	0.094	-
Po+Fe oxide	1.09	59.98	5.73	0.41	32.8	54.98	0.095	-
Po+Soil AC	0.85	59.73	5.38	0.45	33.58	70.39	0.090	-

ThC: *Trichoderma harzianum* inoculated wheat straw without inorganic material; Th+Fe-oxide: *Trichoderma harzianum* inoculated wheat straw co-composted with Fe-oxide; Th+Soil AC: *Trichoderma harzianum* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series). TvC: *Trametes versicolor* inoculated wheat straw without inorganic material; Tv+Fe-oxide: *Trametes versicolor* inoculated wheat straw co-composted with Fe-oxide; Tv+Soil AC: *Trametes versicolor* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series). PoC: *Pleurotus ostreatus* inoculated wheat straw without inorganic material; Po+Fe-oxide: *Pleurotus ostreatus* inoculated wheat straw co-composted with Fe-oxide; Po+Soil AC: *Pleurotus ostreatus* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series).

Figure 3.7 Fourier transform infrared spectra of wheat straw and bulk compost samples from: Tv compost: *T.versicolor* inoculated wheat straw without inorganic materials addition (non co-composted) and Tv+Fe oxide compost: *T.versicolor* inoculated wheat straw supplied and co-composted with Fe_2O_3 at T1.

3.3.4.3 Cross Polarization/Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS[13]C NMR)

Solid-state ^{13}C NMR spectra of WS and composted WS materials are dominated by signals in the chemical shift region between 60 and 110 ppm (65-75% of the total C-intensity), typically assigned to O-alkyl C in carbohydrate-derived structures (Chen et al.,2003; Knicker et al., 2005; Scotti et al., 2013) (Fig. 3.8). Clear signals associated to the presence of lignin-products are given in the chemical shift region of O-substituted aromatic C between 120-160 ppm (Knicker et al., 2005; Spaccini and Piccolo, 2009; Habets et al., 2013). Signals of C3 and C5 in syringil units and C3 and C4 of guaiacyl units are present at 153 and 148 ppm, respectively which may be derived from phenolic aromatic compounds in lignin complexes (Table 3.2) (Lüdermann and Nimz, 1973; Wilson, 1987; Knicker et al., 2005). Signals from the remaining aromatic C including lignin structures and modified lignin units were detected at 110-160 ppm (Chen et al., 2003). Besides, some olefinic C may contribute to this region (Antilén et al., 2014). The total contribution of this region comprises between 3.5-5 % of the total spectra for WS and composted WS (inoculated with *T. versicolor*), respectively. Composted WS almost doubled the total aromatic C content from ~4 % in WS to ~9 % in *T. versicolor* inoculated composts, especially when it was supplied with Fe_2O_3 at day 56 (TV+Fe-oxide T1 treatment) by the end of the composting process (126 days). The extracted HS showed that the main contribution to the ^{13}C NMR spectra was associated with the 60-110 ppm region (carbohydrates) and the 110-160 ppm region (including the phenolic- and aromatic-C) (Table 3.3). In addition, between 140-160 ppm, signals corresponding mostly to phenolic-C (aryl C), with some potential resonance contribution of olefinic-C (unsaturated hydrocarbons) substituted by O, N or C were observed (Fig. 3.9) (Antilén et al., 2014). There was a clear contribution to the NMR spectra by the 110-140 ppm region assigned to unsubstituted aryl C. In addition, Figures 3.8 and 3.9 show a higher contribution of the C=O region in composted treatments.
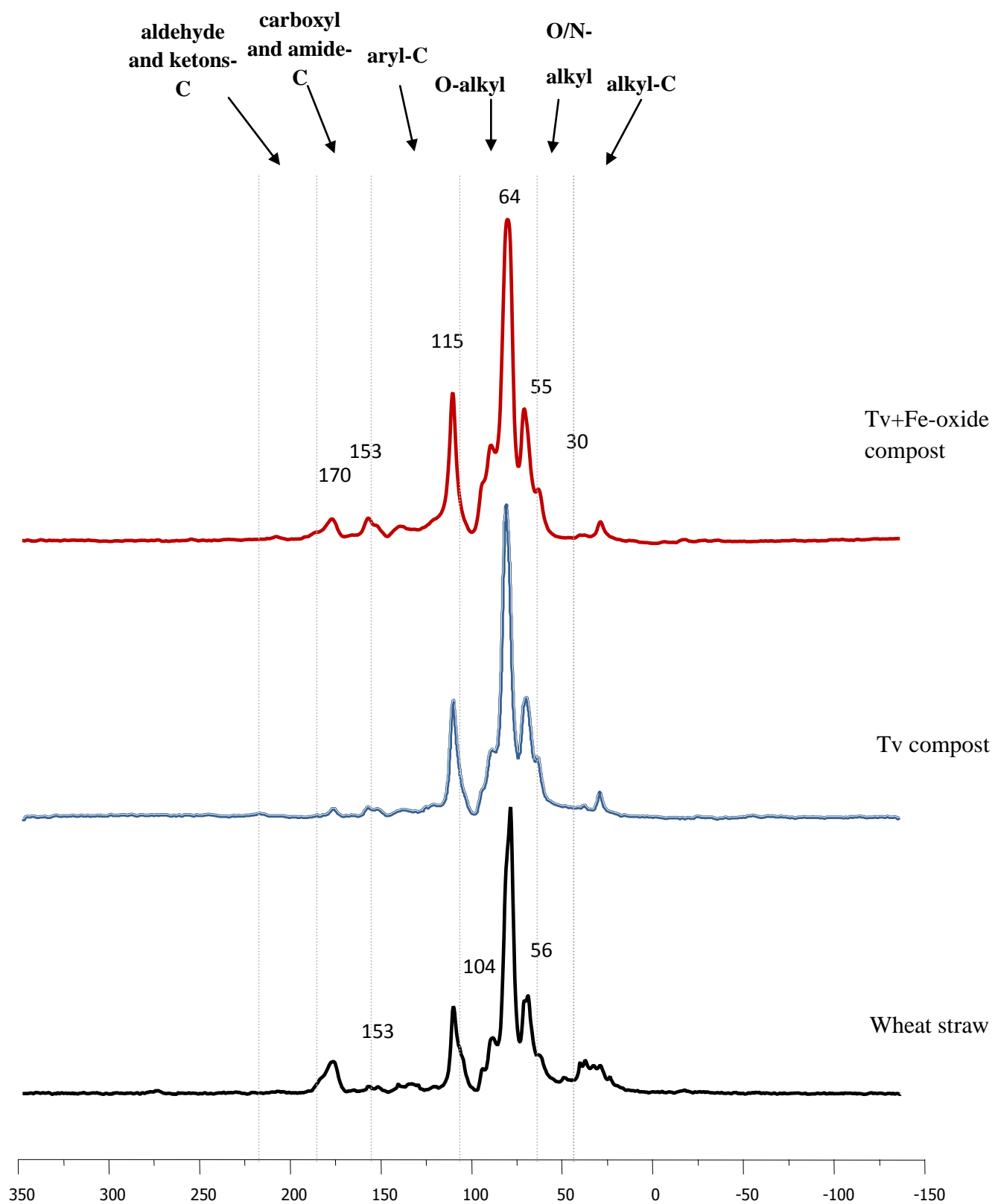
Figure 3.8 Solid state ^{13}CNMR spectra of wheat straw and bulk compost samples from: Tv compost: *T.versicolor* inoculated wheat straw without inorganic materials addition (non co-composted) and Tv+Fe oxide compost: *T.versicolor* inoculated wheat straw supplied and co-composted with Fe_2O_3 at T1.
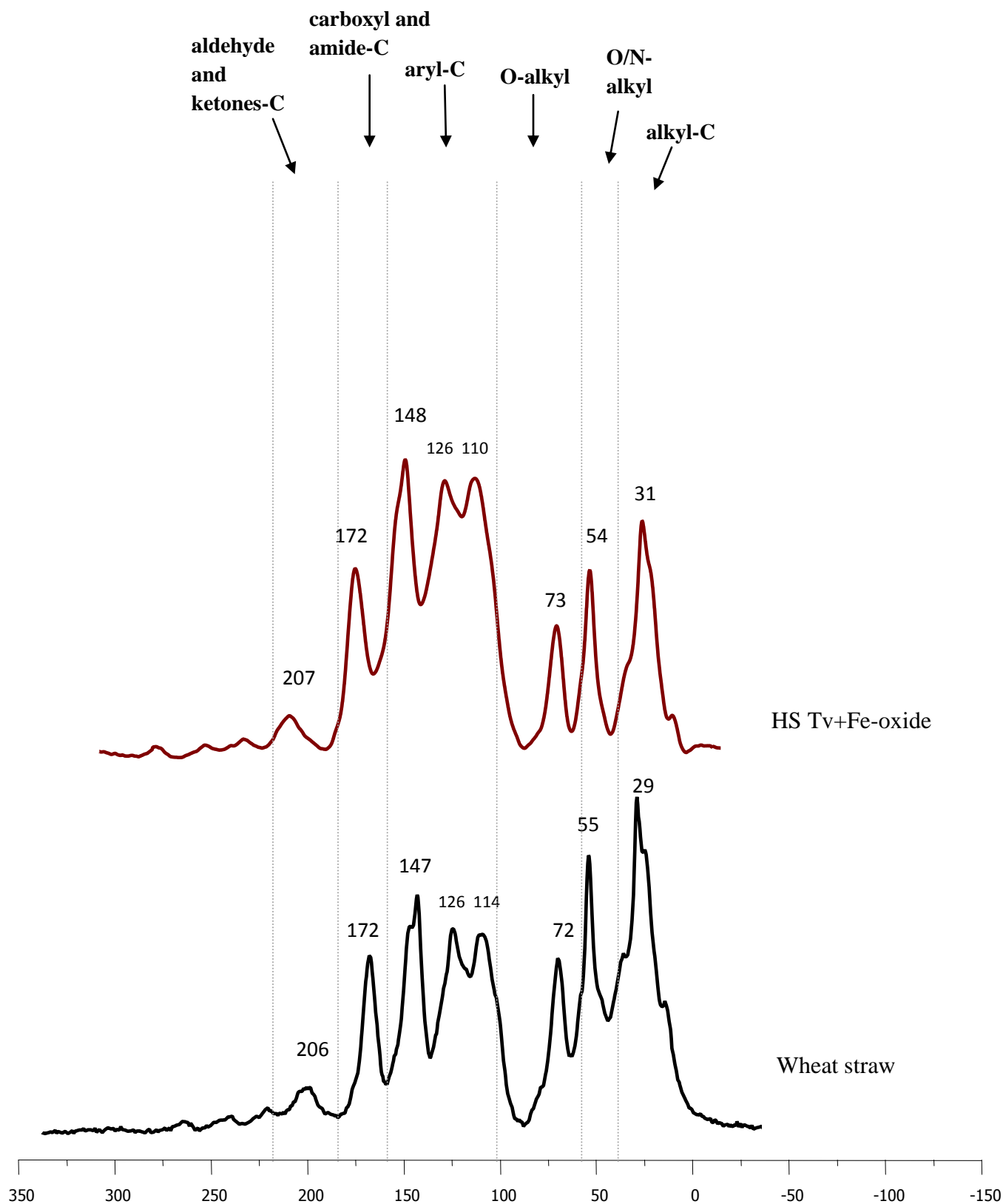
Figure 3.9 Solid state ^{13}CNMR spectra of the alkali soluble fraction of wheat straw and humic like-substances extracted from Tv+Fe-oxide compost (HS Tv+Fe oxide).

Table 3.2 Relative intensity distribution (%) in solid-state ^{13} CNMR spectra of non degraded wheat straw (WS), bulk samples of composted wheat straw (Tv compost; Tv+Fe oxide; Tv+Soil AC) and humic like-substances extracted from WS (WS-NaOH or alkali soluble fraction) and WSC (Tv+Fe-oxide).

Treatment	Ketone C, aldehyde C (245-185 ppm)	Carboxyl C, amide C (185-160 ppm)	Aromatic C, olefinic C (160-110ppm)	O-alkyl C^a(110-60 ppm)*	N Alkyl C methoxyl C(45-60ppm)	Alkyl C (0-45 ppm)	Aryl C unsubstitued (140-110 ppm)	Phenols O-aromatic C (160-140 ppm)	Ratio Aromaticity	Al/Ar ratio
WS	0.41	6.42	4.88	66.48	8.26	13.55	3.51	1.37	0.05	17.9
Tv	0.12	6.32	7.04	66.08	8.21	12.23	4.97	2.07	0.07	12.2
Tv+Fe oxide	0.59	1.71	8.77	72.98	8.61	7.34	6.35	2.42	0.1	10.1
Tv+Soil AC	1.22	1.16	5.96	75.56	9.54	6.56	4.17	1.79	0.06	15.1
HS Tv+Fe	1.4	6.64	33.81	24.43	14.36	19.36	21.11	12.7	0.36	1.8
WS-NaOH	5.26	8.72	31.64	16.02	10.35	28.01	19.56	12.08	0.38	1.7

*spinning side bands were added to this region.

WS: non-degraded wheat straw; Tv compost: wheat straw inoculated with *Trametes versicolor*; Tv+Fe-oxide: wheat straw inoculated with *Trametes versicolor* and co-composted with Fe_2O_3; Tv+Soil AC: wheat straw inoculated with *Trametes versicolor* and co-composted with b horizon of an Andisol (Pemehues soil series). HS Tv+Fe: humic like-subtances extracted from Tv+Fe-oxide compost and WS-NaOH: Alkali soluble fraction from non-degraded wheat straw.

3.3.4.4 Content of functional groups of OM

Table 3.3 shows the content of functional groups in the OM of WS and composted WS material. Wheat straw co-composted with Fe_2O_3 and inoculated with *P. ostreatus* (PO+Fe-oxide T1 treatment) showed the highest values for total acidity (15.4 meq g^{-1}) and phenolic OH group (14 meq g^{-1}) for bulk compost (Table 3.3). Similarly, WS inoculated with *T. versicolor* and co-composted with Fe_2O_3 showed a high content of total acidity and phenolic-OH. Conversely, *T. harzianum* and Fe_2O_3 compost treatment (Th+Fe-oxide T1) showed the lowest acidity values. In comparison, the HS extracted from WS inoculated -

Table 3.3 Acidic functional groups (meq g^{-1}) of bulk compost samples and humic like-substances extracted from selected treatments.

Treatment	Total Acidity	Carboxylic group COOH	Phenolic-OH group	Carbonyl group C=O
		meq g^{-1}		
Compost samples				
ThC	6,22	0,80	5,42	
Th+Fe-oxide	2,80	0,98	1,82	
Th+Soil AC	8,02	0,53	7,48	
TvC	4,17	0,61	3,56	
Tv+Fe-oxide	13,86	0,36	13,49	
Tv+Soil AC	14,96	0,90	14,06	
PoC	8,47	1,74	6,73	
Po+Fe	15,40	1,40	14,00	
Po+Soil AC	8,20	1,28	6,91	
Humic like-substances samples				
ThC	5,00	2,02	2,98	4.4
Th+Fe-oxide	6,29	1,97	4,31	5.2
Th+Soil AC	7,10	1,36	5,74	5.3
TvC	6,74	1,61	5,13	5.1
Tv+Fe-oxide	12,66	2,29	10,37	4.49
Tv+Soil AC	10,45	2,20	8,25	3.8
PoC	12,30	2,26	10,03	5.46
Po+Fe-oxide	16,47	2,34	14,12	4.4
Po+Soil AC	9,18	1,35	7,82	3.4

ThC: *Trichoderma harzianum* inoculated wheat straw without inorganic material; Th+Fe-oxide: *Trichoderma harzianum* inoculated wheat straw co-composted with Fe-oxide; Th+Soil AC: *Trichoderma harzianum* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series). TvC: *Trametes versicolor* inoculated wheat straw without inorganic material; Tv+Fe-oxide: *Trametes versicolor* inoculated wheat straw co-composted with Fe-oxide; Tv+Soil AC: *Trametes versicolor* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series). PoC: *Pleurotus ostreatus* inoculated wheat straw without inorganic material; Po+Fe-oxide: *Pleurotus ostreatus* inoculated wheat straw co-composted with Fe-oxide; Po+Soil AC: *Pleurotus ostreatus* inoculated wheat straw co-composted with the b horizon of an Andisol (Pemehue soil series).

- with *P. ostreatus* and supplied with Fe_2O_3 showed the highest content for total acidity, carboxylic and phenolic-OH (Total acidity was 16.5 meq g^{-1}; carboxylic was 2.3 meq g^{-1}; and phenolic-OH was 14.1 meq g^{-1}). Humic like-substances extracted from *T. versicolor* and Fe_2O_3 compost showed high values for acidic functional groups as well (12.67 meq g^{-1} for total acidity, 2.3 and 10.4 meq g^{-1} for carboxylic and phenolic-OH respectively). It should be noted that the composted WS inoculated with the different fungal strains studied showed major phenolic group contents than carboxylic groups. Moreover, ketonic-C associated to carbonyl groups showed a high content in both Control treatments; *T.versicolor* and *P. ostreatus* inoculated compost with no inorganic materials addition (Table 3.3).

3.3.4.5 TEM/ED and SEM imaging

TEM images (Fig. 3.10 c, d) showed conglomerates and aggregates of crystalline materials into the organic matrix of HS extracted from compost (Tv+ Fe-oxide T1 treatment) which were interacting bound or immerse to the OM. The electron diffraction pattern (ED) confirms with clear rings the presence of crystalline materials contained into the organic matrix of HS (Fig. 3.10 e, f). TEM images of HS (at pH 4.0) extracted from wheat straw compost co-composted with Fe_2O_3 showed spheroidal particles at scales of 20-100 nm which coalesce to form aggregates (Stevenson and Schnitzer, 1982). TEM images at scales of 1 to 200 nm showed iron nanoparticles (~20 nm) incorporated into the HS matrix and also forming nano- to micro-size aggregates highlighting the ED pattern corresponding to crystalline compounds as well.

Besides, at pH 4.0, the SEM analysis showed that globular structures of the extracted HS prevailed, some of them aggregating within a large network (Fig. 3.11a). Conversely, at pH 8.0, long tubular and dendritic shaped structures were observed (Fig 3.11b). The HS extracted from compost showed the formation of dendritic conglomerates with Fe inclusions even under a low pH, indicating the possible formation of stable organic-mineral complexes. At high pH (8.0), there were mainly linear structures rather than aggregates (Fig. 3.11b).
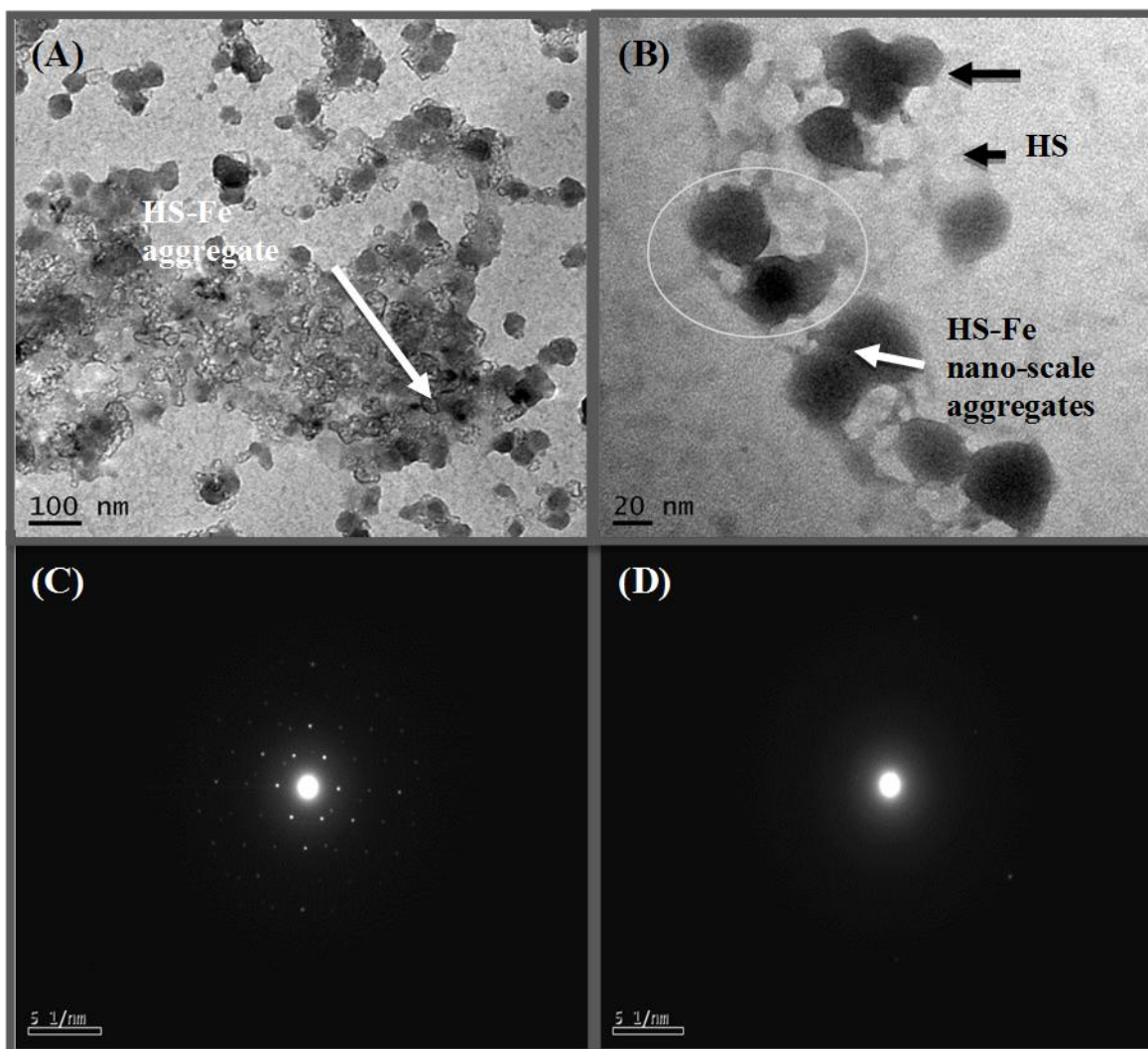
Figure 3.10 Transmission electron microscopy (TEM) micrographs of humic like-substances (HS) extracted from wheat straw based-compost (Tv+Fe-oxide). (A and B) particles of HS at 100 and 20 nm wherein a conglomerate of crystalline materials into the HS matrix are observed; (C and D) Electron diffraction pattern (ED) of the inorganic materials (iron) into the organic matrix of HS extracted from compost.
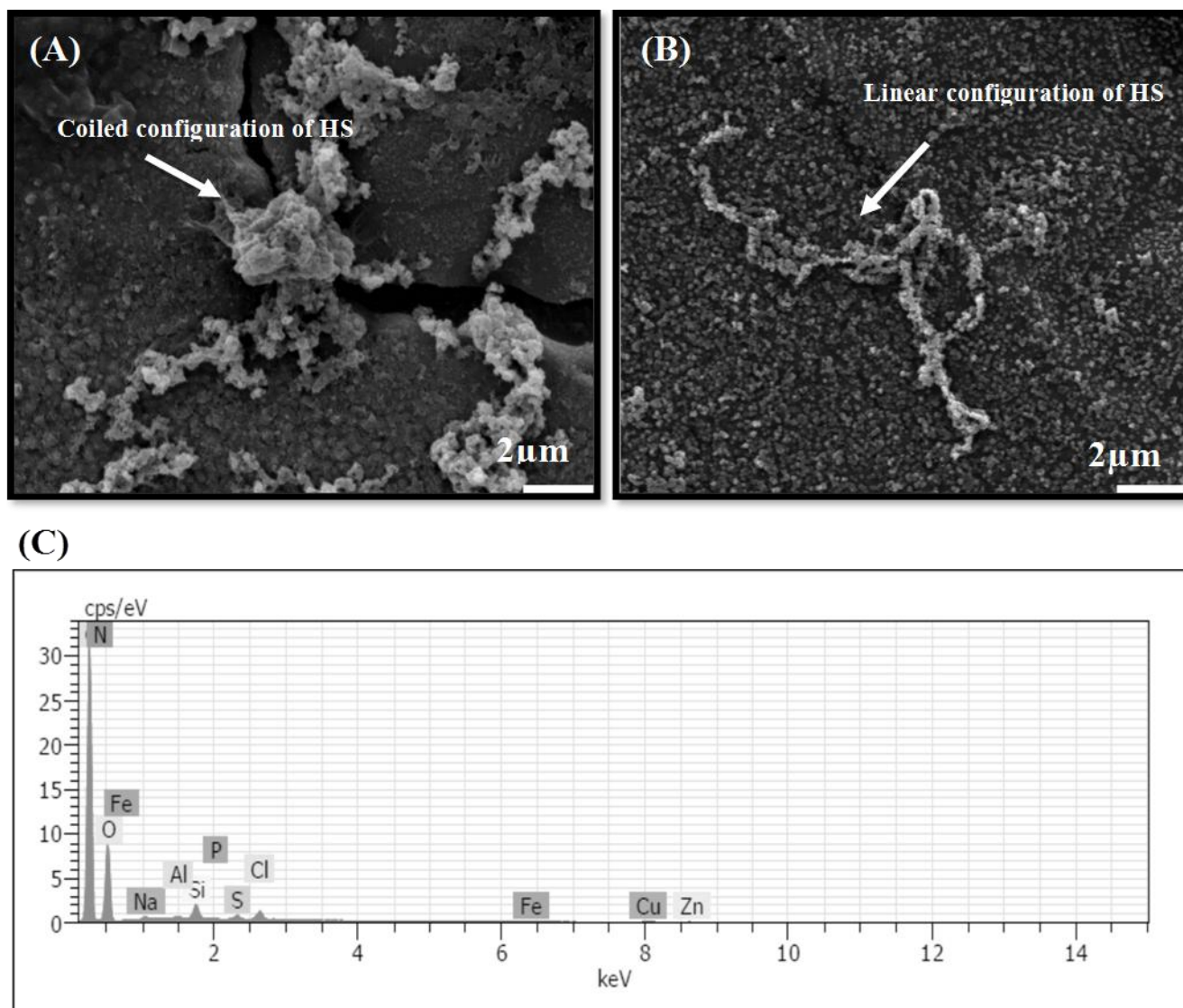
Figure 3.11 Scanning electron microscopy (SEM) micrographs of humic like-substances (HS) extracted from composted wheat straw co-composted with Fe_3O_2 (Tv+Fe-oxide) adjusted at (A) pH 4.0 and (B) pH 8.0, respectively (scale 2 μm). Formation aggregate and coiled configuration of HS is showed in (A) and long chains of HS can be showed in (B). (C) X-ray Energy Dispersive spectroscopy (X-EDS) spectrum of HS.

3.3.4.6 X-EDS analysis

The elemental distribution obtained by X-EDS spectroscopy showed different elements and metals which were found in the HS extracted from Tv+ Fe-oxide compost (Table 3.4). In this sense, it should be noted the presence of Fe and Zn with values of 0.10 and 0.5 % respectively.

3.4 Discussion

3.4.1 Enzymatic activities

There are saprophytic fungi which are capable of polymerizing and depolymerizing lignin and HS using their enzymatic systems (Zavarzina et al., 2011; Takhur et al., 2013; Zeng et al., 2014). Production of the ligninolytic enzymes such as LAC and MnP is common among selectively lignin-degrading basidiomycetes such as white-rot fungi (Grinhut et al., 2007; Sahadevan et al., 2016). In this study, our results suggest that *P. ostreatus* and *T. versicolor* are the most efficient fungi in producing enzymes involved in WS degradation (Tortella et al., 2005; Arriagada et al., 2009; Zhang et al., 2016). The inoculation of WS with *P. ostreatus* and *T. versicolor* increased the activities of LAC (~3.0 U L^{-1}) and MnP (~0.350 U L^{-1}) relative to those effects shown by other inoculated treatments as *T. Harzianum* (1.9 U L^{-1}) for LAC and MnP (0.180 U L^{-1}) activities. It should be noted that treatments inoculated with *T. versicolor* also showed important β-glucosidase activity (~600 μmol pNP g^{-1} h^{-1}) when metallic oxides were incorporated to the composting process. The β-glucosidase enzyme is associated with the transformation and decomposition of cellulose and ligno-cellulose polymers (Haddadin et al., 2009). In this study, the enzymes related to depolymerization and oxidative decomposition of WS (LAC and MnP) were probably produced at the beginning of the process when pH in piles was preferentially acidic (from ~3.8 to 5.5), however, patterns of enzyme evolution are inconclusive and do not show a clear tendency (SD; annexes 1, 2, 3). The potential effect of the oxidative processes in terms of WS decomposition could be associated to the first stage of composting wherein oxidative decomposition carries out (day 21).

Table 3.4 Elemental distribution in humic like-substances (HS) extracted from wheat straw based-compost (Tv+Fe-oxide*) supported by X-ray Energy Dispersive spectroscopy (X-EDS).

Elements	(wt. %)
Carbon (C)	63.01
Nitrogen (N)	0.28
Oxygen (O)	31.48
Sodium (Na)	0.36
Magnesium (Mg)	--
Aluminium (Al)	0.21
Silicon (Si)	1.65
Phosphorus (P)	0.08
Sulfur (S)	0.42
Chlorine (Cl)	1.16
Potassium (K)	--
Calcium (Ca)	--
Titanium(Ti)	--
Manganese (Mn)	--
Iron (Fe)	0.10
Copper (Cu)	--
Zinc (Zn)	0.54

*Tv+Fe-oxide: HS extracted directly from *Trametes versicolor* inoculated wheat straw co-composted with Fe_2O_3.

Despite evolution curves of the ligninolytic enzymes were inconclusive; there were changes of the involved OM at the end of the process which were observed via chemical and spectroscopic analyses. Chemical analyses showed differences of C, H, O and N contents in the elemental analysis at the end of the process between the different inoculated treatments (Table 3.1). Moreover, phenolic-OH group and aromaticity was higher in *P.ostreatus* and *T. versicolor* (~12 meq g^{-1}) inoculated treatments and was confirmed by CPMAS^{13}CNMR spectra with an increased signals associated to aromatic-C (160-110 ppm) and ketonic-C (185-220 ppm) which lead to the assumption that a possible mechanism of OM decomposition occurred through oxidative process.

In this regard, oxido-reductases are considered essential to catalyze depolymerization/decomposition reactions of biomass and soil OM (Burns, 1987; Zavarzina et al., 2011). When these enzymes are secreted by saprophytic fungi in soils and litter, decomposition of organic residues, may lead to the formation of unstable reactive compounds, which can undergo subsequent polymerization. The generation of radicals (*e.g.*, phenoxyl radicals, amino radicals, aryl cation radicals), and the possible incorporation of nitrogenous compounds into sugar-phenol systems, produced during the decomposition of phenolic/non-phenolic and amino acids compounds, may contribute to subsequent polymerization leading to the humification of OM (Stevenson, 1994; Zavarzina et al., 2004; Grinhut et al., 2007). Zavarzina et al. (2004) showed that LAC activity alone extracted from *Panus tigrinus* fungus is capable of performing depolymerization and polymerization reactions of HS synthesis under *in vitro* conditions. These transformations seemed to respond to several factors, such as, initial molecular weight distribution and hydrophobicity among others. Ralph and Catcheside (1998) reported that *Phanerochaete chrysosporium* and other white-rot fungi are able to polymerize and depolymerize alkali-solubilized fractions of brown coal (Ralph and Catcheside 1994; Ralph et al., 1996; Hofrichter and Fritsche, 1996). Mn(III), a phenol oxidant produced by Mn-peroxidase-catalysed oxidation of Mn(II) (Glenn et al. 1986), appears to be responsible for depolymerization and to a lesser degree, of the polymerization of brown coal macromolecules (Catcheside 1988). Phenolic hydroxyl groups in coal molecules are presumably oxidised by Mn(III) to phenoxy radicals, which subsequently couple (cross-coupling), forming coal polymerizates.

On the other hand, Zavarzina et al. (2004) noticed that some type of humic acids may inhibit the activity of LAC enzyme due to the effect of carboxylic groups from OM. Moreover, studies carried out by Lorenzo et al. (2005) and Baldrian et al. (2005) have reported the effects of some organic compounds and heavy metals on LAC (LacII, extracted from *T. versicolor*), MnP and other cellulolytic enzymes. In this sense, it should be noted that, under the studied conditions of composting, iron oxide supplied treatments showed the highest activities in all the studied enzymes.

Under our experimental conditions the activity of LAC and MnP enzymes was lower than the enzymatic activity reported by other several studies (Sahadevan et al., 2013, 2916; Zhang et al., 2015). For this reason, it can lead to the hypothesis that besides affecting fungi colonization and the activity of the enzymes, the inorganic materials (clays and metallic oxides) and its combination with fungi inoculated treatments could promote largely the WS decomposition, stabilization and OM protection than the isolated effect of fungi and its enzymatic systems. In this regard, authors as Baldrian et al. (2005) reported an increase of lignocellulosic biomass degradation as the effect of metals in lignocellulosic substrates colonized by saprophytic fungi. Therefore, in this composting study, LAC and MnP activities from saprophytic fungi may have slightly contributed to depolymerization/polymerization reactions depending as we mentioned above, on the existing environmental conditions (Ralph and Catcheside, 1998).

In this sense, statistical analysis showed that the ligninolytic enzyme activities were not significantly correlated with the humification index (E_4/E_6 ratio) under the studied experimental conditions (r= -0.348 for LAC; r= -0.379 for MnP). Moreover, these results may be explained by the heterogeneous spatial distribution of microsites, and the diversity of microorganisms involved during composting. Alternatively, the data obtained from this composting study may indicate that depolymerization and polymerization reactions occur simultaneously during the whole duration of the compost study. Schnitzer and Monreal (2011) indicated that a microbial and biochemical humification pathway in soils may proceed via synthesis of polyketides. In the latter process, intracellular and complex multi-enzyme systems from different microorganisms as the analyzed in this study may cooperate to catalyze process.

There was no significant correlation between the ligninolytic enzymes (LAC and MnP) and pH. The pH is a fundamental variable for fungal growth. Some fungi and enzymes have a limited optimum pH range for mycelia development and activity, thus they are sensitive to pH modifications during composting. It has been reported that an optimal range of pH between 3.8 and 5.5 (Zavarzina et al., 2011) is required for the fungal and enzymatic processes involved in lignin-cellulose depolymerization and decomposition (Haddadin et al., 2009). Thus, the pH results from this study, may suggest a decomposition activity and depolymerization stages in the first phases of composting (days 1 to 14 in *T.versicolor* treatments), as the pH ranged from ~3.8 to 5.5 as we mentioned above.

3.4.1 Composting process

Most of the criteria used to evaluate the composting process and compost stability has been based on studying biochemical, physical and chemical properties of OM. These properties reflect the metabolic activity of microorganisms involved in the composting and humification processes mediated by biotic and abiotic components and pathways. The composting temperature was evaluated every two weeks. It increased from ~19 °C at the beginning of composting (day zero) which is associated with the meshophilic phase in composting and wherein fungi colonized the substrate, to a maximum of ~42 °C (around day 28) which is associated with the thermophilic phase. Thereafter, the temperature decreased gradually to ~19°C towards the end of the composting (102 days). In solid state fermatation systems, temperatures in a wide range (25 to 30°C) positively influence the WS decomposition by white-rot fungi. A small number of fungi reached their growth and decomposition of lignin at 22 °C (*Collybia radicata, Pleurotus serotinus*) but the majority of fungi showed best growth and substrate decomposition at 30°C (Zadrazil et al., 1985; Jalc, 2002).

The slow temperature increase can be also attributed to the high C/N ratio of WS (~90:1), which attains high temperatures more slowly than composts derived from other organic residues with lower C/N ratios (Haddadin et al., 2009). From a practical viewpoint, since the initial C/N ratio of the organic residues is an important factor to help speed the composting process, the mixing of WS with legume based wastes, animal manures or

nitrogen containing-fertilizers should be considered in WS composting. Nevertheless, caution need to be exercised as lower C/N ratios may produce net mineralization at the end of the thermophilic phase in detriment of the humification. Baca et al. (1992) reported that the low C/N ratio (35:1) of cotton residue may not have had any positive effect on the composting time and quality of the end product. Another important parameter in composting systems is pH. Under our experimental conditions, pH increased from ~4 to ~8.5 during 126 days of composting. Composts prepared with some plant residues normally reach a high pH. Authors like Avinmelich et al. (1996) noticed that mature compost should have a pH near neutral. In our composting system, low initial pH was measured probably due to the production of organic acids at the first stages of composting by the rapid stimulation of microbial activity and due to a decrease of NH_4^+ content (Chen, 2003; Khan et al., 2009). The increase of pH values can be attributed to the degradation of organic acids, organic matter mineralization and production of ammonia and NO_3^- as composting progressed (Haddadin et al., 2009). A rapid degradation of easily available OM during the early stages of composting initiates a sequence of events leading to an intensive acidification and slow composting process at the beginning (Wang et al., 2016). The generation of low molecular weight organic acids reduces composting pH and the acidic pH may inhibit some microbiological activity and cause acidic odour emission (Sundberg et al., 2004; Sundberg and Jönsson, 2005).

The humification index E_4/E_6 is widely used by soil and composting scientists for characterizing OM and reflect the degree of stability, and the poly-condensation of aromatic structures in OM both in soils and bulk compost samples (Zbytniewski and Buszewski, 2005; Khan et al. 2009). This is an operational procedure introduced by Kononova (1961) to characterize degree of aromaticity in OM by comparing light absorbance at ~472 nm (E4, blue light) with that at ~664 nm (E6, red light). Poly-condensed aromatic rings absorb more light at 664 nm (denoting higher degree of humification) than ring systems with a lesser degree of condensation (denoting lower degree of humification) (Kleber and Johnson, 2010). Campbell et al. (1967) noticed an inverse relationship between the E_4/E_6 ratio and the radiocarbon dating of soil OM concentration in alkaline extracts. These authors interpreted these results as an indication that the older, more ''humified'' material is the more highly condensed and more aromatic

in nature. It has been convened that an E_4/E_6 ratio value of < 5 represents humified soil organic matter and some composting systems (Gieguzynska et al. 1998; Khan et al., 2009).

In this study, at the end of the composting process, the E_4/E_6 ratio of extracted HS from the treatments receiving Fe_2O_3 was ~8 and lower than ~13 measured in the untreated (Table 3.1). The latter suggests an incomplete humification of the composted WS, but also points out the potential role of Fe_2O_3 as a catalyst and complexing agent in the humification/stabilization process of WS. Thus, we can suggest that HS extracted at the end of composting are more poly-condensed than OM in WS; and may represent a higher molecular weight than alkali-solubilized fractions extracted at the beginning of composting. It should be noted that E_4/E_6 ratio is governed primarily by the particle size and weight of these materials as well. There are secondary relationships between the E_4/E_6 ratio and pH, O, C, COOH contents and total acidity (Chen et al., 1977).

In general, we may suggest that when fungi and inorganic materials were added to WS, its OM decomposed and underwent catalytic transformation of lignocellulosic materials, leading to the formation of low molecular weight and unstable compounds which would produce E_4/E_6 ratios > 5 or higher. As composting progressed and metallic oxides such as Fe_2O_3 /Al_2O_3 and Soil AC were added to the WS, molecules with a higher stabilization state, size and/or molecular weight were produced, likely followed by inorganic catalysis facilitating polymerization/condensation reactions similar to OM turnover in soils (Sollins, 1996; Jastrow et al., 2007; Zavarzina et al., 2011) (Table 3.1). In spite of the fact that most of the humification process is biologically driven, there is also significant evidence for abiotic condensation and polymerization reactions at mineral surfaces in soils (Jastrow et al., 2007) and compost (Senesi and Plaza, 2007; Brunetti et al., 2008; Bolan et al., 2012; Chan et al., 2016; López-Cano et al., 2016). We hypothesized that the addition of inorganic materials to WS is a key factor in its OM humification/stabilization via physico-chemical protection of OM. Our results warrant further research on the role of inorganic materials in the humification and stabilization of OM from WS during composting.

Jastrow et al. (2007) described that mineral phases present in soil are key factors in the transformation and stabilization of OM. Several authors have reported that clay minerals (e.g., allophane) and Fe, Al and Mn oxides could serve as catalysts for the

formation of HS from mixtures of phenols, amino compounds and sugars under *in vitro* conditions. These inorganics act as catalyst of various condensation, oxidative polymerization, ring cleavage, decarboxylation and dealkylation reactions (Wang and Huang, 1991; Gonzalez-Martinez, 2002; Senesi and Plaza, 2007; Brunetti et al., 2008; Hardie et al., 2010; Truc and Yoshida, 2011; Qi et al., 2012.). One of these non-enzymatic reactions could be the condensation of amino acids and reducing sugars to form melanoidins (insoluble brown nitrogenous containing compounds) (Gonzalez-Martinez, 2002). In soils and possibly composts, the amino compounds and reducing sugars are readily available from the lysis of microorganisms, whereas the quinones are the result of oxidation of polyphenols derived from lignin and other plant materials as well as from microorganism oxidation (Jastrow et al., 2007).

Metallic oxides such as Mn oxides (*e.g.,* birnessite) and iron oxides which occur in soils and sediments are very active in promoting the polymerization of phenolic compounds. These minerals act as Lewis acids which accept electrons from diphenols like hydroquinones leading to the formation of a semiquinone radical involving a single electron transfer. Then, semiquinone may couple to each other to form stable HS polymers (Huang, 1999). In humic like-fraction isolated from olive milled wastes catalytically treated with MnO_2 resulted in a loss of aliphatic materials and an increase of extraction yields, oxygenation, acidic functional groups, aromaticity and carbohydrate moieties as characterized by chemical and spectroscopic analyses. In our study, similar features were observed under spectroscopic analyses highlightining the increase of aromaticity (Table 2) and the loss of aliphatic materials (Fig. 3.8 and 3.9). Thus, increased OM humification appears to result from the treatment of organic wastes with metallic oxides (Senesi and Plaza, 2007; Brunetti et al., 2008).

In addition, Fenton reactions, involving the abiotic polyphenol oxidation by inorganic materials like iron oxides are also important to OM humification. This advanced oxidation process involves the generation of highly oxidizing radical species like hydroxyl radicals ($\bullet OH$) through the catalytic reaction of Fe(II) to Fe(III) in presence of H_2O_2, which are capable of decompose and transform a wide range of organic materials (Garrido et al., 2010). There are different materials capable to catalyze the Fenton and Fenton-like reactions such as zeolite, activated carbon and clay minerals (Garrido et al., 2010). Thus,

considering our composting systems, it is expectable that Fenton-like reactions are carried out in the aerobic composting of WS when metallic oxides (Fe and Al oxides) and Soil AC were applied, catalyzing the OM decomposition and transformation reactions of WS. Moreover, some fungi posses non-enzymatic degradation mechanism which are mostly assisted by oxidation through production of free hydroxyl radicals (•OH) (Guillen et al., 1992; Suzuki et al., 2006). In fact, many white and brown-rot fungi have been shown to produce hydrogen peroxide (H_2O_2) which enters the Fenton reaction and results in release of •OH. These free radicals attack polysaccharides as well as lignin in plant cell walls in a nonspecific manner providing some cleavages which make it easier for the lignocellulolytic enzymes to penetrate (Dashtban et al., 2009). These chemical and compositional changes may be observed during WS composting under our experimental conditions.

On the other hand, micromolecular interactions between OM matrix and inorganic materials may promote the physicochemical protection to microbial decomposition products through the formation of inner-sphere complex and by microaggregation phenomena at the nano to micro-size scale as we can observe in the micrograph obtained by SEM and TEM microscopy from HS extracted from WSC (Fig. 3.10 and 3.11). Under this context, it is expected that the OM in the studied composting systems treated with metallic oxides and Soil AC could be subject to increased stabilization by these micromolecular interactions.

3.4.3 Elemental and spectroscopic characterization of compost and humic like-substances

In general, the content of C and H range from ~5 to ~56 % (wt/wt) for HS extracted from lignocellulosic residues (Almendros et al., 1992; Vane et al., 2004) and soils (Mollisol) (Schnitzer and Schulten, 1998; Schnitzer and Monreal, 2011). The HS extracted from *T. versicolor* and Fe-oxide compost (Tv+Fe oxide T1), contained similar proportion of C and H (~45%C, 5% H for bulk compost samples and ~53% C, 5% H for HS), and approximately 10% to 20% (~33 to 39 %) more O and less N content than in typical HS extracted from soils (Mollisol) (Schnitzer and Schulten, 1998). The final C/N ratio of HS extracted from composted WS presented a wide range of values among treatments. For example, WSC inoculated with *T. versicolor* presented C/N ratio values ranging from ~32

to 72, with a much narrow C/N ratio observed in the extracted HS (~27 to 30) at the end of composting. The C/N ratio determines the microbial capacity to degrade the substrate (Fiahlo et al., 2010). However, some organic residues as plant residues contain a high proportion of lignin that can hinder the process. This ratio has been used to indicate the stabilization of OM (Chen, 2003) to microbial decomposition and helps to predict the stability of compost in soil (Fiahlo et al., 2010).

There is no consensus regarding the optimum C/N ratio defining well humified composts. Some authors suggest that values near to 10 indicate slow microbial activity and result in stabilization of the material (Chefetz et al., 1996; Provenzano et al., 2001). For Tomati et al. (2002), stabilized compost possesses a C/N ratio between 11 and 22. According to Chefetz et al. (1996), the C/N ratio is not a reliable indicator of compost maturity for some composted materials (Fiahlo et al., 2010). Based in our study, the C/N ratio is highly dependent on the initial contents of C and N in the residue (WS ~90:1); therefore, lignocellulosic materials could produce mature compost with high C/N ratios and relatively stable HS. Moreover, the substantially lower H/C ratios (used for alliphaticity) for both bulk compost samples and HS suggest the presence of more aromatic structures, at the end of composting. Our results indicate that on the basis of E_4/E_6 ratios and relative to untreated WS, the OM in the composted WS had a higher level of condensation at the end of process. The latter reflects some degree of aromatic condensation, and various pools of OM with partial humified states (Chen et al., 1977; Senesi et al., 2007; Khan et al., 2009).

In addition, the level of compost aromaticity and maturity indicated by the C/N and E_4/E_6 ratios can be strengthened by characterizing the OM by FTIR and CP/MAS ^{13}C NMR spectroscopy. The analyses by FTIR and ^{13}C NMR of OM in the WS, composted WS and extracted HS reveal the presence of oxygen-containing functional groups, as COOH, COO$^-$, phenolic-OH and C=O and that their content varied and increased with composting treatments. The latter groups appear to indicate the oxidative effect of different treatments evaluated since these groups results from OM degradation through oxidative process (Hastrup et al., 2012; Scotti et al., 2013). The increased presence of ketonic-C (Figure 3.8 and 3.9) (185-220 ppm), and the high content of carbonyl groups in HS extracted from WS (3.5 to 5.5. meq g^{-1}) which is in the range of humic acids from soils and marine ecosystems (Rashid, 1985), is consistent with the hypothesis that a path of HS synthesis occurs via

polyketide synthesis from the biotic transformation of simple organic substrates (Schnitzer and Monreal, 2011). The presence of phenolic units in WSC and the extracted HS (Figure 3.7, 3.8, Table 3.3) may indicates decomposition of the ligno-cellulosic complex by the action of phenoloxidases but also through the action of inorganic catalyst (Ghosh et al., 2011). Moreover, the band attributed to aromatic C=C, C=O stretching of amide groups, quinonic C=O and/or C=O of H-bonded conjugated ketones (1650 cm^{-1}) becomes relatively more intense which is typical of composted materials (Senesi and Plaza, 2007). These observations are complementary and supported by the higher relative aromaticity determined by the CP/MAS ^{13}C NMR analysis (Fig. 8 and 9; Table 2). Analysis by IR and FTIR provide useful information on the outer surfaces of HS and molecules related to functional groups and reactions that may occur at these surfaces with metals, pesticides and other xenobiotics, especially those associated to COOH and COO$^-$ groups (Schnitzer and Monreal, 2011). In Figure 7 the shoulders at 1700- 1720 cm^{-1} (C-O stretch of C=O and COOH of organic acids) and peaks in the 1630-1640 cm^{-1} region (C-O stretch of COO^{-1}), observed in the HS extracted from composted WS, indicate potential for strong interaction and formation of highly stable iron-HS complexes.

On the other hand, the acidic functional groups (*e.g.,* phenolic and carboxylic) quantified in the aerobic composting systems were similar to those of fulvic acids and HS extracted from compost and soils. This is an important aspect to take into consideration because organic waste treatments must ensure the formation of adequate amounts of HS that chemically and physico-chemically resemble those in native soil (Senesi and Plaza, 2007). The total acidity content of HS extracted from composted WS (5 meq g^{-1} to 16 meq g^{-1} for *T. harzianum* and *P. ostreatus* inoculated treatments respectively) are in the range of values described for soil HS (Table 3.3). The phenolic-OH content in composted WS was, however, higher (14 meq g^{-1} for *T. versicolor* inoculated treatments) than that reported for soil HS extracted from Mollisols (Schnitzer and Monreal, 2011). Conversely, carboxylic content of HS was lower compared to soil HS, nevertheless, they are in the range of HS from composted organic residues for example, Olive leaves + solid fraction of olive oil wastewater and commercial *sphagnum* peat (~2 to 5 meq g^{-1}) (Clemente and Bernal, 2004).

Considering our data, it can be suggested that the relative increase in phenolics may be associated with the production of intermediates resulting from the decomposition stage

of lignin (Grinhut et al., 2007), and phenolic substances produced by microorganisms (Ghosh et al., 2011). Hence, when WSC was inoculated with saprophytic fungi and supplied with inorganic materials, the binding properties of bulk compost samples and HS were altered to enhance sorption and exchange capabilities, for example, by increasing the content of acidic or ketonic functional groups. Thus, the studied composting treatments represent important management practices and techniques to aerobically transform and stabilize WSC.

3.4.4 Humic like-subtances observations and visual characterization

Finally, the micrographs of HS extracted from WSC treated under different pHs (4.0 and 8.0) showed aggregates at micro to nanoscale wherein molecules tended to form small spheres coalesces in dendritic structures to gradually agglomerate into large size clusters (Fig. 3.11). This morphology could be influenced by transition metals as potential complexing agents who were at least partly responsible for some of the aggregation observed in the solution phase (Bryan et al. 2001). Alvarez-Puebla et al. (2004) indicated that under increasing pHs humic acids tend to increase their net negative charge, due to the continuum ionization of acidic groups and hence their solubility. They noticed that at pH 4.0 or superior most of the strong carboxylic acidic groups in HS are ionized increasing the surface charge and the amount of adsorbed metals, thus promoting the precipitation and aggregate formation of the HS-metal complex. In addition, TEM images showed the interaction and possible sorption processes at nano-scale of metals into the OM matrix of HS (Fig. 3.10) which may be confirmed by the FTIR spectra. This spectroscopic analysis showed the presence of shoulders at 1700- 1720 cm^{-1} and peaks in 1630-1640 cm^{-1} region from HS, wherein transition metals like Fe could be sorted to the OM, suggesting the formation of highly stable Fe-HS interactions as well.

Although other analyses should be conducted to describe the stabilization processes and potential interactions of metallic oxides and clay minerals into the inorganic amended-compost systems, the techniques here performed which include chemical analyses, FTIR, CPMAS^{13}CNMR spectroscopy and TEM/SEM microscopy are powerful tools which may help to clarify the stabilization of composting sytems through the characterization of the

organic materials even down to the nanometer scale. These analyzes allowed us to determine compost changes, structural composition, colloids size, and morphology among others. Considering this and the obtained data, our chemical, spectroscopic and microscopic analyzes may suggest that similar physicochemical interactions associated to OM stabilization in soils (*i.e.,* humification, microaggregation, sorption interactions with minerals) are being carried out by the OM from soil-less systems as compost when inorganic materials are incorporated to the system.

3.5 Conclusions

Experimental results showed that under the experimental conditions of this research, the use of saprophytic fungi and inorganic materials enhanced the composting process of the WS residue. The studied composting treatments appear as interesting biotechnological tools capable of enhancing WS composting. The supply of inorganic materials, such as Fe_2O_3, to WS, promoted OM stabilization during aerobic composting by acting as a catalyst and compost protectors (OM complexing agents). Moreover, the combined addition of both saprophytic fungi and inorganic materials also promoted the humification and stabilization of WS. Results obtained from enzyme assays together with the use of the state-of-the-art spectroscopic analytical techniques FTIR and CP/MAS ^{13}C NMR and the microscopic techniques TEM and SEM were essential to help explaining the components and processes involved in depolymerization and decomposition of OM, as well as in the humification and stabilization of OM during WS composting.

Chapter IV

Effects of Wheat Straw based-Composts (WSC) in Cu-contaminated soils

Associated to specific objectives 2 and 3

Manuscripst associated to this chapter:

- Use of saprophytic fungi and inorganic materials for enhancing the aerobic composting of wheat straw. II (Paper to be submitted to Waste Management).

- Chemical structural characteristics of wheat straw and composted wheat straw inoculated with Trametes versicolor and treated with Fe₂O₃, and their effect on organic matter in a metal contaminated soil (Paper in preparation to be submitted to Soil Biology and Biochemistry).

Abstract

Wheat straw based - composts (WSC) produced in a previous stage using microorganisms as inoculants and inorganic materials (metallic oxides/clay minerals), were selected to evaluate their effects in copper (Cu) contaminated soils were evaluated. Decomposition experiments by monitoring the release of CO_2 and the effects of WSC in chemical and biochemical properties of Cu-contaminated soils were conducted. Copper basal contaminated (BC): ~330 ppm and spiked soils (SS): ~830 ppm) were mixed with WSC and were placed in respiration jars containing CO_2 traps. These jars were incubated and analyzed at different times during 90 days of experiment. β-glucosidase and dehydrogenase (DHA) enzymatic activities were performed. The lability of Cu was determined through a sequential extraction procedure. Additionally, ^{13}C NMR spectroscopy was performed to bulk samples after 1 year of soil-WSC incubation. Results indicated that the microbial respiration (CO_2 release) was higher in WSC- amended soils. Higher respiration values were observed at day 7 (~160 mg C-CO_2 kg^{-1} for BC and ~200 mg C-CO_2 kg^{-1} for SS) and showed a slightly decrease as the experiment progressed. Under our experimental conditions, WSC treated with metallic oxides exhibited the lower microbial respiration (~50 mg C-CO_2 kg^{-1}) in soils than non-treated compost. Dehydrogenase activity showed a similar tendency than CO_2 release. Higher activities were showed at day 1 and 7 (~3 and 2 µg INTF g^{-1}h^{-1}, respectively), decreasing as the incubation progressed. However, DHA activity was highly affected by increasing Cu levels in SS. The greater β-glucosidase activity was reached at day 45 by Fe-oxide treated WSC (Tv+Fe-oxide). Compost addition reduced the easily exchangeable fraction and increased the Cu-bound to the OM fraction (~10 to 15%). NMR spectra showed clear evidence about the presence of lignin and cellulose-products as effect of the compost addition. Our results suggest that stabilized WSC have an important effect on microbial activities and Cu-availability in Cu-contaminated soils. Thus, the use of novel strategies for compost stabilization can be a suitable tool to increase the organic C levels and decrease the availability of Cu in contaminated soils.

Keywords: Composting contaminated soils, lignocellulosic residues, mining, organic matter stabilization, and soil remediation.

4.1 Introduction

Composting is considered an important way for carbon (C) stabilization involving the partial humification of the organic matter (OM). During composting, organic compounds are transformed to more stable and complex OM, which chemically and biologically resembles humic substances (HS) (Dinel et al., 2004). The resulting humified OM is a potentially suitable amendment for agricultural, contaminated and degraded soils (Kästner and Miltner, 2016). Compost addition in general produces positive effects on crop yields. The incorporation of composts into soils may improve the water retention capacity and enhance the soil porosity. The pH buffering capacity of soils, the increase in the cation exchange capacity (CEC) and the soil organic matter (SOM) content are other beneficial effects of compost addition (Senesi, 1989; Castaldi et al., 2005; Scotti et al., 2015). Since some organic compost may be quickly mineralized into the soil (Chang et al., 2009; Godbout et al., 2010) alternative methods to stabilize C by using inorganic materials such as clay minerals and metallic oxides have been developed for agricultural waste composting. These inorganic materials can interact with OM by catalyzing the humification process or acting as compost protectors through inter-molecular interactions such as OM-mineral complexation, thereby, preventing or decreasing its microbial decomposition with the concomitant increase of C stock in soils (Brunetti et al., 2008; Bolan et al., 2012; Chan et al., 2016).

On the other hand, indiscriminate waste disposal practices have led to significant build up in soils of a wide range of toxic elements (TE) such as metal(oid)s arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se), and zinc (Zn) (Bolan et al., 2014). There are a lot of different methods useful for cleaning soil including physical, chemical and biological methods. The application of physical or chemical methods, like washing the soil with halogenated substances and heating it in high temperatures, chemical extraction, or electro remediation (Hempel and Thoeming 1999; Renneberg and Dudas 2001; Thoming et al. 2000) is mostly limited by the economic point of view (Smolinska, 2015). The utilization of biological techniques appears as interesting approaches from an economical and environmental point of view (Meier et al., 2011). In this sense, compost characterized for its high content of OM as well as its high concentrations of macro and micronutrients are widely used as soil ameliorant.

Mature composts are considered an important source and technology for soil remediation of TE in contaminated soils (Senesi and Plaza, 2007; Park et al., 2011). Some advantages of their utilization in contaminated soils may include, the increasing of colloidal humified OM, the increasing of the buffering capacity that allows adjusting the pH of soils, the increasing of microbial activities and the improvement of physical and physic-chemical soil properties (Crecchio et al., 2004; Guo et al., 2006; Alguacil et al., 2011; Olivier et al., 2011; Garrido et al., 2012; Moreno-Jiménez et al., 2016) which are essential in the remediation of degraded and contaminated soils. In general, due to its chemical and biological properties and its high capacity in binding compounds, compost decrease the (bio)availability of metal(oid)s through various process and reactions that include immobilization, precipitation, oxidation/reduction, methylation/demethylation and rhizosphere modification (Park et al., 2011; Jones et al., 2016; Moreno-Jiménez et al., 2016). Mature compost do not remove metals and pollutants from soils, but transform them into less soluble or insoluble forms (Macnair et al., 2000; Mench et al., 2003; Kumpiene et al., 2008; Bolan et al., 2014).

The immobilization is referred to sorption reactions, promoted by the addition of exogenous OM (Compost) which may induce metal retention through an increase of the surface charge and by the incorporation of binding compounds such as organic compounds having unshared pairs of electrons (*e.g*., enolate, amine, azo, ring N, carboxylate, ether, and carbonyl groups) (Chen et al., 2006; Gondar and Bernal, 2009). In mining contaminated soils, where transition metals (*e.g*.,Fe, Mn, Zn, Cu) and toxic heavy metals (*e.g.,* Hg, Pb, Ni, Cd) can be found, sorption and desorption processes are fundamental for keeping its levels of availability. As we mentioned above, the sorption processes involves mainly the negatively charged surfaces of OM as well as clay minerals, and metal oxides or hydroxides with which metallic cations as Cu can establish interactions that go from weak Van der Waals type interactions to stronger bonds involving the formation of inner sphere complexes (Sposito, 2008; Garrido et al., 2012). The presence of contaminants and other metals, changes in OM, pH, cation exchange capacity and redox potential can alter the magnitude of sorption processes (Sparks, 2005; Rooney et al., 2006; Park et al., 2011; Garrido et al., 2012). Moreover, all TE, metal and xenobiotic binding reactions strongly relate to the chemical nature and reactivity of the OM. These properties, however, change

during composting and biodegradation of the organic materials (Chen et al., 2006). Thereby, the chemical nature of compost and an adequate composting process is essential for being used in contaminated soil.

In a previous stage of this thesis, wheat straw based-composts (WSC) were produced using saprophytic fungi and co-composting them with inorganic materials (Fe-Al oxides and clay minerals) in order to promote and enhance the C stabilization on WSC. Results showed that the combination of saprophytic fungi and inorganic materials modified the OM structure and composition and promote the C stabilization in wheat straw composting. Thus, the effects of WSC in chemical and biochemical properties of contaminated soils, and how the WSC addition affect the soil microbial activity and the Cu availability in Cu-contaminated soils were studied as presented in this chapter. In accordance, the objectives of this study were: (i) to analyze the mineralization and C stabilization of wheat straw based- composts (WSC) in Cu-contaminated soils and (ii) to evaluate the effects of wheat straw based-composts (WSC) on physico-chemical, microbiological and biochemical properties of Cu-contaminated soils and determine the immobilization of Cu after compost application.

4. 2 Material and methods

4.2.1 Study area and soil sampling

The Puchuncaví valley in the coastal area of central Chile has been exposed to emissions of sulfur dioxide and metal-rich particles from the Ventanas smelter since 1964. Thereby, the soils exposed to the smelter emissions are acidic (pH_w=4.7) as consequence of sulfur acid, and Cu-contaminated (up to 680 mg Cu kg^{-1} soil; 330 mg Cu kg^{-1} for this study) (Ginocchio, 2000; Ginocchio et al., 2004). In smelter dust, Cu is mainly present as chalcocyanite ($CuSO_4$), a highly soluble compound (Neaman et al., 2009). The copper contaminated soils from Puchuncaví Valley (central Chile, 32°46' 172" S; 71°28'17" W) correspond to Alfisol (Los Maitenes soil series) and were obtained at Los Maitenes site area which is located approximately 2 km east of the Ventanas smelter and has the greatest metal concentration in the Puchuncaví valley (González and Bergqvist, 1986). Soils were

sampled randomly and collected in July 2014. Bare soil (0-20 cm) from this representative ecosystem was selected as previously described by Cornejo et al. (2008) and Meier et al. (2011). Soil was homogenized and after being air-dried, it was passed through a 2-mm nylon mesh. Part of the selected soils were spiked as described Ginocchio et al. (2006) with increasing amounts (500 mg Cu kg^{-1}) of copper salts often used in standard soil toxicity assays, $CuSO_4$ X $5H_2O$ Suprapur (Merck, Darmstadt, Germany) in order to prepare mixtures with different total copper concentrations. Thus, soils were divided in basal contaminated soils (BC) (330 mg Cu kg^{-1}) and spiked soils (SS) (830 mg Cu kg^{-1}).

4.2.2 Composting process and wheat straw based-compost (WSC)

Wheat straw based-composts (WSC) were produced at pilot scale under greenhouse conditions during 126 days of aerobic composting. For a detailed description of the process, treatments and selection, see chapter 3 (section 3.2.1 and 3.2.2). Briefly, wheat straw piles were initially inoculated with different fungal strains corresponding to *Coriolopsis rigida* (Berk. Et Mont.) Murrill, *Trametes versicolor* (L.:Fr.) Pillat, *Trichoderma harzianum* Rifai and *Pleurotus ostreatus* (Jacq.) P.Kumm. Additionally, iron oxide (Fe$_2$O$_3$; Sigma Aldrich®), aluminum oxide (Al$_2$O$_3$; Sigma Aldrich®) and the b horizon of and Andisol (Pemehue soil series, 25 % of allophane content) were added to different composts as inorganic catalysts of the process. These inorganic catalysts were incorporated at different times of the composting process in a proportion 2 % w/w of total WS (Brunetti et al., 2008). Produced WSC showed clear differences by chemical/spectroscopic analyzes in both bulk compost and humic like-substances (HS) in terms of composition, chemical structure and functional groups (see chapter 3). These differences were used as selection criteria.

4.2.3 Decomposition experiment; Microbial activities

With the objective of determine the microbial activity and potential decomposition of WSC when applied to soils, a decomposition experiment wherein WSC were incubated with a Cu-contaminated soil was conducted. Decomposition experiment was carried out by monitoring the release of CO_2 using respiration flasks (Bolan et al., 2012). Wheat straw

based-composts were selected at the end of the first experiment (Chapter 3) using different chemical and spectroscopic analyzes and the inorganic-amended ones (most stabilized) which correspond to: *i*) Tv+Fe-oxide compost: WS inoculated with *T. versicolor* and co-composted with iron oxide at 2% w/w; *ii*) Tv+Al-oxide compost: WS inoculated with *T.versicolor* and co-composted with aluminum oxide at 2% w/w; *iii*) Tv+Soil AC compost: WS inoculated with *T. versicolor* and co-composted with the horizon b from an Andisol (Pemehue soil series) at 2% w/w (Gadepelle et al., 2008); and the less stabilized and non-amended with inorganic materials *iv*) TV compost: inoculated with *T. versicolor* and without inorganic materials, were incubated with the Cu-contaminated soils. Additionally, a control treatment (with no compost addition) (C) was incubated (Fig. 4.1).

Composts were mixed and incubated with Cu -contaminated soil samples at a rate of 50 g C kg^{-1} soil (equivalent to 25 Mg per ha, at 5 cm of depth), and were placed in a respiration jars containing a CO_2 trap (3-dm^3; 20 mL NaOH 0.5 M) following the design described in Bolan et al. (2012). Soil samples were separated in BC (~330 mg Cu kg^{-1}) and SS (~830 mg Cu kg^{-1}) as we mentioned in the previous section (4.2.1). Thus, 10 treatments and 3 replicates were defined for this experimental design, wherein 5 treatments were incubated with BC soils and 5 with SS. These jars were incubated in darkness at 24 °C and kept with 60% of water capacity. The water content of the incubations was controlled by weighting at every sampling. Evaporation losses were compensated by adding deionized water. Traps were removed at various intervals over a period of 90 days for the analysis of CO_2 release. Every sampling time, jars were aerated to promote aerobic conditions as well. At the same time, in order to analyze enzymatic activities and Cu availability and for not modifying the conditions of the decomposition experiment, a parallel experiment under equal conditions and treatments was carried out. pH of incubations was measured as well at days 7, 75 and 90 (Sadzawka et al., 2006). To clarify the experimental stages see flux chart diagram (Fig.1). Finally, in order to analyze SOM changes in structural composition (^{13}CNMR), the incubations were kept for one year under greenhouse conditions.
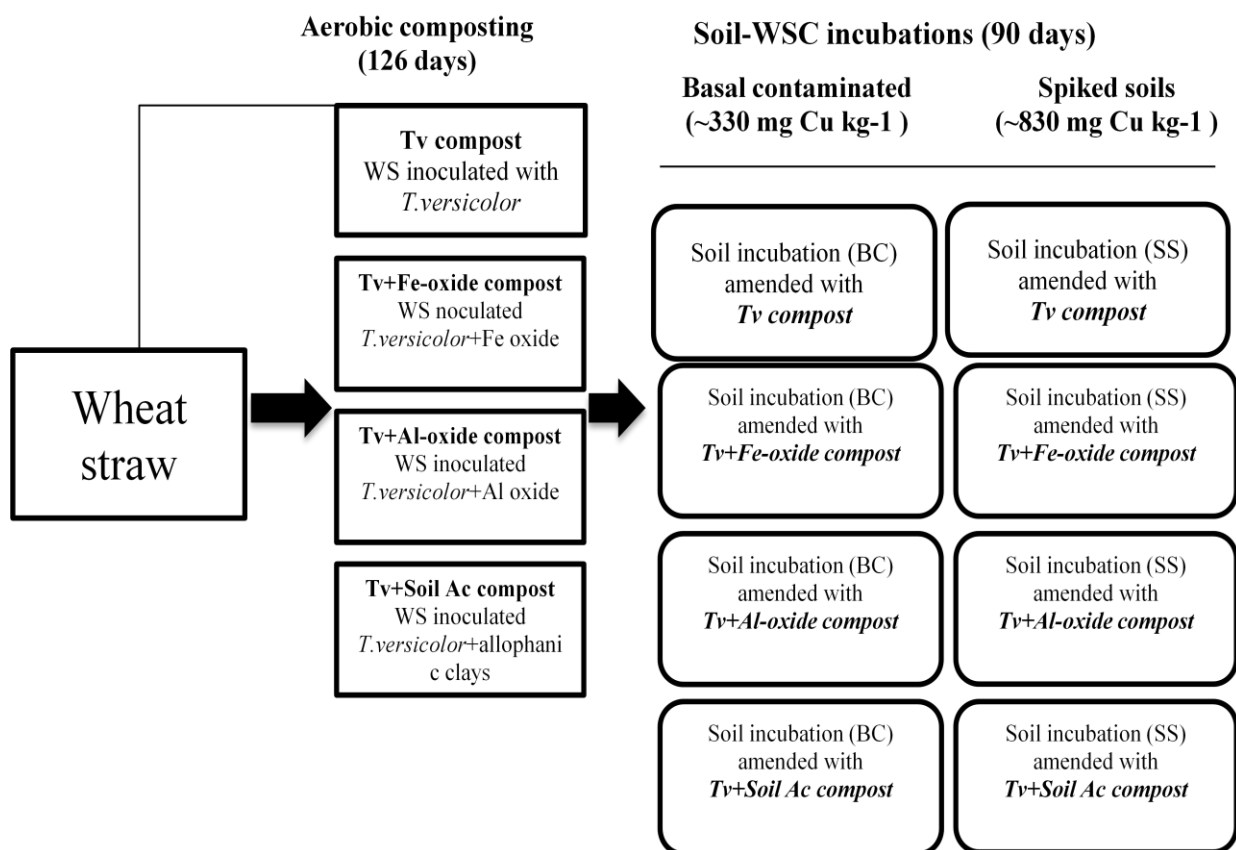
Figure 4.1 Experiments and treatments flux chart diagram

4.2.3.1 Soil respiration (CO$_2$ Release)

Jars (3 per treatment) were analyzed at different times for 90 days (at days 1, 7, 15, 30, 45, 60, 75 and 90) and the evolved CO$_2$ was measured in a NaOH trap. An aliquote of 5 mL obtained from each CO$_2$ trap was mixed with 5 mL of 0.5 M BaCl$_2$ (Alef and Nanipieri, 1995). After the precipitation of the trapped CO$_2$ as BaCO$_3$, the amount of evolved CO$_2$ was determined by back-titration of unreacted NaOH using 1 M HCl (Bolan et al., 2012). Moreover, some drops of an indicator (Phenolphthalein 60% v/V) were added to the solution. The amount of released C was calculated by using the following equation: respiration (mg C-CO$_2$) = (B-S) x N x E x (vtr/vt1) wherein B: mL of blank; S: mL of sample; E: conversion factor (22= mg of CO$_2$); N: Normality of acid; vtr: volume of NaOH in CO$_2$ trap and vt1: volume of NaOH used for the back titration.

4.2.3.2 Enzymatic assays

The evolution of both enzymatic activities was determined at different times as follows: soil dehydrogenase activity (DHA) was evaluated at days 1, 7, 15, 30, 45, 60 and 90. β-glucosydase enzyme activity was assessed at days 15, 45 and 60. Soil DHA was determined following the method described by Skujins (1976), with the modifications described in García et al. (1997). The method is based on the extraction and colorimetric determination of the intensely colored iodo-nitrotetrazolium formazan (INTF) produced from the reduction of colorless 2-iodophenyl-3-*p*-nitrophenyl- 5-phenyltetrazolium chloride (INT) in soils. For this, 1 g of field-moist soil was mixed and exposed to 0.2 mL of 0.4% INT in distilled water for 20 h. The test tubes were sealed with rubber stoppers and incubated at 22°C in the dark. INTF formed was extracted with 10 mL of tetrachloroethylene-acetone, by shaking vigorously for 1 min and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophoto-metrically at 490 nm against the blank (Medina et al., 2006). The calibration curve were made adding 0, 1, 2 and 5 mL of INTF solution and 13.5 mL of the extracting solution into test tubes and mixing each tube thoroughly. The calibration concentrations were: 0, 100, 200 and 500 μg per test (Alef and Nannipieri, 1994).

The β-glucosidase activity was assayed by the determination of *p*-nitrophenol (pNP) released by the catalytic action of the enzyme using *p*-nitrophenil-β-d-glucopyranosyde (pPNG) as substrate (Tabatabi, 1982). Therefore, 1g of moist and sieved (2mm) soil (from soil-WSC incubations) was mixed in Erlenmeyer flasks (50 mL) with the reaction mixture. Reaction mixture contained 4 mL of MUB buffer (pH 6.0), 0.25 mL of toluene and 1mL pPNG (25mM). Each tube was stoppered, and the content was mixed thoroughly by shaken and incubated for 2 h at 37 °C. After the incubation, when samples were cooled down, 1 mL of 0.5 M CaCl solution and 4 mL of 0.5 M Na_2CO_3 buffer (pH 11.0) were added in order to stop the reaction. The flasks were swirled, centrifuged at 3500 rpm for 10 minutes, and filtered (Whatman filter No.2v). Each measurement of color intensity was performed at 398 nm. For calibration curves, each test tube contained a concentration of 0, 20, 50, 100 and 200 μg of pNP.

4.2.4 Sequential extraction of Copper

To evaluate the effects of WSC on Cu availability/immobilization, a Cu sequential extraction were performed using the procedure suggested by Tessier et al. (1979). The Cu was separated in the following fractions: easily exchangeable (E), bound to carbonates (CA), bound to Fe and Mn/Al oxides (Ox), organically bound (OM) and residual fraction (R). The sequential extraction was conducted in 50 mL polyethylene centrifuge tubes using 1 g of soil. Exchangeable fraction was obtained using 1M $MgCl_2$; CA fraction obtained by applying 1M CH_3COONa/CH_3COOH (pH 5.0); OX fraction by adding 0.04 M NH_2OH-HCl in 25 % (*v/v*) CH_3COOH (pH 2.0) and OM fraction extracted by 30% H_2O_2/0.02 M HNO_3 (pH 2.0) followed by 3.2 M CH_3COONH_4/20% (v/v) HNO_3 (Fig. 4.2). After each extraction step the supernatant liquid was separated from the solid phase by centrifugation at 4000rpm for 15 min and was filtered through 0.45 μm cellulose acetate paper. It was then decanted into polyethylene vessels acidified with HNO_3 and stored at 4°C before analysis (Park et al. 2011). The remaining residue was washed with 10 ml of Milli-Q water, and the washings were discarded after centrifugation. The samples were analyzed for Cu using Atomic Absorption Spectrophotometer UNICAM (Pietrzak and Mcphail, 2004).

SOIL

8 mL (1 M MgCl$_2$ pH 7)

1 h at 25°C → 4000rpm x 15 min (centrifuge) → Easily exchangeable (supernatant)

PELLLT

← 10 mL H$_2$O (Wash cycle)

4000rpm x 15 min (centrifuge) → Discard (supernatant)

8 mL (1 M CH$_3$COONa/CH$_3$COOH pH 5)

5 h at 25°C → 4000rpm x 15 min (centrifuge) → Bound to Carbonates (supernatant)

PELLLT

← 10 mL H$_2$O (Wash cycle)

4000rpm x 15 min (centrifuge) → Discard (supernatant)

20 mL (0.04 M NH$_2$OH·HCl in 25% (v/v) CH$_3$COOH pH 2)

6 h at 96°C → 4000rpm x 15 min (centrifuge) → Bound to Fe and Mn oxides (supernatant)

PELLLT

← 10 mL H$_2$O (Wash cycle)

4000rpm x 15 min (centrifuge) → Discard (supernatant)

3 mL (0.02 M HNO3)

5 mL (30% H$_2$O$_2$ pH 2)

2 h at 85°C

PELLLT

3 mL (30% H$_2$O$_2$ pH 2)

5 mL (3.2 M CH$_3$COONH$_4$/20%(v/v) HNO$_3$) + 15 mL H$_2$O

3 h at 85°C → 4000rpm x 15 min (centrifuge) → Bound to organic matter (supernatant)

PELLLT

← 10 mL H$_2$O (Wash cycle)

4000rpm x 15 min (centrifuge) → Discard (supernatant)

20 mL(HNO$_3$/HCl)

Digestion in microwave 2 h → Residual

Figure 4.2 Scheme of Cu sequential extraction in soil according to methodology described by Tessier (1979).

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4.2.5 Changes on SOM: Spectroscopic analyzes

At the end of the incubation (90 days) the chemical characterization of bulk incubation samples and extracted HS included functional groups and the transformation of C types in OM were performed.

4.2.5.1 Analysis of main functional groups of SOM by Fourier Transform Infrared (FTIR) spectroscopy

The functional groups of OM and its changes in soil-WSC incubations were characterized by Fourier transform infrared (FTIR) spectroscopy after 90 days of incubation. For this, the humified fraction (HS extracted with 0.5 M NaOH; for extraction procedure see section 3.2.4 in Chapter 3) were prepared and analyzed at the Department of Chemistry, Universidad de La Frontera, Temuco, Chile. Briefly, HS were ground and sieved at 450 nm, dried overnight at 70°C, mixed with 400 mg KBr and then compressed into pellets. Spectra were obtained by using Varian Scimitar 1000 FTIR. For this analysis the HS were defined as C (control), H1, H2, H3 (from soil-WSC incubation) and H4 (from WSC).

4.2.5.2 Analysis of chemical structural features of SOM by Cross Polarization/Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (CP/MAS ^{13}C NMR) spectroscopy

Structural changes of C-types in OM of selected bulk soil samples from incubations were determined using CP/MAS ^{13}C NMR. For this, soil-WSC incubations were kept for one year and the analysis was performed at the end of this period. In addition, soil samples were purified using a successive sample cleaning with HF (10%) solution in order to remove the paramagnetic materials from soil samples. The ^{13}C solid-state NMR spectra were obtained with a Bruker Ascend^{tm} 400 MHz wideboard spectrometer operating at a ^{13}C resonance frequency of 150.93 MHz. The cross polarization magic angle spinning (CPMAS) technique was applied with a spinning speed of 14 kHz. The ^{13}C chemical shifts were calibrated relative to tetramethylsilane (=0 ppm) with glycine (176.04 ppm). Approximately 10,000 single scans with pulse delays between 300 and 500 ms were

accumulated for each spectrum. Spectra regions were divided as follow: alkyl-C (0-45 ppm), N-alkyl/methoxyl C (45-60 ppm), O-alkyl C (60-110 ppm), aryl C (110-160 ppm), carboxyl/amide C (160-185 ppm) and aldehyde and ketones C (185-245 ppm) (de la Rosa et al., 2014).

4.2.6 Statistics

All data sets were subjected to two-way ANOVA to determine the main effects of compost addition, contaminant level, and all the possible interactions among the factors, for each sample stage. Repeated measurements analyses were performed to analyze the global effect of the above mentioned factors. Significance level was established at $p < 0.05$. Statistical analyses were performed with the SPSS software v.10.0 (SPSS, Inc., Chicago, Il.).

4.3 Results

4.3.1 Characteristics of the amendments

Wheat straw based-compost were characterized in chapter 3 (see sections 3.3); however, some general chemical features are provided. The amendments contain mainly C (~45%), a smaller proportion of N and S (~0.6 to 1.4 % and 0.4 to 0.5 respectively). pH assayed at the end of the wheat straw composting showed that the stabilized WSC were alkaline (pH: ~8.5). The nature of functional groups in WSC was identified by FTIR spectroscopy (Chapter 3; Fig.3.7) and chemical analysis. The peaks around 3650 cm^{-1} indicate the presence of hydroxyl groups. The band at 1580 cm^{-1} corresponds to the stretching vibrations of conjugated C=O bonds in aromatic rings (Cao and Harris 2010). The bands around 870 cm^{-1} are due to the contribution from C–H bond vibration in aromatic compounds (Park et al., 2011) and was confirmed by the relative intensity distribution of ^{13}CNMR spectra wherein the contribution of aromatic C (110-160 pm) was higher in WSC than untreated wheat straw (Chapter 3; Fig. 3.8). The presence of the above mentioned surface functional group activities are responsible for the adsorption of metals. In addition,

chemical analyzes showed the high contribution of these functional groups, wherein Tv+Fe-oxide compost and Tv+Soil AC showed the higher total acidity (15 and 13.9 meq g^{-1} respectively) and phenolic-OH (~13.5 and ~14.1 meq g^{-1} respectively).

4.3.2 Effects of WSC on microbial activities

The pH of soil increased with the WSC application (Table 4.1). Soil incubation amended with Tv+Fe-oxide compost showed the highest pH (~6.5) on BC soils. Otherwise, Control soil without WSC application was the lowest (~4.7). Soils treated with the other WSC (Tv compost, Tv+Al-oxide and Tv+Soil AC) increased pH up to 5.7 in BC soils. The pH in SS showed an slightly increase in WSC amended soils (up to 5.6 in soil treated with Tv+Fe-oxide compost). Application of WSC significantly increased soil respiration (CO_2 release) and DHA especially in BC soils. Soil respiration showed the highest level when Tv compost was added to BC soils. The higher values of soil respiration were observed at the beginning of the incubation experiment at day 7 (~150 mg C-CO_2 kg^{-1} for Tv+Fe-oxide treatment) and day 45 (~155 mg C-CO_2 kg^{-1} for Tv treatment) in BC (Fig. 4.3). In SS an irregular evolution was observed and higher values were obtained at days 7 (Tv compost) and 30 (Tv+Fe-oxide compost) (~250 and 180 mg C-CO_2 kg^{-1} respectively). Otherwise, a clear tendency was observed and the low soil respiration was concentrated at day 90 (~50 mg C-CO_2 kg^{-1}) in both BC and SS corresponding to the end of the incubation experiment (Fig. 4.3). At this time the mean values of soil respiration were significantly higher in WSC amended soils (Tv compost: 150 mg C-CO_2 kg^{-1}) than control soils (30 mg C-CO_2 kg^{-1}). However, it should be noted that the soil respiration of soil incubations with WSC treated with metallic oxides (Fe-Al-oxides) and clay minerals (Soil AC)(~90 mg C-CO_2 kg^{-1}) were significantly lower than the WSC untreated ones (Tv compost) (Fig. 4.4). In addition, increasing levels of Cu in SS did not affect the soil respiration (Fig 4.3 and 4.4).

Table 4.1 pH (water) of soil-WSC incubations (BC:~330 mg Cu kg^{-1}; SS: ~830 mg Cu kg^{-1}) at days 7, 75 and 90.

	Day 7		Day 75		Day 90	
Wheat Straw based-compost	BC	SS	BC	SS	BC	SS
Tv+Fe oxide	6.44	4.83	6.29	5.15	6.59	5.6
Tv+Al-oxide	5.52	4.48	5.55	4.48	5.7	5.01
Tv+Soil Ac	5.5	4.49	5.48	4.57	6.04	5.18
Tv compost	5.6	4.76	5.46	4.81	6.27	5.4
Control	4.79	4.18	4.67	3.92	4.69	4.35

Tv: soil incubations (BC and SS soils) amended with Tv compost (wheat straw inoculated with *T. versicolor* without inorganic material); Tv+Fe-oxide: soil incubation (BC and SS soils) amended with Tv+Fe-oxide compost (wheat straw inoculated with *T. versicolor* co-composted with Fe-oxide); Tv+Al-oxide: soil incubation (BC and SS soils) amended with Tv+Al-oxide compost (wheat straw inoculated with *T. versicolor* co-composted with Al-oxide) and Tv+Soil AC: soil incubation (BC and SS soils) amended with Tv+Soil AC compost (wheat straw inoculated with *T. versicolor* co-composted with the b horizon of an Andisol; Pemehue soil series). Basal contaminated soils (BC)(~330 mg Cu kg^{-1}) and Spiked soil (SS)(~830 mg Cu kg^{-1}).

Evolution of soil respiration
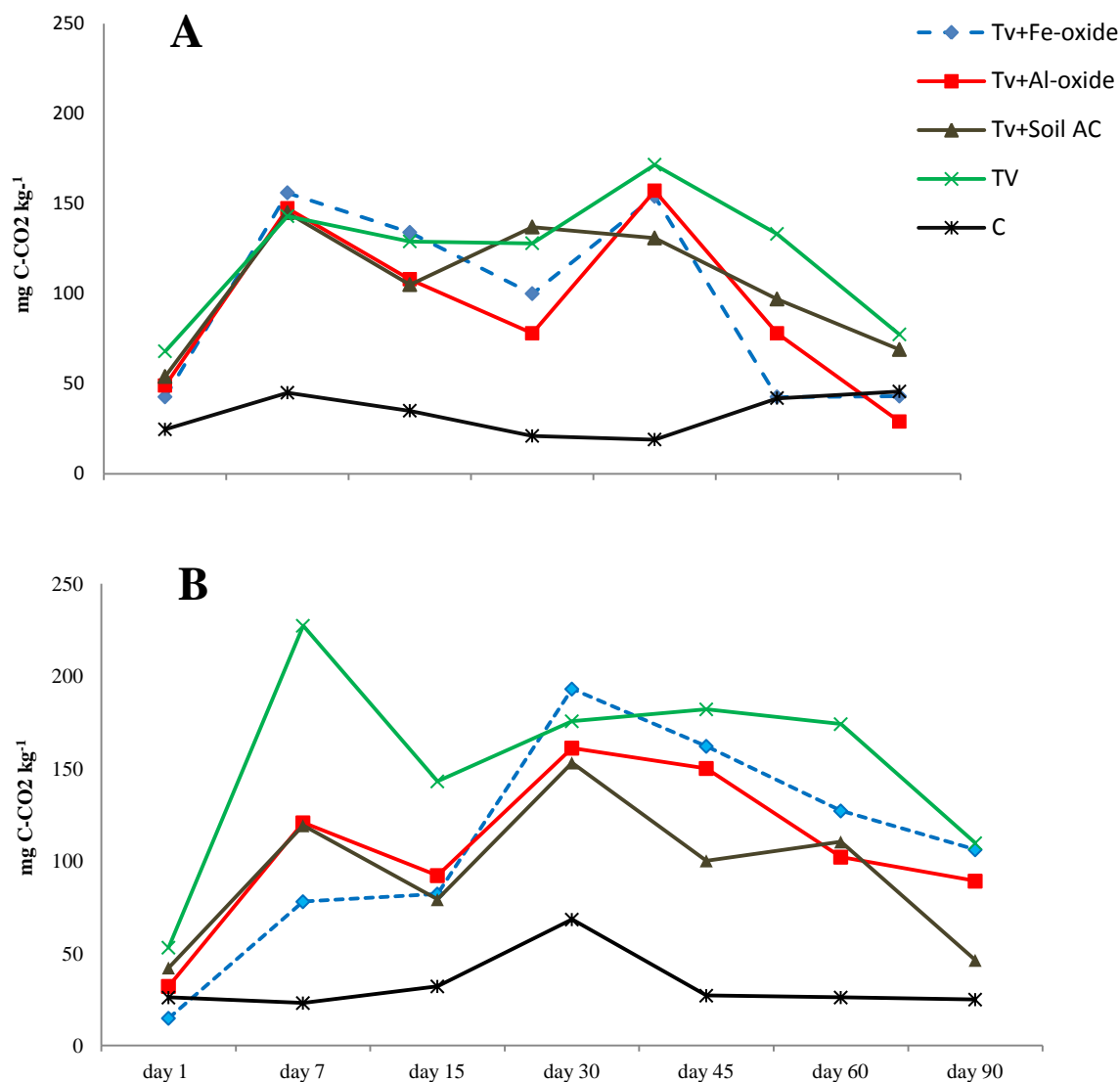
Figure 4.3 Evolution of soil respiration (CO_2 release; mg C- CO_2 kg^{-1}) in soil-WSC incubations. A) BC: Basal contaminated soil (~330 mg Cu kg^{-1}); B) SS: Copper spiked soil (~830 mg Cu kg^{-1}). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*).

Figure 4.4 Mean CO_2 release (mg C- CO_2 kg^{-1}) of soil-WSC incubations. Basal contaminated soil (BC) (~330 mg Cu kg^{-1}) (black bars); Copper spiked soil (SS) (~830 mg Cu kg^{-1})(grey bars). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*). Different letters indicate a significant differences (Tukey *P<0.05*).

The activity of DHA increased with the addition of WSC. The DHA activity found to decline in all the incubations as the experiment progressed, and the maximum was recorded at the beginning of the incubation experiment between days 7 to 30 (~3 µg INTF $g^{-1}h^{-1}$) in BC soils with the highest DHA activity observed in Tv+Al-oxide compost amended soils (Fig. 4.5 A). The inputs of WSC favor the overall soil oxidative activity in some treatments (Fe and Al-oxide, ~1.3 µg INTF $g^{-1}h^{-1}$) under BC soil, specially at the beginning of incubation experiment (Fig 4.5 A and 4.6). However, the increasing levels of Cu in SS significantly affected and reduced the DHA activity in all the evaluated treatments. Conversely, increasing levels of Cu in SS did not affect the activity of β-glucosidase enzyme. The addition of WSC to both BC and SS soils increased the activity of this enzyme. Significant differences between WSC amended soils and Control treatments without compost addition were observed. Higher values were observed at day 60 with ~19 µmol pNP $g^{-1}h^{-1}$ (Tv+Fe-oxide) in SS incubations. Otherwise, the lowest β-glucosidase enzyme activity was found at day 15 wherein the activity was ~3 µmol pNP $g^{-1}h^{-1}$ (Tv+Soil AC) in BC soils (Fig. 4.7). The high mean value of the β-glucosidase activity was observed in Tv+Fe-oxide treatments (11 µmol pNP $g^{-1}h^{-1}$) and the low one was obtained in Control treatments without compost addition (2 µmol pNP $g^{-1}h^{-1}$).

4.3.4 Availability and redistribution of copper

Sequential extraction results showed that the addition of WSC reduced easily exchangeable fraction and increased organic bound fraction of Cu (Fig. 4.9). Copper in the treatments without WSC amendments were primarily associated with the easily exchangeable fraction, accounting for over ~40% of total Cu in SS. Copper was strongly bound to the soil since more than ~70% in BC soils and 65% in SS of its total content was accounted for in oxide bound, organic bound and residual fractions after 90 days of incubation. The addition of compost decreased the sum of easily exchangeable and carbonates bound fractions as experiment progressed in both BC and SS soils from ~45% (Control treatment in SS) to around 25% (Tv+Al-oxide in SS) in comparison with controls. The application of these organic amendments (WSC) increased organic bound Cu from 10% to 20% (Tv+Soil AC) in BC soils, and around two times in SS in comparison to Control treatments at the end of the experiment (90 days).
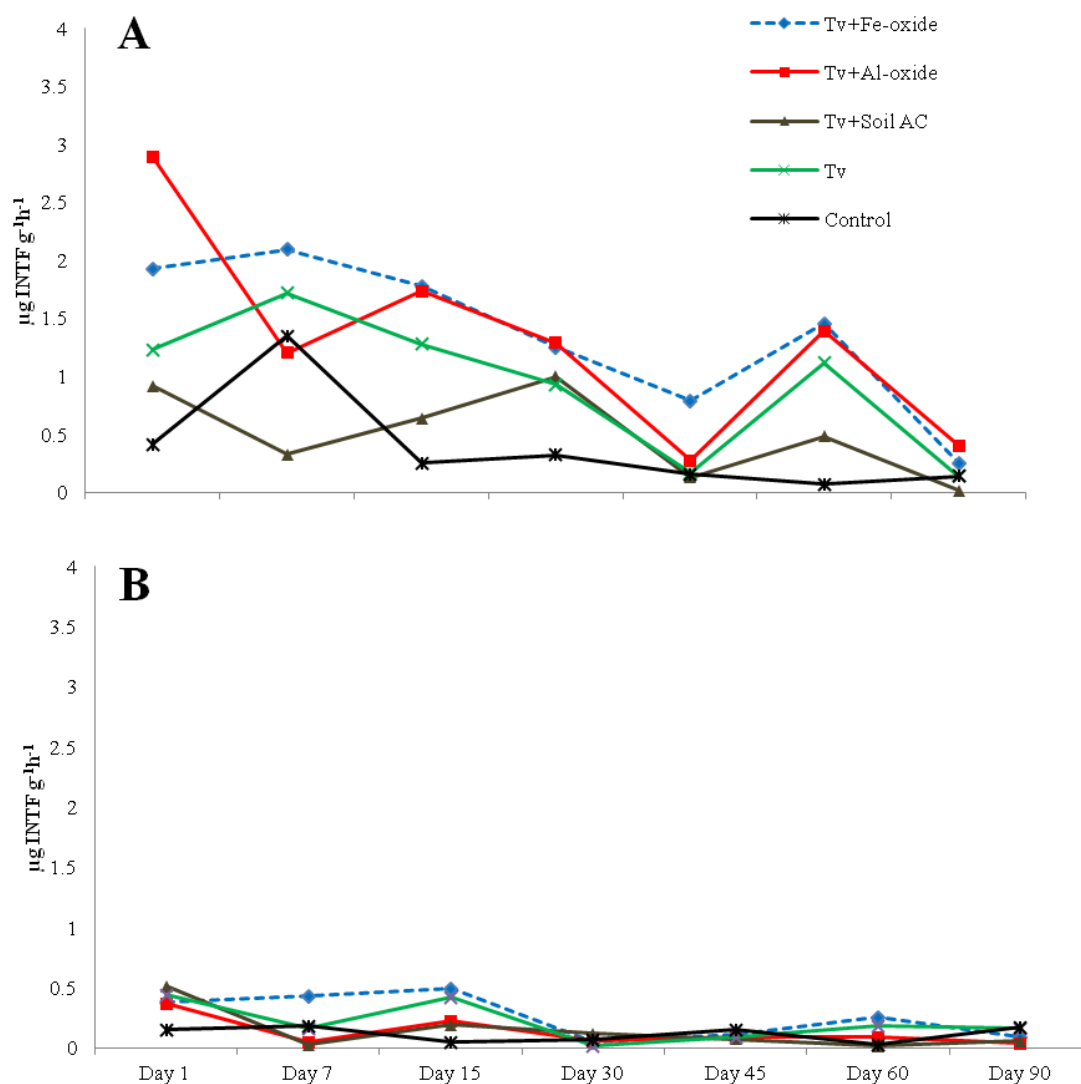
Figure 4.5 Evolution of dehydrogenase activity (DHA) (µg INTF $g^{-1}h^{-1}$) in soil-WSC incubations. A) BC: Basal contaminated soil ($\sim$330 mg Cu kg^{-1}); B) SS: Copper spiked soil ($\sim$830 mg Cu kg^{-1}). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*).

Figure 4.6 Mean DHA activity (μg INTF $g^{-1}h^{-1}$) of soil-WSC incubations.   Basal contaminated soil (BC) ($\sim$330 mg Cu kg^{-1}) (black bars); Copper spiked soil (SS) ($\sim$830 mg Cu kg^{-1})(grey bars). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*). Different letters indicate a significant differences (Tukey *P<0.05*).

Evolution of β-glucosidase activity
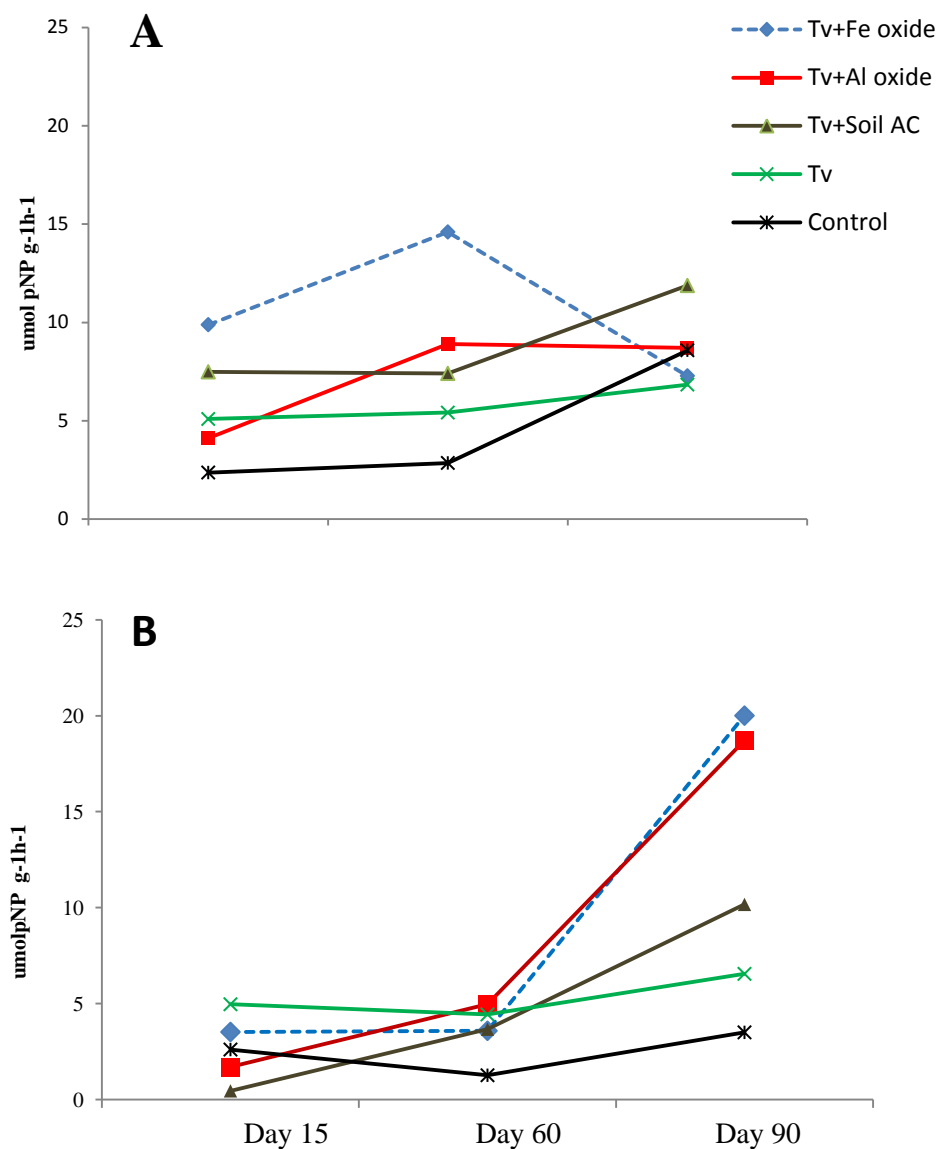
Figure 4.7 Evolution of β-glucosidase activity (μmol pNP $g^{-1}h^{-1}$) in soil-WSC incubations. A) BC: Basal contaminated soil (~330 mg Cu kg^{-1}); B) SS: Copper spiked soil (~830 mg Cu kg^{-1}). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*).

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Figure 4.8 Mean (μmol pNP $g^{-1}h^{-1}$) of soil-WSC incubations. Basal contaminated soil (BC) (~330 mg Cu kg^{-1}) (black bars); Copper spiked soil (SS) (~830 mg Cu kg^{-1}) (grey bars). C: soil incubation with no compost addition; Tv: soil incubation amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials); Tv+Soil AC: soil incubation amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series); Tv+Al-oxide: soil incubation amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide) and Tv+Fe-oxide: soil incubation amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide) (*n=3*). Different letters indicate a significant differences (Tukey *P<0.05*).

Figure 4.9 Sequential fractionation and distribution of Cu in soil-WSC incubations. A) Basal contaminated (BC) soil (330 mg Cu kg^{-1}) 90 days after WSC application; B) Copper spiked soil (SS) (830 mg Cu kg^{-1}) 90 days after WSC application. TvFe treatment=soil amended with Tv+Fe-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Fe-oxide); TvAl: soil amended with Tv+Al-oxide compost (wheat straw inoculated with *Trametes versicolor* and co-composted with Al-oxide); TvS: soil amended with Tv+Soil AC compost (wheat straw inoculated with *Trametes versicolor* and co-composted with the b horizon from an Andisol; Pemehue soil series) Tv= soil amended with Tv compost (wheat straw inoculated with *Trametes versicolor* without inorganic materials) and C: soil incubation with no compost addition (*n=3*).

4.3.5 Changes on SOM: Spectroscopic analyzes

4.3.5.1 Functional groups by (FTIR) spectroscopy

The FTIR spectra showed the main absorbances of HS from soil-WSC incubations (From BC soils: C, H1, H2 and H3) and HS extracted from compost (Tv+Fe-oxide compost; H4) which are mainly associated to functional groups concerned in the adsorption process caused by the lignin and cellulose decomposition in soil (Fig. 4.10). In general, the FTIR spectra corresponded to a deep peak at 3360-3450 cm^{-1} (H-bond to -OH groups), a distinct peak at 2920-2925 cm^{-1} (aliphatic -CH_3 stretch), weak peak at 2849 cm^{-1} (-CH_2 aliphatic C), pronounced peaks in the 1630-1710 cm^{-1} region (C-O stretch of -COO^{-1}), small peaks at 1450-1465 cm^{-1} (-CH_3, -CH_2, -asymmetric bending), 1420-1425 cm^{-1} (C-OH deformation of -COOH and -COO^{-} symmetric stretch), 1375-1385 cm^{-1} (C-CH_3, C-CH_2 deformations), and at 1235 cm^{-1} (C-O stretch or OH deformations of -COOH). Finally, a very strong peak could be detected near 1050-1070 cm^{-1} due to C-O stretching of cellulose. Also, it must be highlighted the signals corresponding to shoulders at 1700-1720 cm^{-1} that belong to C-O stretch of -C=O and -COOH (Ammar et al., 2014) of uronic acids, strong signals between 1500-1515 cm^{-1} (C=C aromatic rings of lignins), peaks at 1360-1365 cm^{-1} (CH_2OH aliphatic -OH), 1265-1270 cm^{-1} (C-O stretching and -OH deformations of alcohols and phenols), weak peaks at 1125 cm^{-1} (alcoholic -OH), and strong peaks at 1035-1070 cm^{-1} due to C-O stretching of cellulose (Fig. 4.10).

4.3.5.2 Chemical structural features by (CPMAS^{13}CNMR) spectroscopy

We analyzed the SOM from bulk samples of soil-WSC incubations from both BC and SS, however, just the spectra obtained in BC soils is showed (Fig. 4.11). Figure 4.11 shows the analyzed ^{13}C NMR spectra for Control soil, soil amended with WSC inoculated with *T. versicolor*, and soil amended with WSC inoculated with *T. versicolor* and treated with Fe_2O_3. In general, the ^{13}C NMR spectra of bulk soil and bulk samples from soil-WSC incubations showed relatively strong signals due to alkyl C between 45 and 0 ppm region; specifically, one sharp signal at 30 ppm. Lower resonances due to C in methoxyl sugars and

amino acids in 60-45 ppm shift region were observed as well, being higher in the Tv compost amended soils. Also, stronger signals arise from C in carbohydrates and aliphatic structures bearing OH groups (110-60 ppm) highlighting important peaks in the 72 to 74 ppm region. The spectra also showed signals between 160 and 110 ppm (aromatic C), 170-150 ppm (phenolic C), and a more prominent resonance at 173 ppm (carboxyl-C), similar to resonances for OM in whole soils (Schnitzer and Preston, 1986).

The spectrum of Control soil was characterized by broad and low resolution peak signals (Figure 4.11). Moreover, discernible chemical shifts in the aliphatic, aromatic, and carboxylic regions (*e.g.,* at 30, 73, 123 and 173 ppm respectively) for Control are showed. In contrast, the spectra from both WSC amended soils (Tv Composts and Tv+Fe-oxide), produced after 1 year of incubation, exhibit well-defined signals, especially those associated with the O-substituted aromatic C in lignin and modified lignins (160-120 ppm), and carbohydrates (ppm region). In Figure 4.11, the signals at 153 and 148 ppm correspond to C3 and C5 syringil units, respectively. Considering the high aromaticity and presence of carbohydrates in samples, the signals peaks at ~56 and 72 ppm could also suggest that the aromatic moieties have a high content of N-alkyl and O-alkyl C molecules. The N-alkyl C may include both amine and amide functional groups (Adani et al., 2006). Clear differences in terms of relative distribution were found between the Control for both (BC and SS) samples and WSC amended soil samples. It should be noted that in WSC amended soil samples (amended with Tv compost, Tv+Soil AC andTvFe treatments) the relative proportion of aliphatic-C structures (110-0 ppm) was large as a consequence of the exogenous OM addition (WSC) with high content of cellulose among others (Table 4.2).
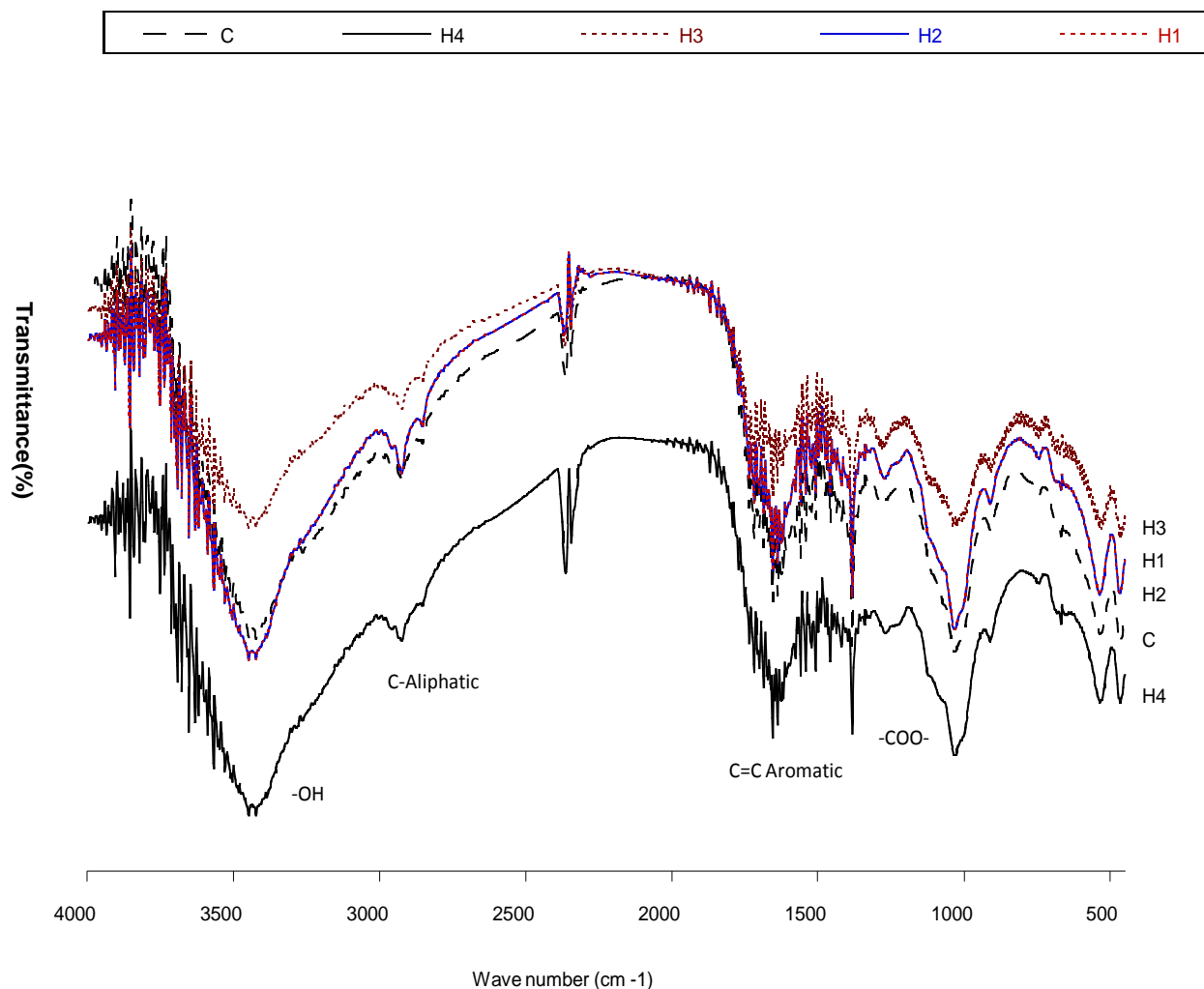
Figure 4.10 Fourier transform infrared spectra of humic substances (HS) extracted from Cu- contaminated soils (BC) amended with WSC. C: HS extracted from soil with no compost addition; H1: HS extracted from soil amended with Tv compost (wheat straw inoculated with *T. versicolor* and without inorganic materials); H2: HS extracted from soil amended with Tv+Fe-oxide compost (inoculated with *T.versicolor* and co-composted with Fe_2O_3); H3: HS extracted from soil amended with Tv+Soil AC compost (wheat straw inoculated with *T.versicolor* and co-composted with b horizon of an Andisol; Pemehue soil series) and H4: HS extracted directly from Tv+Fe-oxide compost.
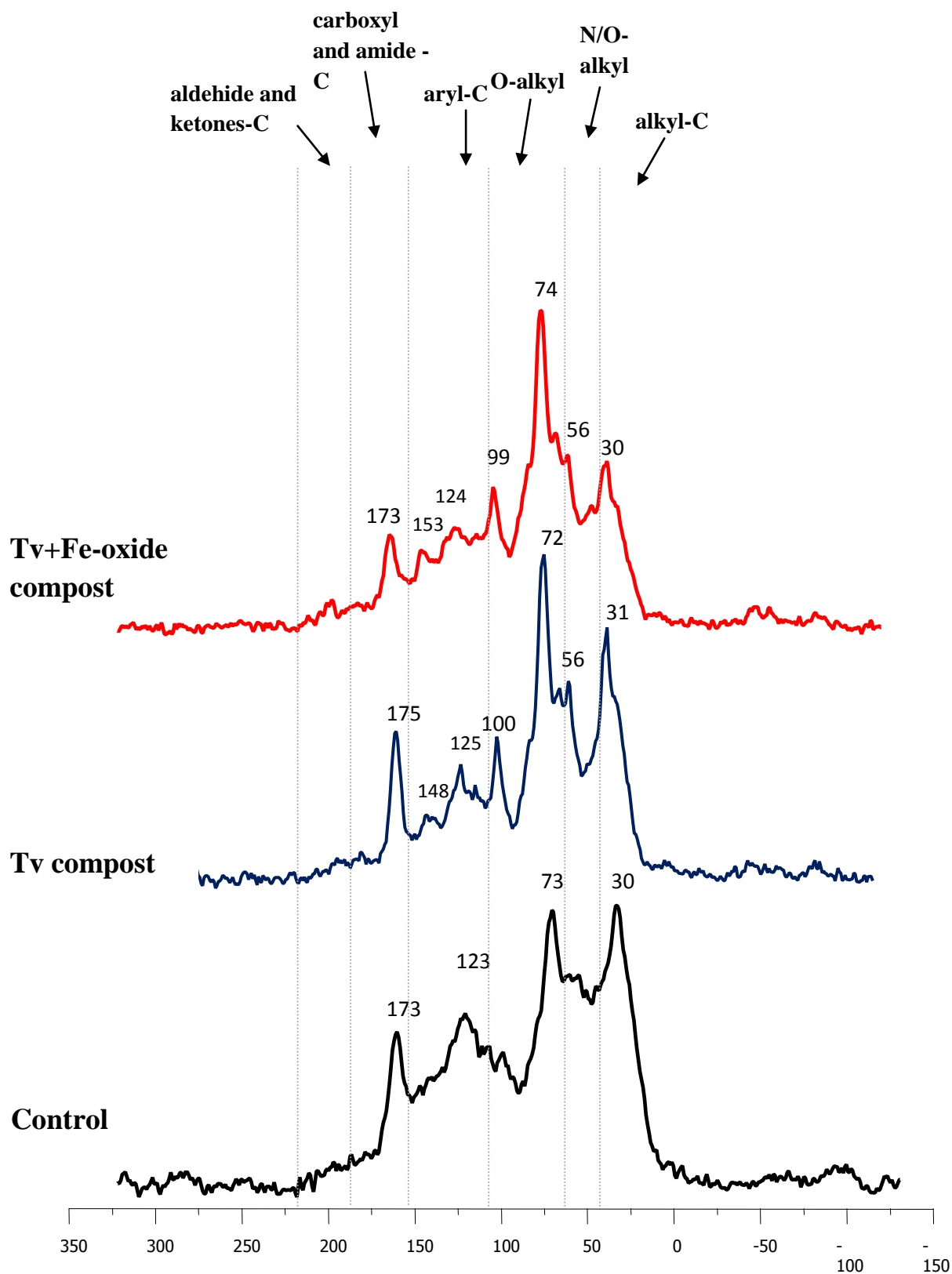
Figure 4.11 Solid state ^{13}CNMR spectra of bulk samples from Control without compost addition (BC soil); Soil amended with Tv compost (BC) and Soil amended with Tv+Fe oxide compost (BC).

Table 4.2 Relative intensity distribution in solid-state [13] C NMR spectra of bulk samples from soil-compost incubations and non-amended soil.

Treatment	Ketone C, aldehyde C (245-185 ppm)	Carboxyl C, amide C (185-160 ppm)	Aromatic C, olefinic C (160-110ppm)	O-alkyl C[a](110-60 ppm)*	N Alkyl C methoxyl C(45-60ppm)	Alkyl C (0-45 ppm)
BC-Tv compost	2.24	6.86	18.89	37.41	10.77	23.83
SS-Tv compost	3.55	8.12	16.93	35.93	10.26	25.21
BC-Tv+ Soil AC	2.06	5.85	17.05	42.45	10.73	21.86
SS-Tv+Soil AC	1.44	5.9	16.36	43.03	10.52	22.75
BC-Tv+Fe-oxide	3.48	6.64	19.14	40.45	10.03	20.26
SS-Tv+Fe-oxide	3.14	6.3	16.9	40.61	10.13	22.92
BC-Control	2.67	8.52	23.05	30.03	10.30	25.43
SS-Control	3.65	8.3	25.04	30.13	9.9	22.98

Tv: soil incubations (BC and SS soils) amended with Tv compost (wheat straw inoculated with *T. versicolor* without inorganic material); Tv+Fe-oxide: soil incubation (BC and SS soils) amended with Tv+Fe-oxide compost (wheat straw inoculated with *T. versicolor* co-composted with Fe-oxide); Tv+Al-oxide: soil incubation (BC and SS soils) amended with Tv+Al-oxide compost (wheat straw inoculated with *T. versicolor* co-composted with Al-oxide) and Tv+Soil AC: soil incubation (BC and SS soils) amended with Tv+Soil AC compost (wheat straw inoculated with *T. versicolor* co-composted with the b horizon of an Andisol; Pemehue soil series). Basal contaminated soils (BC)(~330 mg Cu kg^{-1}) and Spiked soil (SS)(~830 mg Cu kg^{-1}).

4.4 Discussion

4.4.1 Decomposition experiments: Soil respiration (CO_2 release)

Wheat straw based-compost application significantly affected the soil respiration assayed as CO_2 release. Control treatments without compost application showed the lower values of mean CO_2 release (~35 mg C-CO_2 kg^{-1}) associated with lower levels of OM content in soil (<2%). Conversely, Tv compost amended soil had the highest soil respiration reaching up to ~160 mg C-CO_2 kg^{-1} (Fig. 4.4). The increase in the soil respiration might be attributed to reduced toxicity of Cu as effect of its immobilization (changes in pH and the exogenous OM incorporation) and due to the increase in nutrients and DOC, thereby enhancing microbial activity and decomposition of SOM.

The soils amended with different types of organic fertilizers (composts, manures) behaved quite differently in terms of microbial activities and soil respiration. Flavel and Murphy (2006) noticed that C mineralization varied between various organic amendments in soils that include poultry manure, green waste compost, straw compost and vermicompost which they attributed to the differences in C quality parameters as measured by ^{13}C NMR spectroscopy. In this sense, a recent study conducted by Shah et al. (2016) showed important differences in soil incubations amended with organic sources such as cow manures and sheep-wheat straw compost among others. They reported that wheat based compost (15% of wheat straw and sheep manures) showed the lower values of soil respiration in agricultural amended soils reaching its peak of soil respiration (C-CO_2 release) at day 25, with a cumulative CO_2 emission of ~200 mg C-CO_2 kg^{-1} which was significantly lower than the other organic sources such as cow manures (914 mg C-CO_2 kg^{-1}). Similar results were obtained in this study wherein the maximum cumulative soil respiration was ~160 mg C-CO_2 kg^{-1} in Tv compost amended soil and the maximum soil respiration was observed at day 7 with ~230 mg C-CO_2 kg^{-1} by the same treatment (Tv compost amended soil) in SS conditions. Addition of such high lignocellulosic material to a compost leads to slower decomposition, so that less CO_2 is emitted from soil incubations. Shah et al. (2016) attributed the stability of the compost (with the higher C/N ratio) to the

fact that wheat straw contains a high fraction of lignin. It is, therefore, quite resistant to mineralization due to strong chemical bonds (McCrady 1991).

Bolan et al., (2012) reported that the addition of stabilizing agents, as goethite, gibbsite and allophanic clays decreased the rate of decomposition of organic amendments as poultry manure compost and poultry manure biochar applied to soils. It is important to recognize that despite we did not evaluate the rate of decomposition among the studied WSC, and their potential half life ($t_{1/2}$) in soils, the soil respiration allow us to measure the effects of compost in microbial activities and their potential stability in order to determine the most stabilized and "recalcitrant" amendment. Considering this, under our experimental conditions we found significant differences between the WSC amended soils related to soil respiration in soil-WSC incubations. As we have mentioned above, soils amended with Tv compost (without inorganic materials) in both BC and SS soils showed the higher CO_2 release than the other inorganic co-composted amendments with ~200 mg C-CO_2 kg^{-1} at the beginning of the incubation experiment (Fig. 4.3) and a mean value (cumulative soil respiration) of ~160 mg C-CO_2 kg^{-1} (Fig. 4.4). Wheat straw based-composts co-composted with metallic oxides (Tv+Fe/Al-oxides treatments) in BC soils and Tv+Soil AC compost (co-composted with clays) for SS showed the lowest soil respiration (~100 mg C-CO_2 kg^{-1} and ~90 mg C-CO_2 kg^{-1}respectevely), suggesting a potential effect of the addition of oxides and clays on compost properties, stabilization and degradation in soils as other authors had noticed (Bolan et al., 2012; Barthod et al., 2016). This aspect is noticeable, because according to the parameters used for selection (functional groups, C/N, E_4/E_6 ratio and spectroscopic analysis among others), the co-composted WSC (Tv+Fe-oxide, Tv+Al-oxide and Tv+Soil AC) were more stable and protected in comparison with no co-composted ones.

Decrease of CO_2 release in WSC amended soils treated with inorganic materials may be attributed to differences in the distribution of C fractions between the co-composted and non co-composted materials. Truc and Mashida. (2005) observed that application of Ca-zeolite increased the C accumulation of humic fractions as well as the degrees of humification and aromaticity of the humic acids by the effect of the combined application of Ca-zeolite and organic amendments to the soil. These results suggest possible complexations between oxy zeolite components and organic acid that take form organo-

metallic (Bolan et al., 2012). It has been noticed that Fe/Al-oxides and clay minerals (allophanic clays) immobilize soil C though different molecular interactions such as inner-sphere ligand-exchange reactions which, together with physical protection mechanisms, would play a key role in increasing the mean residence time of different C forms in soils (Dahlgren et al., 2004; Jastrow et al., 2007; Matus et al., 2008; Calabi-Floody et al.,2011; Bolan et al., 2012; Garrido and Matus, 2012; Fernández-Ugalde et al., 2016), thereby preventing or reducing the microbial decomposition (Day et al., 1994; Wada, 1985). Volcanic soils (incorporated to the Tv+Soil AC treatment) have a large content of OM (Broquen et al., 2005). In this kind of soils the preservation of OM is influenced by a number of processes including: protection of OM by complexation with iron, aluminum and allophane; reduced bacterial activity that results from the presence of free iron and aluminum; low pH and poor availability of nutrients to soil microorganisms involved in OM degradation (Parfitt, 2009).

Sikora (2004) noticed that iron-rich residue addition decreased the rate of decomposition of SOM which they attributed to a decrease in soil microbial activity. In the same way, studies of Scheel et al. (2008) and Schwesig et al. (2003) showed that Al decreases the biodegradability of DOC thereby increasing C stabilization which they attributed mainly to the reduced bioavailability of DOC after Al-induced precipitation. In composting systems, authors like Bolan et al. (2012) have observed the effects of allophane addition in a reduction of the decomposition rate (poultry manures-based amendment) and an increase of their half life ($t_{1/2}$) when applied to soils as well as we observed in our incubation experiment (~90 mg C-CO$_2$ kg^{-1} for Tv+Soil AC). Furthermore, in studies conducted by Brunetti et al. (2008) and Qi et al. (2012), the stabilizing effect of Fe(III)-and Mn(IV)-oxides was also attributed to the fact that the metallic oxides can act as oxidants (lewis acids) and catalyst promoting reactions like the poly-condensation of humic precursors. According to Brunetti et al. (2008) the humic acid (HA)-like fraction isolated from fresh olive mill wastewater (OMW) was affected due to the metallic-oxides (Mn oxide) addition, which were able to catalyze the OM humification in these organic residues and produce more stabilized amendments with a potentially longer half life ($t_{1/2}$)in soils. Similar results were found in our previous study (chapter 3).

Our results suggest a protection effect of the inorganic materials (Al-Fe oxides and soil allophanic clays) in WSC and then when applied to soils, promoting a reduction in the microbial activities and soil respiration (50% less than no co-composted WSC ~Tv compost~) by mechanisms and reactions above described. In a previous study (see chapter 3), we observed the effect of Fe-oxides and Soil AC in compost properties and OM from the humified fraction extracted from compost samples. These interactions and OM transformations (*i.e.,* changes in chemical structure, organo-mineral sorption, aggregation phenomena) in composting systems may confirm the stabilization of OM by the effect of the studied inorganic materials.

It has been reported that metal contamination inhibits microbial activity, thereby affecting the decomposition of OM with a direct effect in compost decomposition. For example, Sauve (2006) and Zeng et al. (2006) have shown a significant decrease in soil respiration at increasing levels of Cu and Zn (285 and 500 mg $C-CO_2$ kg^{-1} soil, respectively) (Bolan et al., 2012). In this regard, metals including Zn, Cu and Pb among others tend to form both soluble and insoluble complexes with OM, thereby inhibiting the supply of free C for decomposition (Bolan et al., 2011; Kunhikrishnan et al., 2011). Nevertheless, caution need be exercised due to under our experimental conditions there was not significant differences between WSC amended soils in both BC and SS soils. Authors like Hattori. (1996) have concluded that the recalcitrance of the organic materials (*e.g.,* plant debris) towards microorganism degradation should also be considered (Quenea et al., 2009). Thus, residues treated in our study was mainly affected by the co-composting treatments suggesting a decrease on CO_2 release to ~100 mg CO_2 kg^{-1} in both Tv+Soil AC treatment in basal contaminated soil and Tv+Al-oxide treatment in the spiked soil.

4.4.2 Enzymatic activities

Enzymatic activities are considered as major factors contributing to overall soil microbial activity, and soil quality (Nannipieri, 1994). The increase of enzymatic activities in soils is involved in an increase of the availability of nutrients to plants, which in turn have a positive influence on soil fertility (García et al., 1997) even in contaminated soils. Soil enzymes have been recommended as standard biochemical indicators to assess the quality

of metal-contaminated soils; however, it is important to differentiate between extracellular and intracellular enzymes (Zhang et al., 2013). The dehydrogenase activity, which is only present in viable cells and essentially depends on the metabolic status of the soil biota, may be considered a direct measure of soil overall microbial activity, while β-glucosidase can also occur extracellularly (Parelho et al., 2016). In our study, DHA activity was more sensitive to conventional trace metal contamination (Cu). The addition of WSC increased the enzymatic activities in comparison to Control treatments without compost addition (Figs. 4.5, 4.6, 4.7 and 4.8). Nevertheless, when Cu was added at increasing levels SS (830 mg Cu kg^{-1}) to soils the DHA activity was significantly decreased or even inhibited (Fig. 4.5 B). Authors have described the high sensitive of DHA to increasing levels of contaminants. In studies wherein high levels of Cu (150-450 mg kg^{-1}) were added to different type of soils, the DHA activity was inhibited in contrast to other enzymes (*e.g.*, urease) suggesting a high sensitivity of DHA enzymes exposed to high Cu levels (Wyszkowska et al., 2006). In addition, the use of INT methods in Cu contaminated soils could be self-defeating since the presence of Cu affects the absorbance of the reaction product INTF (Obbard, 2001).

The higher levels of DHA activity at the beginning of the incubation experiment in BC soils (3 μg INTF g^{-1}h^{-1}) is attributed to a higher availability of OC, nutrients and stimulated microbial activity as effect of the WSC addition, decreasing as the experiment progressed and labile forms of OM decline. However, mean values of DHA were significant lower than the reported in several studies (<100 μg g^{-1}h^{-1}) (Zhang et al., 2013; Parelho et al., 2016) probably due to the forms of C applied in this study. Significant correlations have been observed between SOM and enzyme activities (Bergstrom et al., 1998). Interestingly, not only the amount of SOM is important but most of all its quality, as OM affects the supply of energy for microbial growth and enzyme production (Fontaine et al., 2003). Furczak and Joniec (2007) showed that stimulation of DHA was accompanied by an increase in the number of the microbial groups and improvement under other living conditions (aeration and moisture). Thus, in general, our results indicates that lower level of metals (Cu) and higher levels of OM may lead to stimulate soil microbes to synthesize DHA, promoting microbial metabolic activity (Casida, 1964; Zaman et al., 2002; Zhang et al., 2013; Parelho et al., 2016). Furthermore, Levyk et al. (2007) demonstrated that acidic

conditions in the pH range between 1.5–4.5 resulted with strong DHA inhibition in relation to alkaline soils. Thus, pH increasing by WSC application could stimulate the activity of DHA as well.

The β-glucosidase activity indicates the transformation and hydrolysis of carbohydrates from OM by the action of microorganisms in soils. In this study, compost application increased this hydrolytic activity even under contaminated conditions (SS: 830 mg Cu kg^{-1}). The effect of WSC application can be confirmed comparing Control treatments and WSC amended incubations wherein β-glucosidase activity was significant higher indicating an increase in the microbial activity when these organic amendments were incorporated (Medina et al., 2006). The β-glucosidase activity showed a slight increase as the experiment progressed in all the evaluated treatments (~5 to 20 μmol pNP g^{-1}h^{-1}) under BC and SS soils. These results are in accordance with Tica et al. (2011) and Burges et al. (2015) who found a positive correlation between β-glucosidase activity and metal concentrations for metal contaminated soils. This enzymatic activity has a crucial role in the soil C cycle since it catalyses the hydrolysis of various β-glucosides during the decomposition of organic materials (Schinner et al., 1996), producing hydrolysis products which are then important sources of energy for soil microorganisms; in this respect, the β-glucosidase activity has an important role in TE contaminated soils, where microorganisms have a higher C requirements for repair and maintenance (Lamb et al., 2009). Indeed, an increase in the bioavailable fraction of TE as Cu leads to a deterioration of the environmental conditions for the exposed soil microorganisms and, concomitantly, increases their C requirement (Burges et al., 2015). The highest β-glucosidase activity in SS conditions was observed at day 90 in Tv+Fe-oxide treatment (~20 μmol pNP g^{-1}h^{-1}) and the highest mean value under the same treatment (~12 μmol pNP g^{-1}h^{-1}) (Fig. 4.6 and 4.7). Wyszkowska et al. (2010) reported that, despite of being slightly affected by increasing Cu concentrations (doses of 150 and 450 mg Cu kg^{-1}), β-glucosidase was the most resistant enzyme when they compared its tolerance between different ones as well as we can confirm in this experiment. Nevertheless, if we compared our β-glucosidase activity values with agricultural systems such as soils under long-term conventional farming practices (mean value 545 μg pNP h^{-1} g^{-1} dry soil) (Avellaneda-Torres et al., 2013), the activity was clearly lower. Considering this, the evidence suggests that in metal contaminated soils the β-

glucosidase is strongly affected and decreased (Parelho et al., 2016). On the other hand, generally, enzyme activities tend to increase with soil pH (Błońska, 2010; Moeskops et al., 2010). In this regard, β-glucosidase enzyme activity could be affected by pH modifications as well. In this study, pH decreased to ~4.8 when high levels of Cu (addition of 500 mg Cu kg^{-1}; ~830 mg Cu kg^{-1}) were added to soils (SS) which probably affected and decreased the enzyme activity as well (Control in SS: ~2 µmol pNP g^{-1}h^{-1}). However, when WSC (*e.g.,* Tv+Fe-oxide treatment; pH: ~6.5) were incorporated there was an increase in pH values with the potential concomitant effect on the enzyme activities (Geiger et al., 1998a).

Furthermore, it has been considered that a proportion of enzymes in soils are soluble in the aqueous phase and a proportion is physically or chemically bound to mineral, humified fractions of OM and/or organo-mineral surfaces (Burns, 1982; Boyd and Mortland, 1990). Therefore, the smallest soil particles, the clay size phyllosilicates and organic colloids, with their large surface areas, assume a prominent role in soil microbial ecology. Taking into account, we can also relate the differences between treatments (Tv+Fe-oxides, Tv+Al-oixdes and Tv+Soil AC) regarding to enzymatic activities into soils, due to the application of inorganic materials (*i.e.,* Fe/Al-oxides and the b horizon of an Andisol) to WSC. Significant differences were observed between treatments highlighting the Tv+Soil AC amended soils with the lowest enzymatic activities for both DHA and β-glucosidase (mean value of 0.5 µg INTF g-1h-1 for DHA activity and ~6 µmol pNP g^{-1} h^{-1} for β-glucosidase activity). Under this context, authors like Bayan and Eivazi (1999) indicated that the catalytic proteins may be adsorbed to clay size particles and as such either completely deactivated (*e.g.,* α and β-glucosidase in the presence of montmorillonite) or their activity is significantly reduced. In the same study, they reported that some enzyme activities were stimulated by iron oxides. Nevertheless, caution need to be exercised due to the fact that there are some disparities in the literature.

4.4.3 Immobilization and redistribution of copper

Compost with high surface area may enhance the sorption of metals when incorporated into soils. The more recent concern about soil contamination has resulted in organic wastes been used as materials for remediation of contaminated sites (Park et al., 2011; Brown and

Chaney, 2016; Jones et al., 2016; Kästner and Miltner, 2016). The organic wastes and mature amendments applied to soils in general do not remove or degrade TE or organic compounds, but transform them into insoluble or less soluble forms and then less available by mechanisms and reactions of sorption, complexation or co-precipitation (Macnair et al., 2000; Mench et al., 2003).

The application of OM promotes the re-distribution of metals from soluble and exchangeable forms to fractions associated with anionic functional groups of OM (Son et al., 2007). In this regard, we found a decrease in exchangeable Cu associated with the application of all four WSC relative to the unamended samples. In general, the addition of WSC increased the fraction of Cu associated to the OM around two times in comparison with Control treatments in both BC and SS conditions. Moreover, after 90 days of incubation Cu was mainly associated to soil (Ox fraction, OM fraction and residual) under BC and SS conditions accounting with more than the ~65% of its content to these fractions. Nevertheless, compost in general are not completely effective in Cu immobilization probably attributed to the increase of labile C fractions like dissolved organic C which may contribute to Cu mobility (Beesley and Dickinson 2011; Park et al., 2011). Thereby, the use of stabilized OM with a high degree of humification can contribute to reduce the solubility, mobility and availability of metals in soils through the formation of highly stabilized OM-metal complexes (Clemente et al., 2006). Under our experimental conditions the most stabilized WSC (Tv+Fe/Al-oxide, Tv+Soil AC) were associated with a less contribution of the Cu-exchangeable fractions. Nowadays, there is a general trend that application of compost combined with biochar may enhance Cu immobilization. Their combined application have been effective in contaminated soils wherein leachable Cu is likely to decrease due to Cu's well-documented strong affinity for OM (Kumpiene et al., 2008; Jones et al., 2016) and should be considered for future research.

Metals form both soluble and insoluble complexes with organic constituents in soils, and the processes apparently depend on the nature of the OM. As might be expected, the organic component of soil constituents has a high affinity for metal cations because of the presence of ligands or functional groups that can form chelates with metals. With increasing pH, the carboxyl, phenolic, alcoholic, and carbonyl functional groups in SOM dissociate, thereby increasing the affinity of ligand ions for metal cations (Bolan et al.,

2014). When organic amendments as biochar or compost are incorporated to soils the presence and concentration of ligands (functional groups) increase promoting the adsorption of metal cations to the OM which may occur by non specific (simple Coulumbic interactions) and specific adsorption (inner sphere complex formation) processes (Park et al., 2011 a, b; Bolan et al., 2014). Considering this, we suggest that metal-OM sorption processes may occur as result of the increasing content of ligands and this process could be mediated by both categories when WSC are applied.

In addition to the OM, pH plays the most crucial role in determining the availability and bioavailability of metals to biota in soils (Lock et al., 2000; Lakhdar et al., 2009). Increase in pH values can increase the adsorptive capacity of soils and consequently, decrease metal bioavailability. Given this, Table 4.1 showed that soil pH consistently increased in all the amended soils at each of the three time points compared to the unamended soil (4.7 to 6.5). Several studies reported that stabilized organic amendments as mature composts have a positive liming effect when applied to soils with low pHs as the affected by the deposition of materials from copper refineries (Ginocchio et al., 2009; Neamann et al., 2009; Neamann et al., 2012). Their application increases the soil pH, and thus improves the immobilization of heavy metals and nutrient availability (Bolan et al., 2014; Jones et al., 2016; Kargar et al., 2016) as result of increased negatively charged sites as well as the displacing and decrease of H^+ concentration (Kargar et al., 2016).

4.4.4 Compositional changes on SOM and potential interactions with Cu: spectroscopic analyzes

The humic substances extracted from soil-WSC (H1, H2, H3) and from Tv+Fe-oxide compost (H4) support that the humified organic fraction may have a high affinity for metal cations due to the presence of ligands (Harter and Naydu 1995) as COO– and -OH. Previous results of HS extracted from WSC treated with Fe_2O_3 (Tv+Fe-oxide compost) showed higher values for total acidity (12.6 meq g^{-1}), carboxylic (2.29 meq g^{-1}) and phenolic -OH groups (10.37 meq g^{-1}) than non- treated compost (total acidity 6.74 meq g^{-1}; carboxylic 1.61 meq g^{-1} and phenolic -OH 5.13 meq g^{-1}) (Chapter 3) suggesting a positive effect of inorganic additives on physicochemical properties of compost. Alvarez-Puebla et

al. (2004) noticed that the main adsorption mechanism from pH 4.0 was the complexes formation with carboxylic acids due to the increase of asymmetric and symmetric -COO$^-$ stretching and the reduction of the -O-H stretching of H-bound, -C=O stretching and -C-O stretching and -O-H deformation in -COOH. We observed marked decrease in absorptions near to 1710 cm^{-1} (C=O of COOH) and 1230 cm^{-1} (C-O stretch or OH deformation of COOH), and an increase in absorptions near to 1390 cm^{-1} (asymmetric and symmetric stretch of COO, respectively) in HS from soil-WSC when we compared to the HS extracted from composts and Control samples. These variations may suggest the extensive conversion of COOH to COO- after interaction of HS with Cu^{2+} (Senesi et al., 1986). On the other hand, at pH ~6.0 Cu^{2+} can form hydroxides which may be identified at peaks 3445, 3357, and 3316 cm^{-1} in FTIR spectra, also observed under our experimental conditions (on Tv+Fe-oxide amended soils). The peak at 3360 cm^{-1} can be assigned to -O-H stretching from Cu hydroxides such as Cu(OH)$_2$, suggesting the formation of HS-Cu complex as potential mechanism that helps to reduce the mobility of Cu ions in soils.

The solid-state ^{13}C NMR spectra were acquired for bulk incubation samples, and a selection of 12 months incubated samples of WSC and Cu-contaminated soils were analyzed. There were slight differences in spectra between BC and SS soils in both WSC amended and non-amended soil incubations. Representative spectra are shown in Figure 4.11 wherein the spectrum for the WSC (Tv+Fe-oxides)-amended soil shows a distinct peak at ~72 ppm and shoulders at 84 and 64 ppm and indicate that polysaccharides and some cellulose materials were present (Kögel-Knabner, 1997; Knicker et al., 2008; Pronk et al., 2013), which probably originating from remaining WSC in soil, which was confirmed according results showed in Table 4.2. In this study, solid-state ^{13}C NMR indicates that the abundance of different types of C in the spectra of WSC-amended soils decreased as follows: O/N alkyl ~ alkyl C > aromatic-C > carboxyl-C. In general, the addition of WSC to the Puchuncaví soils (in soil-WSC incubations) incorporated organic materials and HS which may be considered as stabilized and resistant to degradation, including modified lignins and lignin-derived structures, carbohydrates, such as crystalline cellulose, and/or other compounds of lipids, cuttins, amino acids and/or long-chain saturated paraffinic structures which were observed in spectras one year after WSC addition. Moreover, these results obtained by the CP/MAS ^{13}C NMR spectroscopy indicated that aliphatic and

polysaccharide structures from HS of organic amendments appear to be partially incorporated into native soil HS (Senesi and Plaza, 2007). Finally, taking the observation of the different regions of ^{13}C NMR spectra into account, WSC treated with the iron oxide (Tv+Fe-oxide) appear to preserve the aromatic and poly-aromatic structures (160-110 ppm) (Table 4.2) in compost (Chapter 3; section 3.4.3) and soil-WSC incubations reaffirming the effect proposed by several authors (Brunetti et al., 2008; Bolan et al., 2012; Qi et al., 2012) which could improve the stabilization of the OM in compost and when applied in soil.

4.5 Conclusions

Results suggest that WSC increase the microbial activities when applied to Cu-contaminated soils. Nevertheless, there were some differences between the different amendments. In this sense, WSC treated with Fe-Al oxides and allophanic clays (Soil AC) showed a lower soil respiration than Tv compost-amended soils and probably the decomposition of the WSC in soils under our experimental conditions was lower as well. Moreover, our results suggest that other factors like contamination levels (among others) can affect the microbial activities, and then the organic amendments decomposition when applied to soils. Nevertheless, we cannot state that the differences in microbial activities are just associated to the addition of inorganic materials to WSC affecting their decomposition. The WSCs modified the behavior and redistribution of Cu in the Cu-contaminated soil by altering their availability and promoting its binding to the OM fraction and soil components (bound to oxides and residual fractions) in both BC and SS soils. The addition of WSC modified the composition of the OM increasing the distribution of polysaccharides and lignin-modified products in bulk soil samples. Results showed that the application of stabilized WSC to Cu-contaminated soils could be considered a potential biotechnological tool for soil amelioration by reducing metal availability. However, studies to analyze other factors are still necessary in order to evaluate their potentials use in contaminated soils.

Chapter V

General Discussion and Conclusions

5.1 General discussion

White-rot fungi are characterized by their unique set of extracellular oxidases and peroxidases that enables them to effectively degrade lignin, one of the principal components of wood, forest litter, straw and other types of plant biomass commonly called lignocellulose (Baldarian et al., 2005; Sahadevan et al., 2016). In the present study we aimed to stabilize wheat straw (WS) in aerobic composting systems using saprophytic fungi through their extracellular enzyme activities and inorganic materials (metallic oxides and clays minerals) in order to produce stabilized wheat straw based-composts (WSC) to be applied as soil amendments in Cu-contaminated soils.

During delignification, and WS degradation three predominant reactions occur and have been widely defined: *i)* side chain oxidation (Cα-Cβ cleavage), *ii)* ring hydroxylation, and *iii)* demethylation (Geib et al., 2008; Zang et al., 2014). This process ultimately generates phenoxyl and phenyl radicals on the substrate, followed by depolymerization or polymeration and stabilization in soils and artificial systems such as composting (Kirk and Farrell, 1987; Ten Have and Teunissen, 2001; Zavarzina et al., 2011). In this study, our results indicated that combination of *Trametes versicolor* and Fe-oxide (Tv+Fe-oxide) applied at T1 (56 days after fungi inoculation) showed the higher enzymatic activities for LAC (~3 U L^{-1}), MnP (~0.350 U L^{-1}) and β-glucosidase (~600 μmol pNP g^{-1}h^{-1}) compared to the other inoculated treatments. Moreover, it was observed that *Pleurotus ostreutus* presented similar values to *T.versicolor* for enzymatic activities. In composting systems here evaluated, there were slight differences in chemical composition of different treatments at the end of this experiment. All treatments showed a high C (in a range of 45% for bulk compost samples and 55% for HS) content and C/N ratio (control WS ~90:1, TvC ~32:1) due to the nature of the raw material used for composting (Vukobratovic et al., 2008). Chemical and spectroscopic analysis showed a high concentration of acidic functional groups specially phenolic-OH (~15 meq/g in Tv+Fe-oxide) and ketonic-C signals (220-185ppm) in bulk compost samples, which were related to oxidative degradation and transformations of WSC. In addition, these analyses allow us to complement E$_4$/E$_6$ ratios and suggest that compost with low E$_4$/E$_6$ ratios and high aromaticity (8%) were the most stabilized amendments.

Authors as Davis et al. (1994) have studied the decay of lignocellulosic biomass (softwood and hardwood materials) by lignin degrading fungi *P. chrysosporium, T. versicolor* and *Dichomitus squalens* (white–fungi) and brown-rot fungi (*P. placenta*) using ^{13}C CPMAS NMR spectroscopy. Their results indicated a small preference for the degradation of amorphous polysaccharides compared to crystalline cellulose by white-rot fungi, which was even smaller on beech wood (Fackler and Schwanninger, 2012). In this study, despite the enzymes associated to WS degradation were not significantly produced, and the oxidative stabilization processes could be probably mainly affected by time and inorganic materials addition, similar preferences could be observed by our CPMAS ^{13}C NMR analyses wherein crystalline cellulose and polysaccharides appear at the final spectra (Chapter 3, Section 3.3). Despite we did not analyze the accumulation of these compounds by chemical methods; our spectroscopic analyses (CPMAS^{13}C NMR) suggest a loss of methoxyl groups and lignin modifications during the aerobic composting of WS (Fackler and Schawanninger, 2012).

In this research, the ligninolytic enzymes were extensively lower than current reported researches (*e.g*., less than 5 U L^{-1} and 1 U L^{-1} for LAC and MnP respectively) (Zeng et al., 2014; Gomes et al., 2016, Sahadevan et al., 2016). Therefore, the biodegradation of the lignocellulosic material, final stabilization and organic matter (OM) transformations of WSC were probably not significantly affected by the inoculation of saprophytic fungi (*T. harzianum*, *C. rigida*, *T. versicolor* and *P. ostreatus*) and their enzymatic systems. In this sense, there was no significant correlations between the studied enzymes and the humification of composted WS, measured as E_4/E_6 ratio (r= -0.348 for LAC; r= -0.379 for MnP). Thus, the biodegradation and OM transformation of WS could be related mainly to the presence of metals and inorganic materials into the aerobic system. In the past, much attention has been paid to the effect of metals on white-rot fungi. In general, the type and composition of the substrate appears to determine the amount of enzymes produced by basidiomycetes. Additionally, the temperature, pH, carbon source and nitrogen affect the growth of the fungus and its ligninolytic activity (Rodríguez-Couto et al., 2002; Cardoso-Silva et al., 2014). Moreover, metals can be toxic for white-rot fungi and affect their growth, the activity of extracellular enzymes and their penetration and nutrition in the soil environment (Baldrian, 2003; Baldrian et al., 2005). Under this context,

the effect of metals on the ligninolytic enzymes such as LiP, MnP and LAC has been addressed by several authors both under laboratory conditions and in non-sterile soil, and cadmium and mercury have been found to be the most toxic (Baldrian, 2005; Baldrian et al., 2000; Pointing et al., 2000). However, some metals such as iron, cadmium and copper were found to increase the activity of LAC in cultures of *P. ostreatus*. Baldrian et al. (2005) noticed in their study which aimed to determine the effects of selected metal ions on the process of WS degradation by *P. ostreatus* that LAC activity was increased by all tested metals, and unlike other white-rot fungi, nevertheless, Mn-peroxidase levels were low in the presence of manganese. Under our experimental conditions, similar effects were observed. Metallic oxides increased all the enzyme activities evaluated, especially those treated with Fe-oxide. When Fe-oxide was incorporated at day 56 (T1) higher mean activities of LAC, MnP and β-glucosidase were observed highlighting it effect on *P. ostreatus* and *T.versicolor* inoculated treatments. One explanation suggested by Baldrian et al. (2005) to this effect might lie in the limited selectivity of the metal-responsive promoter found in the LAC gene of *P. ostreatus* (Faraco et al., 2003), or the fact that LAC can also be induced in other "stress" conditions like the presence of xenobiotic compounds (Mougin et al., 2002) or interspecific competition (Baldrian, 2004).

On the other hand, the use of inorganic materials such as several common soil mineral constituents are proven to be able to promote the transformation in laboratory conditions of sugars, phenols and amino compounds into HS-like materials by acting as catalysts through various condensation, oxidative polymerization, ring cleavage, decarboxylation and dealkylation reactions (Huang, 2000; Jokic et al., 2004; Qi et al., 2012). Some studies have reported the use of these materials as bulking agents on organic waste stabilization and composting systems with the objective to chemically enhance, protect and promote structural changes of the compost mixtures (Senesi and Plaza, 2007; Brunetti et al., 2008; Jolanum and Towprayoon, 2010; Bolan et al., 2012; Chan et al., 2016). Metallic oxides and clay minerals which act as bulk agents of composting are associated to the polimerization of phenolic compounds by virtue of their high oxidation potential and high specific surface reactivity. Moreover, they may act as Lewis acids by accepting electrons from phenolic compounds and form semiquinones that, in turn, may undergo oxidative polymerization and generate HS (Senesi and Plaza, 2007). In addition,

they may participate in Fenton and Fenton like-reactions producing highly reactive radicals involved in the substrate degradation and OM transformation (Garrido et al., 2010). In this study, the addition of Fe_2O_3; Al_2O_3 and Soil clays produced changes in OM from WSC and its interaction was observed even at micro-nano scales (SEM and TEM microscopy; Chapter 3, section 3.3.5) wherein sorption process could be related to the physicochemical protection of WSC. Spectroscopic analyzes such as FTIR and CPMAS[13]CNMR showed an increase on signals associated to aromatic-C (~8%) (160-110 ppm) and Ketonic-C (~0.6%) (220-185 ppm) in composted WS treated with Fe-oxide and support that OM transformation was carried out by oxidative processes during 126 of aerobic composting. In addition, chemical analyzes showed that total acidity, carboxylic and phenolic-OH groups (~16 meq g^{-1}, 2.3 meq g^{-1} and 14 meq g^{-1} in HS extracted from *P.ostreatus* + Fe-oxide treated compost) were higher on Fe-oxide treated WS as well, and allow us to verify how metallic oxides and clay minerals may act as bulking agents of crop residues composting. Thereby, the results obtained in this thesis suggest a potential oxidative mechanism of OM transformation as effect of the inorganic materials supply (Senesi and Plaza, 2007; Brunetti et al., 2008; Qi et al., 2012; Scotti et al., 2013). Besides, SEM/TEM microscopy and X-EDS spectroscopy showed interactions at molecular level of HS extracted from composted WS with inorganic materials, leading to the assumption that aggregation and organo-mineral complex were carried out. These observations allow us to suggest that similar mechanisms of stabilization (OM transformation, HS-Metal complex formation, aggregation) may be present in HS from soil-less systems as WSC. Nevertheless, caution needs to be exercised and future research must be conducted in order to analyze the isolate effects of the inorganic materials.

On the other hand, the effects of WSC (Tv+Fe-oxide; Tv+Al-oxide; Tv+Soil AC and TV composts) were analyzed in Cu-contaminated soils. Basal contaminated soils (BC) (~330 mg Cu kg^{-1}) and spiked soils (SS) (~830 mg Cu kg^{-1}) were incubated with different WSC to evaluate how compost affect microbial activities and the Cu distribution in soil. Copper-contaminated soils amended with WSC behaved quite different in comparison to Control incubations with no WSC addition (Chapter 4). The Tv compost (no co-composted with inorganic materials) presented the highest CO_2 release whereas, WSC treated with inorganic materials (metallic oxides and soil clay minerals) showed less soil respiration

than Tv compost. In general, soil respiration measured as CO_2 release (~250 mg C-CO_2 kg^{-1}) was considerably lower in soil-WSC incubations than agricultural soils amended with organic amendments (*e.g.,* cow manures; ~900 mg C-CO_2 kg^{-1}); however, they are in the range of metal contaminated soils treated and ameliorated with exogenous OM as compost (Burges et al., 2015; Shah et al., 2016). In this study, the slow CO_2 release can be attributed to different factors such as the kind of material, the presence of metals and the low C/N ratios of the WSC even rather than to other factors (Table 3.1, Chapter 3). In this sense, numerous authors like Shah et al., (2016) have reported that the stability of wheat straw based - compost with a high C/N ratio, is attributed to the fact that these organic amendments contains an important fraction of lignin which is quite resistant to mineralization due to strong chemical bonds and the addition of such high lignocellulosic material leads to slower decomposition when applied to soils. Additionally, factors such as the addition of inorganic materials to composts in order to protect them of the mineralization, high levels of Cu among others were important and should be considered as well. However, caution must be take due to our results are not representative and do not completely support the hypothesis that inorganic materials applied to composting reduce the WSC decomposition in soils; thereby future research must be conducted to evaluate and confirm their role.

Soil respiration and the other biochemical parameters evaluated (*i.e.,* DHA and β-glucosidase activities) suggest that the addition of exogenous OM positively influenced the microbial activities in both BC and SS soils, except for DHA activity which were highly affected by increasing levels of Cu (~830 mg Cu kg^{-1}) even up to its inhibition. Several authors have supported the hypothesis that SOM is a positive factor for microorganisms and macro fauna in mining contaminated soils (Alguacil et al., 2011; Neaman et al., 2012; Olivier et al., 2012). In this research, the application of WSC modified the composition of the SOM and its changes were identified and evidenced by the state-of-the-art spectroscopic analyzes like CPMAS[13]CNMR. In this sense, the functional groups of the humified fraction extracted from WSC amended soils support the potential capacity of WSC to immobilize Cu. In the same way, chemical analyzes performed previously in Chapter 3 to WSC suggested their potential as immobilization agents. In addition, ^{13}CNMR spectroscopy allow us to verify that one year after of WSC application to soil, SOM present

clear evidence of the presence of residual lignin fractions and crystalline celluloses probably originating from WSC (Knicker, 2011; Scotti et al., 2013; Pronk et al., 2013).

We can state that Cu had a significant impact on soil quality (Control treatments: less than 50 mg C-CO_2 kg^{-1} for soil respiration; less than 0.5 µg INTF $g^{-1}h^{-1}$ for DHA activity and less than 5 µmol pNP $g^{-1}h^{-1}$ for β-glucosidase activity) in both BC and SS soils. Nevertheless, the application of WSC improved the environmental conditions for biota and promotes the activity of microorganisms even under spiked soils; thus, the microbial activities were not significantly affected in terms of Cu levels. In this regard, soil respiration (CO_2 release) and β-glucosidase activity did not showed significant differences between BC and SS treatments.

It is well-known that metal bioavailability in soil is strongly influenced by many different soil factors, such as pH, OM content and clay content, as well as by metal sorption–desorption mechanisms (Lamb et al., 2009; Burges et al., 2015). In this research, the application of WSC to Cu-contaminated soils as well as produced important changes in SOM, its addition modified the pH of soils. The aerobic composting of wheat straw no co-composted and co-composted with inorganic materials produced alkaline amendments which were in the range of pH: 8.5 (Chapter 3). These changes in pH values were used as criteria and essential indicator of compost stabilization (Senesi and Plaza, 2007; Scotti et al., 2015). When WSC were applied to soil incubations modified the pH of soils from 4.7 to ~6.5 (soil amended with Tv+Fe-oxide compost) in both BC and SS conditions. With increasing pH, the carboxyl, phenolic, alcoholic, and carbonyl functional groups in SOM dissociate, thereby increasing the affinity of ligand ions for metal cations (Bolan et al., 2014). Moreover, the addition of alkaline amendments may have a "liming effect". In this sense, the increase of pH values in WSC amended soils is related to the consequent increase in the Ca^{2+} ion pool in the soil solution and may result in a displacing and low concentration of H^+ ions, and the potential formation of insoluble complexes (Kargar et al., 2016).

On the other hand, soil incubations amended with WSC decreased the exchangeable Cu fractions and increase in around two times the organic bound fraction (up to 25%) in Cu-contaminated soils. Moreover, we found a decrease in exchangeable Cu associated with application of all four WSC relative to the unamended samples (Control treatments without

compost). This may suggest the effects of soil reactions (*e.g.,* pH modifications) and WSC as immobilization agents on the Cu availability. Several studies have shown that organic composts significantly reduce exchangeable Cu compared to the unamended samples (Park et al., 2011; Bolan et al., 2012; Bolan et al., 2014; Jones et al., 2016). In general, the high metal binding capacities of compost have been attributed to high compost humic and fulvic acid concentrations which may enhance the CEC of soils by increasing the negative charges and binding sites (Bolan et al., 2014). Thus, we suggest that when WSC were incorporated to Cu-contaminated soils the presence and concentration of ligands (functional groups) was modified and increased promoting the adsorption of metal cations to the OM which probably occurred by non specific (simple Coulumbic interactions) and specific adsorption (inner sphere complex formation) processes (Park et al., 2011; Bolan et al., 2014).

Thereby, the chemical, biochemical and physicochemical changes of the OM observed during the wheat straw composting allow us to state that saprophytic fungi and inorganic materials although apparently have a alight effect on wheat degradation, their combination positively affect the aerobic composting of wheat straw, promoting the C stabilization and enhancing some physicochemical properties the final composts, which were able in a second stage to modified different soil properties and the metal availability of Cu-contaminated sites as the studied in this thesis. This aspect is essential in our country, since the Cu mining is the principal economic activity and it is well recognized the impact on natural and agro-ecosystems. Even under a framework of climatic change, the topics covered by this Doctoral Thesis can be focused on the feasible alternativity for agricultural wastes reutilization using soils as C-sinks, and in parallel improving the conditions to remediate contaminated soils by using plants and other organisms. Nevertheless, future research should be carried out to clarify the specific role of every factor affecting composting and soil amelioration.

5.2 General Conclusions

Saprophytic fungi and their low enzymatic activities (>3 UL^{-1} for LAC, 0.350 UL^{-1} for MnP and less than ~600 μmol pNP g^{-1} h^{-1}) produced slight chemical changes of wheat straw suggesting a limited effect of the studied fungi on wheat straw biodegradation. However, spectroscopic analyzes exhibited physicochemical and chemical structural changes of bulk samples and humic like substances extracted from the composted mixture suggesting the transformation of the material through oxidative mechanisms which could be directioned by the inorganic materials. In this sense, the combination of saprophytic fungi and inorganic materials produced the most stabilized composts probably by the potential role of the metallic oxides and clay minerals as catalyst of the humification process and compost protectors.

Our evidence suggests that Cu-contaminated soils were negatively impacted in terms of its quality when increasing levels of Cu were incorporated. Nevertheless, the addition of WSC positively affected and enhaced the microbial activity in comparison to unmended soils even under spiked conditions (SS: ~830 mg Cu kg^{-1}). In this regard, the exogenous OM promoted the soil respiration and enzymatic activities related to the potential mineralization of the organic amendments supplied. However, there were slight differences between the different WSC which could be related to the stabilization level and the potential effect of the inorganic materials as compost protectors. Moreover, the SOM presented changes in terms of their structural distribution and humified fraction composition in amended soils even one year after WSC addition, suggesting that the exogenous OM especially those treated with metallic oxides (Fe_2O_3) could be incorporated to the native OM from soils.

The application of WSC helped to redistribute and decreased the availability of Cu probably through sorption and complexation reactions, and also by its effect as "liming agent" wherein the Cu decrease may be related to the pH modification. Moreover, the application of WSC treated with metallic oxides decreased the easily exchangeable fractions and increased the organic Cu bound to soils (oxides, OM and residual fractions) about two times in comparison to unamended soil in both BC and SS conditions, suggesting

an important role of WSC treated with inorganic materials on Cu immobilization even under repeated events of metal contamination (spiked soils).

Finally, we can conclude that inorganic materials used as bulking agents in co-composting process enhance the stabilization of wheat straw during 126 days of composting under our experimental conditions. In addition, we conclude that the WSC here studied positively affected the Cu behavior in a Cu-contaminated soil by decreasing their availability as potential effect of pH changes, sorption reactions and increased OM levels under different levels of contamination. In this context, we suggest that the addition of stabilized WSC to Cu contaminated soils could be a potential tool of soil remediation by immobilizing metals and reducing their availability in recent contaminated soils or in those affected by past contamination events (historical). However, caution need to be excersiced and future research should be performed in order to analyze the long-term effects of WSC treated with the inorganic materials in terms of their composting process and when applied to metal and multimetal contaminated sites.

5.3 Outlook

The information obtained in this Doctoral Thesis enhanced our understanding of the aerobic wheat straw composting and the potential effects of saprophytic fungi, inorganic materials and their combination on the process. In addition, we analyzed the effects of WSC on copper contaminated soils and how their application modified some properties of soils under contamination scenarios. Nevertheless, studies aimed to understand the main mechanisms involved in the transformation and stabilization of wheat straw in aerobic composting systems, the specific role of catalyst, saprophytic fungi and their combined effects whith other microorganisms, are still largely necessary in medium and long-term research. Moreover, studies with the objective to understand the chemistry of metals when these kinds of composts are applied to multi contaminated soils and the fate of metal, exogenous OM, and metal-OM complexes in soils, should be performed. Thus, considering the possible alternatives and future scenarios, to determine the potential of WSC in remediation strategies in combination with soil microorganisms and plants may become an interesting topic for long-term future studies more needed and highlithed under a scenario of climate change. In summary, caution should be taken and further studies about the use of biotic and abiotically stabilized WSC in soil remediation strategies in order to ameliorate contaminated sites, must be conducted before to stablish any recommendation or plan.

5.4 Final acknowledgements

Chapter VI

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6 References

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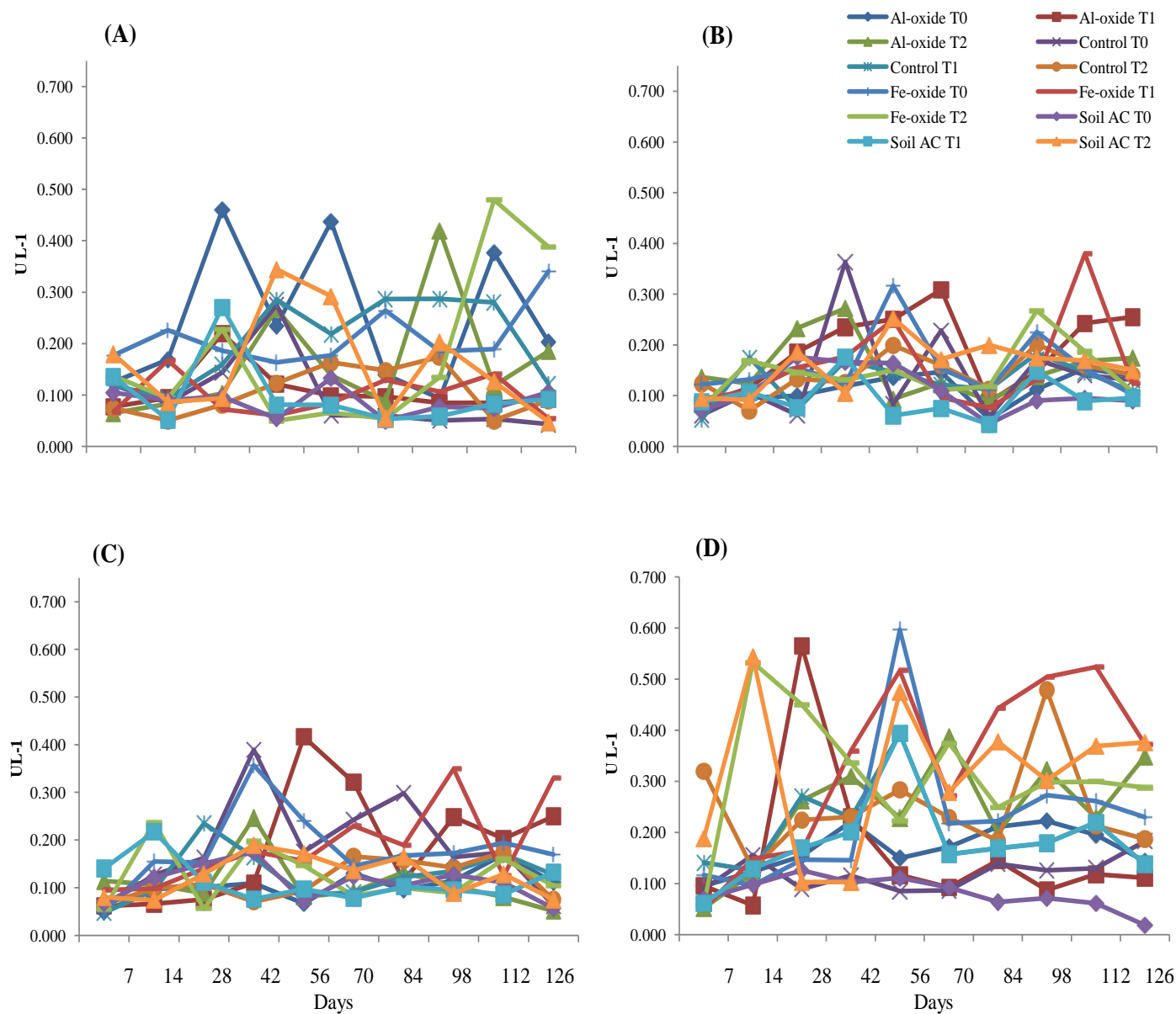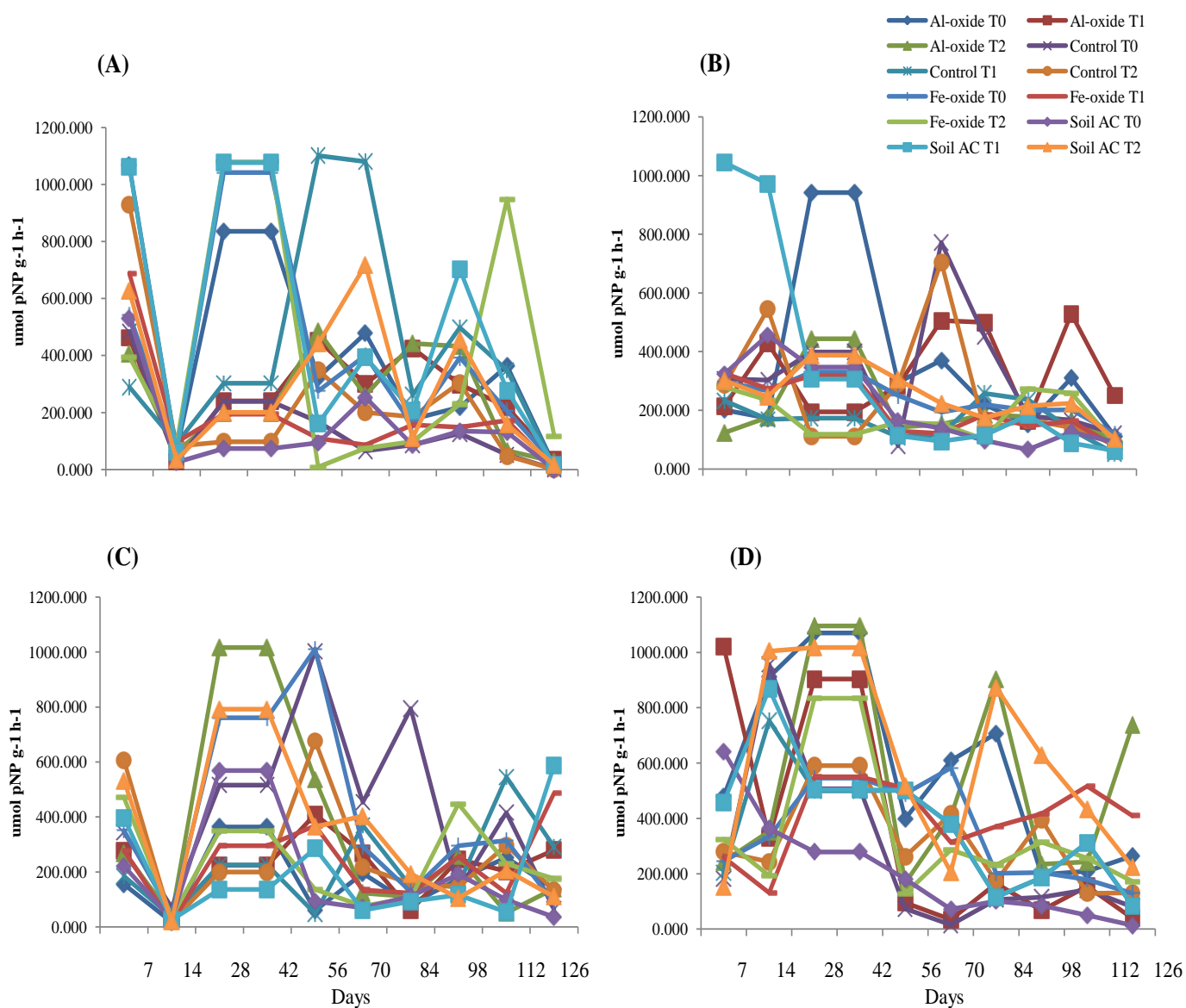
Chapter VII

Supplementary data and annexes
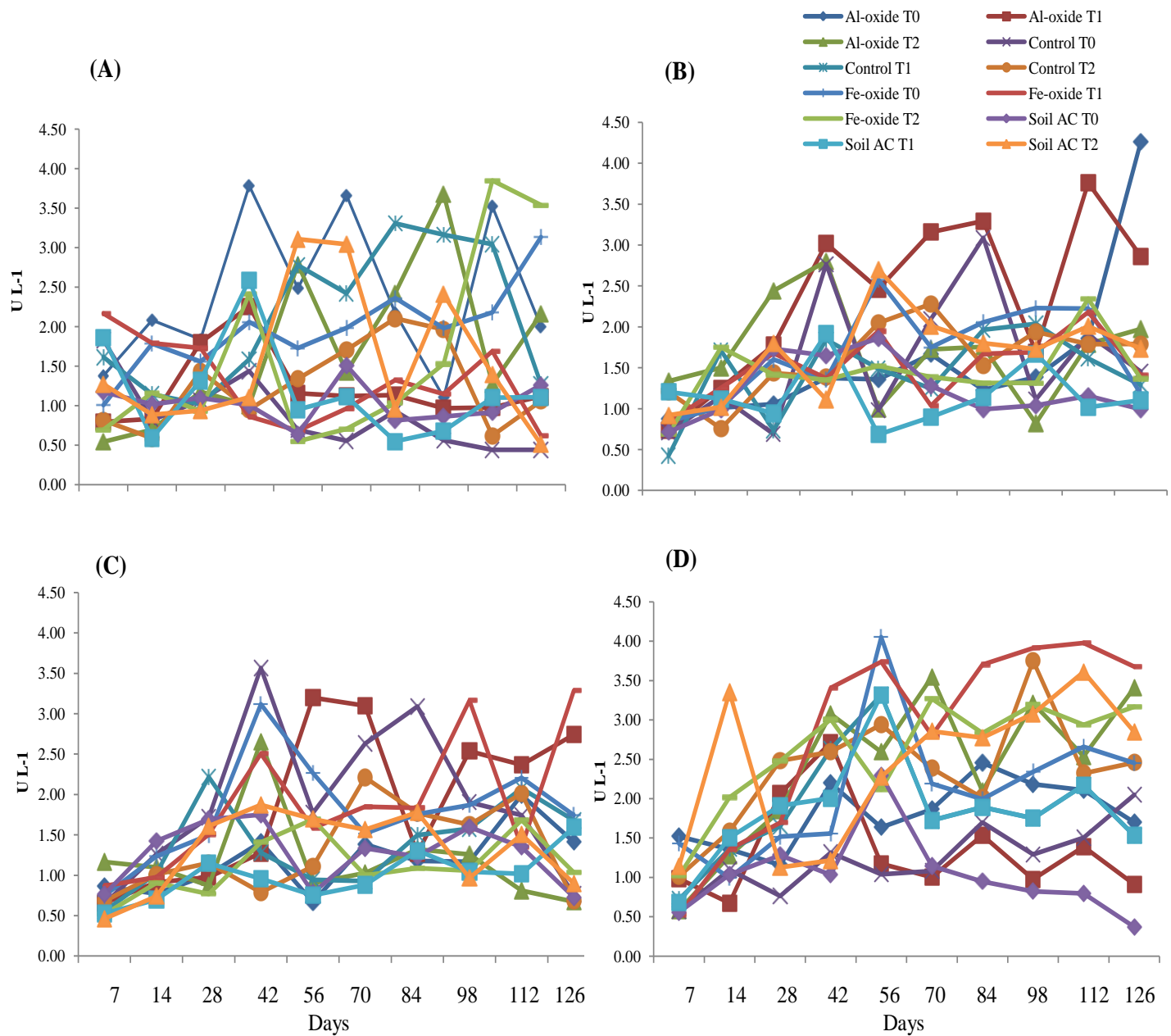
SD1: Evolution of Laccase (LAC) activity during the aerobic composting of wheat straw (WS). A) WS inoculated with *Coriolopsys rigida*, B) WS inoculated with *Pleorotus ostreatus*, C) WS inoculated with *Trichoderma harzianum* and D) WS inoculated with *Trametes versicolor*. Samplings were carried out at different points as composting progressed. Numbers in X axis represent days of composting.

SD2: Evolution of Manganese peroxidase (MnP) activity during the aerobic composting of wheat straw (WS). A) WS inoculated with *Coriolopsys rigida*, B) WS inoculated with *Pleorotus ostreatus*, C) WS inoculated with *Trichoderma harzianum* and D) WS inoculated with *Trametes versicolor*. Samplings were carried out at different points as composting progressed. Numbers in X axis represent days of composting.

SD3: Evolution of β-glucosidase activity during aerobic composting of wheat straw (WS). A) WS inoculated with *Coriolopsys rigida*, B) WS inoculated with *Pleorotus ostreatus*, C) WS inoculated with *Trichoderma harzianum* and D) WS inoculated with *Trametes versicolor*. Samplings were carried out at different points as composting progressed. Numbers in X axis represent days of composting.
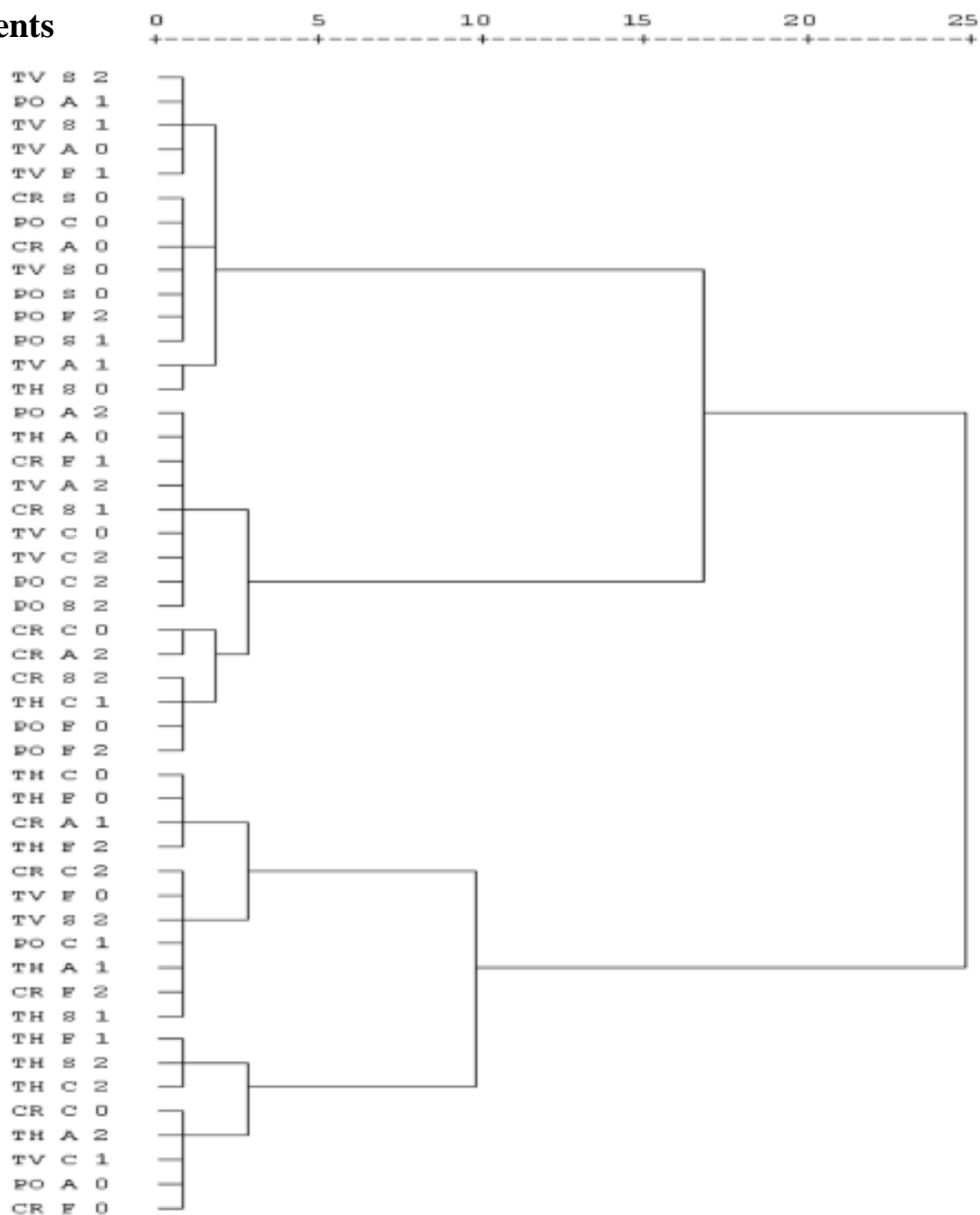
Treatments

SD4: Cluster analysis for different treatments. TV: *T.versicolor*, PO: *P.ostreatus*, CR: *C.rigida*, TH: *T.harzianum*; A: Al-oxide, F: Fe-oxide, C: Controls, S: Soil AC. 0: 14 days after fungi inoculation, 1: 56 days after fungi inoculation and 2: 114 days after fungi inoculation.

List of original papers

- Crop Residue Stabilization and Application to Agricultural and Degraded Soils: A review. Paper published in Waste Management (2015) 42, 41-54.

- Use of saprophytic fungi and inorganic materials for enhancing the aerobic composting of wheat straw I (manuscript to be submitted to Waste Management)

- Use of saprophytic fungi and inorganic materials for enhancing the aerobic composting of wheat straw II (*In preparation*; to be submitted to Waste Management).

- Supramolecular structure of humic substances extracted from a compost-amended mining soil: Main features and their potential effects on Cu-immobilization. (Paper under peer review process - ESPR-S-16-02996, Environmental Science and Pollution Research).

- Chemical structural characteristics of wheat straw and composted wheat straw inoculated with Trametes versicolor and treated with Fe_2O_3, and their effect on organic matter in a metal contaminated soil (manuscript to be submitted to Soil Biology and Biochemistry).

   Others

- Cornejo, P., **Medina, J.** 2014. Compostando y descontaminando. Hacia una gestión integral de los residuos agrícolas. Nuestra Muestra 10, 24-25.

Scientific meetings

- **Medina, J.,** Morales, E., Knicker, H., Meier, S., Borie, F., Cornejo, P. 2015. Stabilization of wheat straw based-composts by using inorganic materials and their effects in a copper contaminated soil. Simposio Nacional de la Ciencia del Suelo, Santiago, Chile.

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- Morales, E., **Medina, J**., Meier, S., Vidal, C., García, S., Borie, F and Cornejo, P. 2014. Use of Saprophytic Fungi and Abiotic Stabilizers to Improve the Humification of Wheat Straw Residues. Effects of compost in Cu-As polluted soils. 2nd International Symposium Soil Plant and Microorganisms, Pucón, Chile.

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