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Research paper

Earthworms increase the potential for enzymatic bio-activation of biochars made from co-pyrolyzing animal manures and plastic wastes

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ABSTRACT

We assessed the enzymatic activation of four different biochars produced from pyrolyzing swine manure and poultry litter, and by co-pyrolyzing these livestock residues with agricultural spent mulch plastic film wastes (plastichars). Enzymatic activation consisted of incubating biochars in soil inoculated with earthworms (*Lumbricus terrestris*), which acted as biological vectors to facilitate retention of extracellular enzymes onto biochar surface. The activity of carboxylesterase –a pesticide-detoxifying enzyme– was measured in non-bioturbed soils (reference), linings of the burrows created by earthworms, casts (feces) and biochar particles recovered from the soil. Our results revealed that: 1) biochar increased soil carboxylesterase activity respect to biochar-free (control) soils, which was more prominent in the presence of earthworms. 2) The maximum enzyme activity was found in soils amended with plastichars. 3) The plastichars showed higher enzyme binding capacities than that of the biochars produced from animal manure alone, corroborating the pattern of enzyme distribution found in soil. 4) The presence of earthworms in soil significantly increased the potential of the plastichars for enzymatic activation. These findings suggest that the plastichars are suitable for increasing and stabilizing soil enzyme activities with no toxicity on earthworms.

1. Introduction

Microplastic pollution is now recognized as one of the key environmental issues that humanity must face in the coming years. Microplastics, i.e., plastic-derived fragments and manufactured plastic polymers both of <5 mm in size (Syberg et al., 2015), are found in many ecosystems worldwide including remote areas (Bergami et al., 2020; Allen et al., 2019). One of the human activities with a significant input of plastic debris in the environment is conventional agriculture. Application of biosolids and composts as soil amendments, and the use of plastics in many agricultural practices (e.g., plastic films for soil mulching and solarization, greenhouse and low tunnel covers, irrigation drip tubes, and packaging plastics, among others) are the major direct inputs of plastic debris in agricultural soils (Espi et al., 2006; van den Berg et al., 2020). It is estimated that the global consumption of plastics in agriculture is around 8 million tons per year, China being the main producer and consumer of plastics (Qi et al., 2020). For example, China increased four-fold the consumption of plastic mulch films since beginning 90s, reaching 1.25 million tons in 2011 (Liu et al., 2014), and it is expected to rise to 2.28 million tons by 2025 (Qi et al., 2020). Polyethylene is the most common polymer used in mulch film production because of economic and practical benefits. However, its low biodegradability together to the high cost of removal after crop season (Ng et al., 2018), lead to accumulation of mulch film debris in agricultural soils with detrimental effects on soil quality and plant growth (de Souza Machado et al., 2019).

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Landfilling, incineration, and recycling are the current options for agricultural mulch film disposal (Qi et al., 2020), although they represent serious risk of environmental pollution or their contribution to reduce plastic contamination is still limited (plastic recycling) (Steinmetz et al., 2016). Alternatively, co-pyrolysis of biomass-plastic blends is a bioengineering option, which its primary goal is to produce bio-oils (liquid by-product) with higher quality (low oxygen content and large amount of aromatic compounds) than bio-oils obtained from pyrolyzing biomass alone (Uzoejinwa et al., 2018; Ryu et al., 2020; Park et al., 2019). Furthermore, co-pyrolysis of biomass-plastic blends consumes less energy than pyrolysis of biomass alone, or even produces supplemental energy for local power use (Ro et al., 2014). Therefore, the use of mulch films as ingredient in co-pyrolyzing biomass emerges as a promising strategy for managing non-biodegradable plastics and produce fuels (bio-oils) which may be an alternative to fossil fuels (Ryu et al., 2020; Abnisa and Wan, 2014). However, less attention has been placed to the potential environmental applications of the solid by-product or char generated from co-pyrolysis biomass-plastic blends. A few studies suggest that the char produced from co-pyrolysis biomass-plastic blends contains a higher carbon content (Sajdak and Muzyka, 2014), higher calorific values (Uzoejinwa et al., 2018) than those of char produced from biomass. Furthermore, higher char yields (Wang et al., 2019) with altered morphology (Chen et al., 2017) are generally achieved by co-pyrolysis biomass-plastic blends. Nevertheless, the impact of the co-pyrolyzed-derived char on soil quality and fertility has not been investigated.

There is a general consensus whereby char is renamed biochar when its use is to be a soil conditioner (El-Naggar et al., 2019). The inherent structural characteristics of biochar (e.g., high open porosity, large surface area) and its high stability in the environment (Lian and Xing, 2017; Wang et al., 2016) have led it to be a potential remediating material in polluted water (Sizmur et al., 2017) and soil (Ahmad et al., 2014). Biochar is also a suitable carrier for immobilizing and stabilizing enzymes for industrial (Cea et al., 2019; Zhang and Hay, 2020) and environmental applications (Sanchez-Hernandez, 2018), which has stimulated research towards improving of biochar reactivity for environmental remediation purposes (Rajapaksha et al., 2016). Such an improvement is generally gained by treatment of feedstock (pre-pyrolysis activation) or biochar (post-pyrolysis activation) with chemical or physical procedures (Sizmur et al., 2017; Tan et al., 2017; Wang et al., 2017).

Although physico-chemical activation is the most common method for improving biochar reactivity, biological activation appears to be an attractive low cost and eco-friendly alternative (Sanchez-Hernandez et al., 2019). Accordingly, mixing biochar with compost is proposed as a viable strategy to biologically activate biochar because of the high microbial activity of compost (El-Naggar et al., 2019; Sanchez-Monedero et al., 2018). Soil organisms such as earthworms seem also suitable vectors for activating biochar with extracellular enzymes (Sanchez-Hernandez, 2018). Earthworms are key organisms in soil organic matter decomposition, and provide favorable conditions for stimulating proliferation soil microorganisms and alteration of their community structure (Frazão et al., 2019). Furthermore, the intense burrowing activity of earthworms contributes to dispersing microorganisms and nutrients in soil (Yang and van Elsas, 2018; Van Groenigen et al., 2019). Indeed, walls of the gallery system that earthworms construct are hotspots for microbial decomposers (e.g., protozoa, springtails, enchytraeids, and heterotrophic protists), and exoenzyme production (Stromberger et al., 2012). For example, the permanent burrows created by the earthworm *Lumbricus terrestris* are a dynamic microenvironment for various enzymatic activities. Many hydrolase enzymes such as aminopeptidases, xylanase, phosphatases, or glucosidases display a higher catalytic activity in these biostructures than in the surrounding soil (Hoang et al., 2016a, 2016b). Therefore, there is a general consensus that earthworms promote multiple beneficial effects in agricultural soils such as the increase in biodiversity and fertility (Plaas et al., 2019), improvement of soil structure (Frazão et al., 2019), and control of soil-borne pathogens (Oldenburg et al., 2008).

Earthworms have also been used for remediating polluted soils by inducing microbial communities degrading pollutants (Rodriguez-Campos et al., 2014; Morillo and Villaverde, 2017), which produce detoxifying enzymes such as laccases, tyrosinases, peroxidases and carboxylesterases (Rao et al., 2014; Harms et al., 2011; Ba and Vinoth Kumar, 2017). Particularly, carboxylesterases are serine hydrolases that detoxify synthetic pyrethroid and organophosphorus (OP) pesticides by two ways: hydrolysis in the case of the former (Sogorb and Vilanova, 2002), and non-catalytic detoxification in the case of the OP pesticides (Chambers et al., 2010). Such a detoxification occurs by phosphorylation of the active site of carboxylesterase by the highly toxic metabolite oxon of OP pesticides, thus forming a stable enzyme-inhibitor complex and consequently inactivating the pesticide (Wheelock and Nakagawa, 2010). Recently, some studies suggest that this esterase activity is also involved in the degradation of polyester plastics (Zumstein et al., 2017) as its activity is generally found high in soils incubated with these polymers (Sakai et al., 2002; Yamamoto-Tamura et al., 2015). Therefore, increasing soil detoxifying enzymes via using earthworms could be an environmentally friendly strategy for promoting the natural attenuation capacity of soils against pollutant input.

This study was prompted to improve biochar reactivity using earthworms (*L. terrestris*) to increase soil carboxylesterase activity. We hypothesized that the biochar produced from co-pyrolyzing animal manure with plastic mulch films (hereafter referred as plastichars) increases carboxylesterase activity in amended soil due to adsorption of the enzyme activity onto the biochar surface. Our hypothesis is supported by some studies that indicated co-pyrolyzing blended organic feedstocks and plastic wastes not only increased the yield of resultant biochar compared to that obtained by pyrolyzing the feedstock alone (Tang et al., 2018), but also modifies the chemical properties of biochar surface during pyrolysis because of the high content of hydrogen and carbon in plastic polymers (Hassan et al., 2016; Zhang et al., 2016). Additionally, the use of plastics in pyrolyzing biomass represents a complementary end-of-life management of these synthetic materials, which are one of the major current environmental challenges (Uzoejinwa et al., 2018; Heidbreder et al., 2019). Therefore, the aims of the study were: 1) to assess the toxicity of a single biochar application to soil by comparing survival rate and body weight changes in earthworms incubated in biochar-amended soils and control (biochar free) soils, 2) to assess the impact of manure-derived biochars and plastichars on soil carboxylesterase activity, and to examine whether such effect depended on the presence of earthworms, 3) to determine if the enzyme activity was retained within the biochar particles, and 4) to assess the change in potential inherent capacity of biochar to bind extracellular enzymes during the incubation in soil. Our study focused on the enzyme carboxylesterase because of its pivotal role in OP inactivation (Sanchez-Hernandez et al., 2015), and its potential involvement in depolymerization of polyesters (Zumstein et al., 2017). Therefore, data in this study could be used to develop *in situ* bioremediation strategies that combine biological processes (microbial proliferation and exoenzyme production) promoted by earthworms, and biochar-based engineering strategies to concentrate and stabilize this enzymatic bioremediation potential.

2. Materials and methods

2.1. Biochar preparation

Partially dewatered swine solid (23% solid) was obtained from a solid-liquid separation system treating flushed manure from a 5600-head finishing swine farm in North Carolina (Ro et al., 2014). Poultry litter (75% solid) was obtained from a broiler farm in South Carolina. Several bundles of plastic mulch film (polyethylene-based mulch film, Polygro VIF, Safety Harbor, FL) used for growing watermelons were

collected from the USDA-ARS Vegetable Laboratory, Charleston, SC. The waste plastic mulch film was melted in a hot cooking oil container (191 °C), cooled, and cut into approximately 5 cm cubical form before blending with swine manure or chicken litter (2:1 plastic:manure, w/w ratio). Animal manure based biochars were blended with waste plastic mulch film cubes were fed into a commercial pyrolysis system (Aemerge, LLC, Indiana). The commercial pyrolysis system had a processing capacity of 68 kg/h and was operated at 538 °C for 30 min. Three different biochar samples were made using the commercial system; biochar made from swine manure alone (SM), biochar made from swine manure + plastic mulch film (SM+Plast), and biochar made from poultry litter blended with plastic mulch film (PL+Plast). Before making the biochar from poultry litter, the company closed the business. Therefore, we made the poultry litter biochar (PL) using a box furnace equipped with a gastight retort (Model 51662, Lindburg/MPH, Riverside, MI) in our laboratory with a standard operating protocol of 620 ºC and 2 h. Although any comparative statements about the PL biochar' enzyme activities could not be made due to its different pyrolysis conditions, we included the data because the 100% mortality of all earthworms exposed to the PL biochar with high pH is worthy information for readers.

2.2. Soil and earthworms

Soil used in this study was collected from the topsoil (15 cm) of a Norfolk loamy sand (Fine-loamy kaolinitic thermic Typic Kandiudults) at the USDA-ARS Coastal Plains Soil, Water, and Plant Research Center (Florence, SC, USA). Except for carbon (C) and nitrogen (N), carried out in our laboratory, the rest of the soil physico-chemical characterization was carried out at the Clemson University, Agricultural Service Laboratory, Clemson, SC. The chemical properties are: C 0.55%, N 0.05%, P (plant available) 25 mg kg^{-1}, K (potassium) 59 mg kg^{-1}, CEC (cation exchange capacity), 3.7 cmol kg^{-1}, pH 5.3. The particle size distribution is 81% sand, 17% silt, and 3% clay.

Earthworms (*L. terrestris*) were purchased from a local commercial supplier (Florence, SC, USA), and kept in a plastic box (25 l) containing the same soil used in the biochar activation trials, maintained in permanent dark and 22 ºC. We used a total of 60 adult earthworms (4.71 ± 1.14 g, mean±SD) in the bioactivation experiments.

2.3. Experimental design

Biochar (SM, PL, SM+Plast, and PL+Plast; 20 g dry mass each one) and 1 kg of wet soil were added to polyethylene plastic bags (17.7 cm × 18.8 cm, Ziploc® brand bags, Johnson, SC) to yield a biochar application rate content of 2.5% (w/w, dry mass). The bags were agitated by hand to distribute biochar particles in soil as evenly as possible. The spiking procedure and exposure set up were similar to that described by Prendergast-Miller et al. (2019). Biochar-amended soils were kept for 24 h in dark and 22 ºC for equilibration. Afterwards, we added two adult earthworms in each plastic bag (n = 8 replicates per treatment), which were previously depurated for 48 h to empty the gastrointestinal tract and thereby record body weight. All plastic bags were vertically placed inside cardboard boxes to form a sandwich-like structure with the scope of avoiding destruction of permanent burrows created by earthworms. Earthworms were fed every 1–2 weeks by adding 2 g of litter on the surface of each test container. The plastic bags were sealed using the zipper to avoid worm escaping, and four holes were made in the headspace of bags to allow air exchange. After 30 d of incubation, plastic bags were laterally open, avoiding alteration of the burrow system generated by the earthworms. The earthworms were removed, rinsed in tap water and kept in Petri dishes for 48 h to collect fresh casts. Subsequently, the earthworm weight was recorded again, after 48 h of depuration, to assess body weight change during the incubation period (30 d).

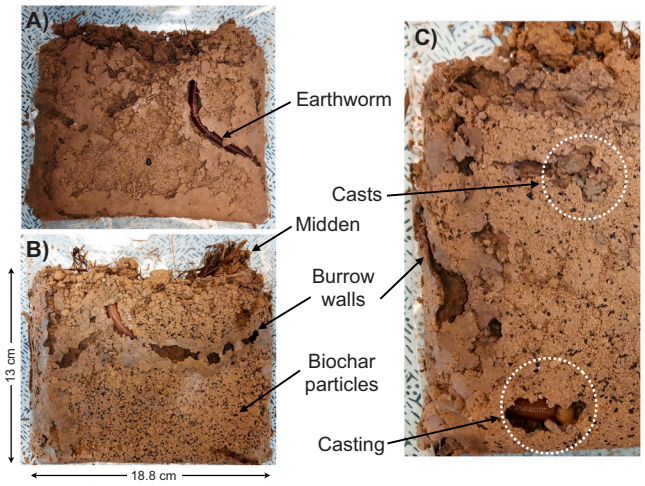

Fig. 1. Some pictures of microcosms used in this study. A) Control (biochar-free) soil inoculated with earthworms. B) Biochar-amended soil inoculated with earthworms. C) Bioturbation of soil by *Lumbricus terrestris*.
Photos by Juan C. Sanchez-Hernandez.

2.4. Sample collection

Samples of burrow walls and non-bioturbed soil (i.e., the soil non biologically reworking by the action of earthworms (Meysman et al., 2006)) were collected from two levels in our microcosm system, at the surface (0–2 cm) and the bottom (10–13 cm) layers (Fig. 1). Samples of burrow walls (~1 cm around wall) were carefully collected using a spatula, and litter buried by earthworms that appeared in the burrow walls was avoided. Non-bioturbed soil was taken from areas of the microcosm that were not altered by earthworms, and these samples were taken as control (Fig. 1). Casts deployed on soil surface were also collected, and refer to "aging casts" in order to distinguish this material from the casts obtained from earthworm incubation in Petri dishes (48 h), which were referred as "fresh casts". All samples were kept at 4 ºC until enzyme measurements that were performed within the week following sample collection.

2.5. Carboxylesterase activity in soil and earthworm cast

Carboxylesterase activity (EC, 3.1.1.1) was measured in aqueous suspensions obtained from casts and soil samples, which were prepared as described in Sanchez-Hernandez (2018). One gram of wet soil was dispersed in distilled water (1:25, w/v) and shaken for 30 min at room temperature (22 °C). In the case of cast-water suspensions (0.25 g cast:1 ml distilled water), it was mixed for only 1 min using a vortex (3000 rpm), which was enough to obtain a homogenous suspension of casts.

Carboxylesterase activity was measured in both soil- and cast-water suspensions by incubation of aliquots (200 µl) for 15 min (20 ºC and dark) in 1.5 ml Eppendorf® tubes containing 375 µl of Tris-HCl 0.1 M (pH = 8.0) buffer and 20 µl 4-nitrophenyl butyrate (2 mM, final concentration) (Sanchez-Hernandez, 2018). The reaction was stopped by adding 250 µl of a solution containing 2% (w/v) sodium dodecyl sulfate, and 2% (w/v) tris (hydroxylmethyl) aminomethane, which enhanced the yellow color intensity of the formed 4-nitrophenolate (Reymond et al., 2009). Tubes were centrifuged at 10,000 rpm for 1 min, and supernatants (250 µl) were poured in 96-well bottom-flat microplates. Absorbance was read at 405 nm using a BioTek® microplate reader (BioTek Instruments, Inc., Highland Park, Winooski, VT, USA). The enzyme activity was expressed as µmol of nitrophenolate formed h^{-1} g^{-1} dry soil (or dry cast). Calibration curves were constructed with serial concentrations of 4-nitrophenol which was added in Eppendorf® tubes containing Tris buffer and soil (or cast) to correct for adsorption of the

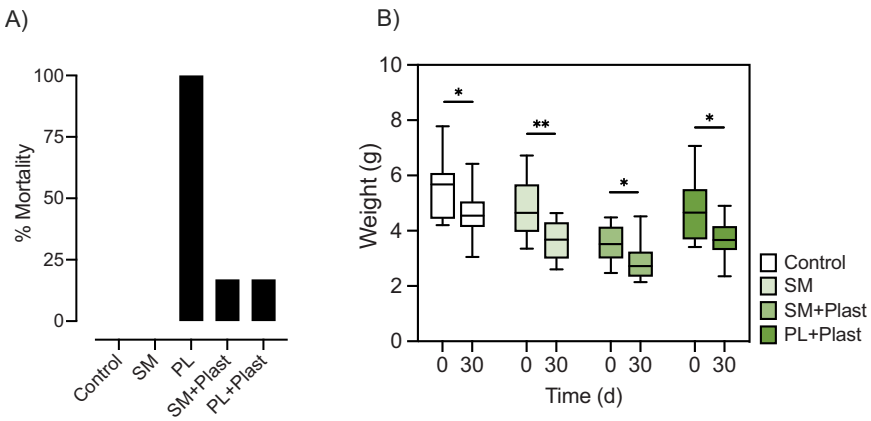

Fig. 2. A) Earthworm mortality after 30 d of incubation in biochar-amended soils. B) Variation of earthworm body weight during 30-d exposure to biochar-amended soils. Box plots indicate the median, the 25th and 75th percentiles (box edges), and the range (whiskers). SM = swine manure-derived biochar, PL = poultry litter-derived biochar, SM+Plast = biochar produced from blended swine manure and mulch films, PL+Plast = biochar produced from blended poultry litter and mulch films. $^{*}p < 0.05$, $^{**}p < 0.005$ (Student's *t* test).

reaction product to soil or cast particles and colloids. Enzymatic measurements of each sample were done by triplicate, and sample absorbance was corrected by background absorbance produced by control (substrate-free).

2.6. Carboxylesterase activity in biochar particles

The esterase activity was also measured with the biochar particles recovered from the soils. The procedure to separate biochar from soil was that described by Lin et al. (2012). The recovered biochar was rinsed multiple times in water to remove fine particles of soil and plant debris particulates. The recovered biochar was kept at 4ºC until the measurement of the enzyme activity within one week of collection.

Carboxylesterase activity was measured in biochar particles as described above with minor modifications. Biochar particles (20–40 mg wet weight) was incubated for 2 min (20 ºC and dark) with 480 µl 0.1 M Tris-HCl buffer (pH = 8.0) and 20 µl 4-nitrophenyl butyrate (2 mM). The time of incubation was lower than that for soil or cast samples because of the high enzyme activity retained in biochar. The subsequent steps of the enzyme assay were the same as described above, however, the calibration curve was constructed in the presence of biochar to correct for adsorption of the reaction product to biochar. The biochar particles used in enzyme assay were dried in thermoblock (80 ºC, 2 h) to obtain the dry mass, and the enzyme activity was expressed as µmol of nitrophenolate formed h^{-1} g^{-1} dry biochar.

2.7. Carboxylesterase adsorption to biochar

Our last aim was to investigate whether biochar capacity to adsorb the enzyme was altered by incubation in soil treated with earthworms. We used a purified carboxylesterase enzyme (24 U mg^{-1} solid, CAS number 9016–18–6, Sigma-Aldrich, USA) to evaluate the adsorption capacity of fresh biochar (no incubation in soil) and biochar recovered from both the burrow walls and non-bioturbed areas of our microcosm units. Adsorption experiments were performed following the method by Jaiswal et al. (2018). The enzyme was prepared at a concentration of 1 U (µmol min^{-1}) ml^{-1} in 50 mM Na-acetate buffer (pH = 5.0). Biochar (50 mg) was incubated in 500 µl of the enzyme solution using Eppendorf® tubes for 30 min at room temperature (22 ºC). Previous incubation assays revealed that this time of incubation was enough to obtain maximum enzyme activity in biochar particles (Sanchez-Hernandez, 2018). Subsequently, the enzyme solution was removed, and biochar particles were rinsed 4 times with distilled water. These biochar particles with adsorbed carboxylesterase enzyme were then used for measuring the activity following the biochar procedure described above.

2.8. Spectroscopic analysis of biochar

2.8.1. Thermogravimetric analysis-fourier transform infrared (TGA-FTIR)

The TGA–FTIR experiment was conducted by a TA Instruments Q500 thermogravimetric analyzer and a Bruker Tensor 27 spectrometer. In this experiment, 5–8 mg of each sample was heated between 20 and 500 °C in the thermogravimetric analysis at a rate of 10 °C/min and under a nitrogen flow rate of 60 ml min^{1}. The resulted volatile decomposition products then traveled through a transfer line to reach the gas cell of the FTIR spectrometer. Although the TGA experiment investigated the thermal degradation of the samples from 20 ° to 800 °C, the FTIR experiment examined the gaseous products released during the main degradation ranging from 100 ° to 500 °C. Both transfer line and gas cell were maintained at 200 °C. When the evolved gases reached the gas cell, they were analyzed by a liquid-nitrogen cooled MCT detector which is equipped with Zn Se window. The gas components were then recorded as the absorption peaks in the 4000–600 cm^{-1} region at a resolution of four wavenumbers. This data was obtained at every 5 °C increment along TGA heating profile and there was a 30 s delay between the timed measurements for the FTIR. When the experiment was completed, the data was analyzed using an Opus software which measures the intensity of the absorption band (representing the functional groups) as a function of temperature. For analytical purposes, an OriginLab 9 software was utilized to retain the three-dimensional images of the FTIR spectra.

2.8.2. Hyperspectral image analysis of biochar

Hyperspectral imaging is state-of-the-art technology that provides both spectral and spatial information of objects simultaneously at one scan with a camera attached with imaging spectroscopy. In this study, an extended visible/near-infrared (EVNIR) hyperspectral imaging system (Micro-Hyperspec, Headwall Photonics, Fitchburg, MA) was used for acquiring reflectance hyperspectral images between 600 and 1700 nm with quartz tungsten-halogen lighting source from biochars, which were contained in 24 wells sample holder. Images were collected by a line-scan mode within 60 s and saved as hypercube format followed by spectral image analysis with an environment for visualizing images (ENVI, Harris Geospatial Solutions, Broomfield, CO) for regions of interest (ROI) data collection and The Unscrambler (Camo Analytics, Oslo, Norway) software for principal component analysis (PCA).

2.9. Chemical properties of biochars

The biochars were characterized for their pH, C, N, S, P, Mg, Ca, and Part 503 metals (As, Cr, Cu, Pb, Mo, Ni, Se, Zn). The pH value of each biochar sample was estimated in triplicate at 5% (w/v) using deionized water after shaking for 90 min and let it sit for 30 min. Single estimates for C, N, S, P, and Part 503 pollutants for each biochar were measured on

Table 1
Selected chemical properties of biochars.

	Feedstock[a]		Biochar[a]			
	SM	PL	SM	PL	SM+Plast	PL+Plast
pH	6.51	7.30	7.38	10.47	7.69	8.02
C (%$_{db}$)	37.61	34.40	31.50	43.30	37.91	39.16
N (%$_{db}$)	4.58	3.24	2.01	3.01	1.22	1.03
S (%$_{db}$)	1.18	0.80	0.24	0.78	0.33	0.22
P (mg/g)	31.2	17.3	28,1	43.1	8.6	7.7
Mg (mg/g)	16.8	8.5	23.0	17.6	4.8	4.3
Ca (mg/g)	32.5	30.2	34.7	59.6	22.3	23.0
As (ug/g)	2.13	3.48	2.45	3.12	2.09	2.01
Cr (ug/g)	14.15	4.17	59.70	9.50	41.49	70.51
Cu (ug/g)	1449	380	950	825	254	214
Pb (ug/g)	2.22	1.19	2.53	1.33	5.04	5.36
Mo (ug/g)	16.44	7.78	28.44	12.83	26.80	29.01
Ni (ug/g)	14.65	8.48	49.37	16.81	25.41	29.24
Se (ug/g)	6.09	1.18	3.92	4.39	0.00	0.71
Zn (ug/g)	3340	593	2123	1913	401	311

[a] SM=swine manure, PL=poultry litter, SM+Plast=blended SM and plastic mulch film, PL+Plast=blended PL and plastic mulch film.

dried basis by Arizona Laboratory for Emerging Contaminants (ALEC, https://www.alec.arizona.edu/): C, N, S using flash combustion, GC separation, and a thermal conductivity detector; P, Mg, Ca, and Part 503 pollutants using the modified USEPA method 3051 and inductively coupled plasma mass spectrometry (ICP-MS).

2.10. Data analysis

Differences in the means of earthworm body weight were detected by the Student's *t* test, whereas the impact of biochar type on the enzyme activity was evaluated by one-way ANOVA followed by *post hoc* Tukey test, after testing data for normality (Shapiro–Wilk test) and homoscedasticity (Levene test). Comparisons of carboxylesterase activity between biochar particles (n = 4 replicates) were run using the nonparametric Kruskal–Wallis test followed by the *post hoc* Mann–Whitney *U* test.

3. Results and discussion

3.1. Impact of biochar on earthworms

In general, biochar at the dose of 2.5% w/w (dry mass) was not toxic to *L. terrestris* during the incubation time (Fig. 2A). The exception was the PL biochar, which caused a 100% mortality after the first week of incubation. This high toxicity still remained even after the biochar was acetone-washed to remove potential organic pollutants that could be generated during pyrolysis. In search for the causes, we investigated if the PL biochar contained higher levels of Part 503 pollutants (As, Cr, Cu, Pb, Mo, Ni, Se, and Zn) than their ceiling concentrations, regulatory limits for biosolids for land application (Table 1). The concentrations of the Part 503 pollutants were all well below the ceiling limits permissible for biosolids to be applied to land (USEPA, 1994). The C, N, and S concentrations of the PL biochar were not much different from the other three biochars (Table 1). However, pH of the PL biochar was more than 2 units higher (pH 10.47) than the other biochars. The marked difference in pH value could be due to the temperature of pyrolysis. The PL biochar was made in a laboratory furnace at 620 °C for 2 h, whereas the other three biochars were obtained from a commercial pyrolysis system with lower temperature and shorter residence time (538 °C for 30 min). Similar increase in pH of PL biochar with pyrolysis temperature was reported by Cantrell et al. (2012). It is well known that higher pyrolysis temperature results in biochars with higher concentrations of Mg and Ca causing the increase in pH (Novak et al., 2014). The concentrations of the two alkali-earth metals, especially Ca, were substantially higher in PL biochar than other three biochars. Therefore, we suspected the high basicity of the PL biochar caused the high mortality of earthworms. Indeed, optimal soil pH values for laboratory culturing of soil dwelling earthworm species ranges between 4.5 and 7 (Lowe and Butt, 2005).

Earthworm body weight significantly decreased ($p < 0.05$, Student's *t* test) in all treatments after 30 d of incubation (Fig. 2B). However,

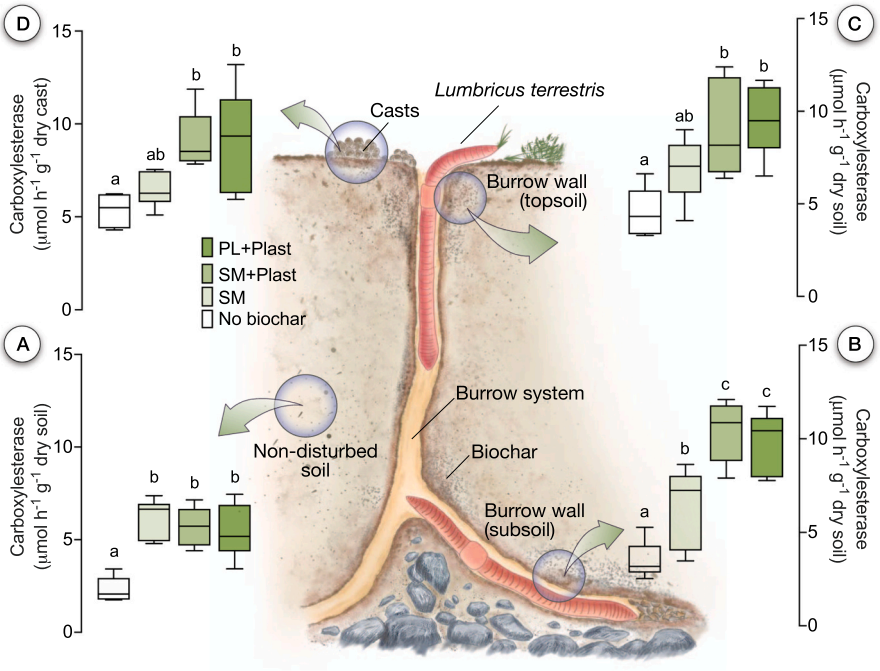

Fig. 3. Soil carboxylesterase activity in biochar-amended soils after 30 d of incubation in the presence of earthworms (*Lumbricus terrestris*), collected from burrow walls (topsoil and subsoil, plots B and C), casts (plot D), and non-bioturbed soil (plot A). Box plots indicate the median, the 25th and 75th percentiles (box edges), and the range (whiskers). Biochar abbreviatures as in Fig. 2. Different letters denote significant differences between treatments ($p < 0.05$, *post hoc* Tukey test).

control earthworms also experienced a significant weight loss (17.4%). Thus, the loss of earthworm weight could not be attributed to biochar toxicity. Furthermore, the percentage of weight loss in control earthworms was rather lower than the validity criterium (30% weight loss) in long-term ecotoxicological experiments with earthworms (Fründ et al., 2010). Therefore, it can be concluded that the dose of 2.5% w/w of both SM and plastichars was harmless to earthworms, at least during the environmental conditions and duration of the assay. This finding also agreed with other related studies which showed biochars such as wheat straw-derived biochar (Elliston and Oliver, 2020; Zhang et al., 2019), spent coffee ground- and pine needle-derived biochars (Sanchez-Hernandez, 2018), or sewage sludge-derived biochar (Paz-Ferreiro et al., 2015), at least at doses <5% w/w, were not toxic to earthworms.

3.2. Carboxylesterase activity in soil and casts

The mean esterase activity of biochar-free (control), non-bioturbed soil was 3.62 ± 0.70 μmol h^{-1} g^{-1} dry soil (mean±SD, n = 6). The addition of biochar caused a significant increase (2.5-fold) of soil carboxylesterase activity in the non-bioturbed soils ($F_{3,19} = 22.2$, $p < 0.001$) respect to biochar-free soil. However, the increase of the soil enzyme activity did not depend on biochar type (Fig. 3A). This result contrasts with our previous research data (Sanchez-Hernandez, 2018), in which the addition of pine needle- or spent coffee ground-derived biochar (2.5% w/w) decreased or unaltered soil carboxylesterase activity. The reason for such discrepancy could be in the sorption of substrates used for enzyme assay to biochar. For example, Bailey et al. (2011) found that adsorption of colorimetric substrates onto the biochar surface underestimated the soil enzyme activity. In the current study, we used an ester substrate (4-nitrophenyl butyrate) which should have a lower adsorption activity to biochar than the substrate (1-naphthyl butyrate) used in our previous study (Sanchez-Hernandez, 2018). We evaluated such assumption by comparing the values of some properties related to the adsorption activity (Log K_{OC} and water solubility for 4-nitrophenyl butyrate = 2.47 and 145.5 mg ml^{-1} at 25 ºC; Log K_{OC} and water solubility for 1-naphthyl butyrate = 3.47 and 23.24 mg ml^{-1} at 25 ºC; data taken from ChemSpider database [www.chemspider.com], and values estimated by the Environmental Protection Agency's EPISuite™). Therefore, 4-nitrophenyl butyrate should be more available to the active site of carboxylesterase than 1-naphthyl butyrate because of the lower organic carbon partitioning coefficient (K_{OC}) and higher water solubility of the former.

Bioturbation of biochar-free soils by *L. terrestris* caused a significant increase of carboxylesterase activity in the burrow walls respect to non-disturbed soil ($F_{2,15} = 6.84$, $p = 0.008$). This observation agree with other related studies that demonstrated the stimulation effect of *L. terrestris* on soil microbial proliferation and exoenzyme production in the burrow linings (Hoang et al., 2016a, 2016b; Sanchez-Hernandez et al., 2019). We found a synergistic effect of earthworms and biochar, which depended on the type of biochar. The enzyme activity was significantly higher in the burrow walls of both subsoil (Fig. 3B) and topsoil (Fig. 3C) of biochar-amended soils respect to the non-bioturbed soil ($F_{2,48} = 6.40$, $p = 0.003$). But more interestingly, the increase of activity was higher with plastichars than in biochar made from pyrolyzing swine manure alone ($F_{2,42} = 9.1$, $p < 0.001$).

Current results clearly show that the drilosphere –the soil environment under the influence of earthworms (Andriuzzi et al., 2013)– of biochar-amended soils displayed a significantly higher carboxylesterase activity than non-bioturbed soil. The mechanism how biochar modulates soil enzyme activity is not still clearly understood. However, empiric data reveal a synergistic effect of biochar and earthworms on soil extracellular enzyme activities (Sanchez-Hernandez, 2018, 2019; Paz-Ferreiro et al., 2014, 2015). One of the potential mechanisms for this synergistic effect could be the bioactivation of biochar in the gastrointestinal microenvironment of earthworms, and further deposition of biochar-rich casts on the burrow walls. Indeed, we found the

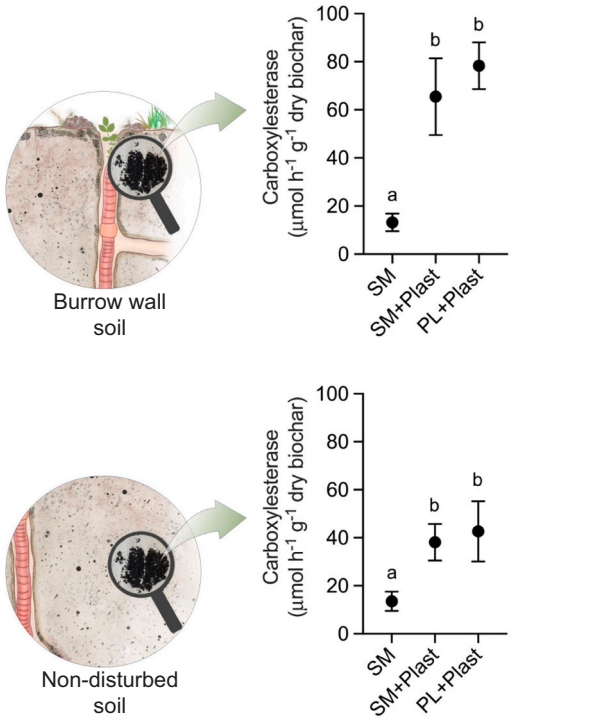

Fig. 4. Soil carboxylesterase activity in biochar particles recovered after 30 d of soil incubation in the presence of earthworms (*Lumbricus terrestris*). Particles were collected from burrow walls and non-bioturbed soil. Different letters denote significant differences between biochars (Mann–Whitney *U* test, $p < 0.05$). Biochar abbreviatures as in Fig. 2.

burrow walls in the biochar-amended soils dark grey color after the incubation period (Fig. 1C). Similar observations were reported by other researchers (Topoliantz and Ponge, 2003, 2005), who showed dark grey casts egested by earthworms exposed to biochar. To provide further evidence that the earthworm gastrointestinal tract contributed to soil carboxylesterase activity, we measured the enzyme activity in the casts collected from the soil surface (aged casts), and casts obtained after keeping earthworms in Petri dishes for 48 h (fresh casts). In aged casts, the activity followed the same trend than that found in the burrow walls. The highest activity levels were found in the casts collected from SM+Plast and PL+Plast treatments (Fig. 3D). However, differences in the enzyme activity of fresh casts were not significant between treatments ($F_{3,19} = 0.014$, $p = 0.62$). These results suggested that the triggering of carboxylesterase activity in biochar-treated soils probably occurred via external microbial-dependent mechanisms instead of digestive enzymes derived from gut symbionts and earthworm themselves.

Soil carboxylesterase activity was not different between subsoil and topsoil burrow walls (Fig. 3B and C). In the literature, soil extracellular enzyme activities generally decreases with soil depth due to a lower microbial activity and nutrient concentration in the subsoil than that of the topsoil (Hoang et al., 2017). Such trend has been also reported in the walls of burrow system created by *L. terrestris*. For example, enzymes such as cellobiohydrolase, β-glucosidase, xylanasa, or chitinase decreased with soil depth (Hoang et al., 2016a). However, our results clearly indicated that synergistic effects from earthworms and biochar kept carboxylesterase activity in the subsoil at levels comparable to that of the topsoil. Immobilization of the enzyme onto the biochar surface was postulated as the most plausible mechanism of the high enzyme activity observed in subsoil burrow walls (Sanchez-Hernandez et al., 2019).

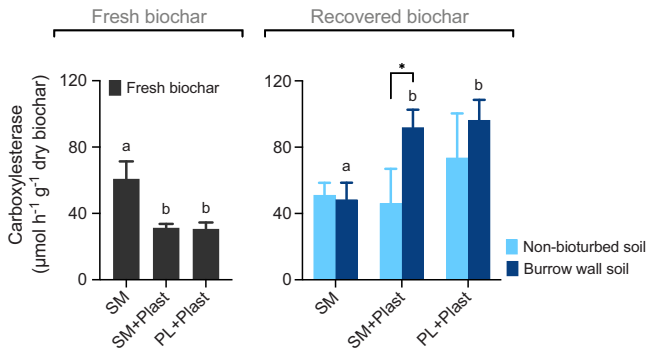

Fig. 5. Enzyme activity in fresh and soil-incubated (recovered) biochars after 30 min incubation with purified carboxylesterase (Mean±SD, n = 4). Different letters denote significant differences between biochar type, whereas asterisks indicate significant differences between biochar location in the microcosm (Mann–Whitney *U* test, $p < 0.05$). Biochar abbreviations as in Fig. 2.

3.3. Carboxylesterase activity in recovered biochar particles

We further explored whether the carboxylesterase activity increase in the burrow walls was due to enzyme binding onto the biochar surface. In the non-bioturbed soil, carboxylesterase activity associated to plastichars was significantly higher than that in the biochar produced from swine manure alone ($F_{2,14} = 18.5$, $p < 0.001$) (Fig. 4). Biochar particles recovered from the burrow walls also showed the same pattern of enzyme variation observed in the non-bioturbed soils ($F_{2,14} = 63.5$, $p < 0.001$). However, whereas there was no statistical difference in the esterase activity of the SM biochar between non-bioturbed and burrow wall soils ($p = 0.88$, Student's *t* test), the activity was significantly higher in the burrow wall plastichars than those from the non-bioturbed soils ($p = 0.009$ for SM+Plast biochar, and $p < 0.001$ for PL+Plast biochar).

Our results show that earthworms increased the retention of carboxylesterase activity onto biochar surface. This finding corroborated previous data from our research group, which evidenced an increase of soil enzyme activities (carboxylesterase, phosphatase, β-glucosidase and arylsulfatase) in both pine needle- and spent coffee ground-derived biochars after two months of soil incubation in the presence of earthworms (Sanchez-Hernandez, 2018). Similar binding of extracellular enzymes to biochar has been also investigated by others. For example, the studies by Cea et al. (2019) and Noritomi (2018) suggested that biochar is a suitable carrier for binding and even increase the activity of enzymes such as lipase and α-chymotrypsin, which may be further used for biotechnological purposes. However, not all enzymes bound to biochar keep their catalytic activity. For example, cellulase and pectinase from soilborne pathogens, were bound to biochar, but the enzymes lost their catalytic activity (Jaiswal et al., 2018). Similarly, the enzymes β-glucosidase and phosphatase partially were inactivated after binding on pine-derived biochar (Foster et al., 2018). Taken altogether, these cited studies and the data in the current study evidence that enzymatic bioactivation of biochar depends on both enzyme type and biochar type.

3.4. Potential enzymatic activity of the co-pyrolyzed biochars

In vitro incubation of both fresh and soil-incubated biochars with purified carboxylesterase revealed that earthworms promoted the enzymatic activities of plastichars. Fresh SM biochar retained a higher enzyme activity ($p < 0.05$, Mann–Whitney *U* test) compare to that of the

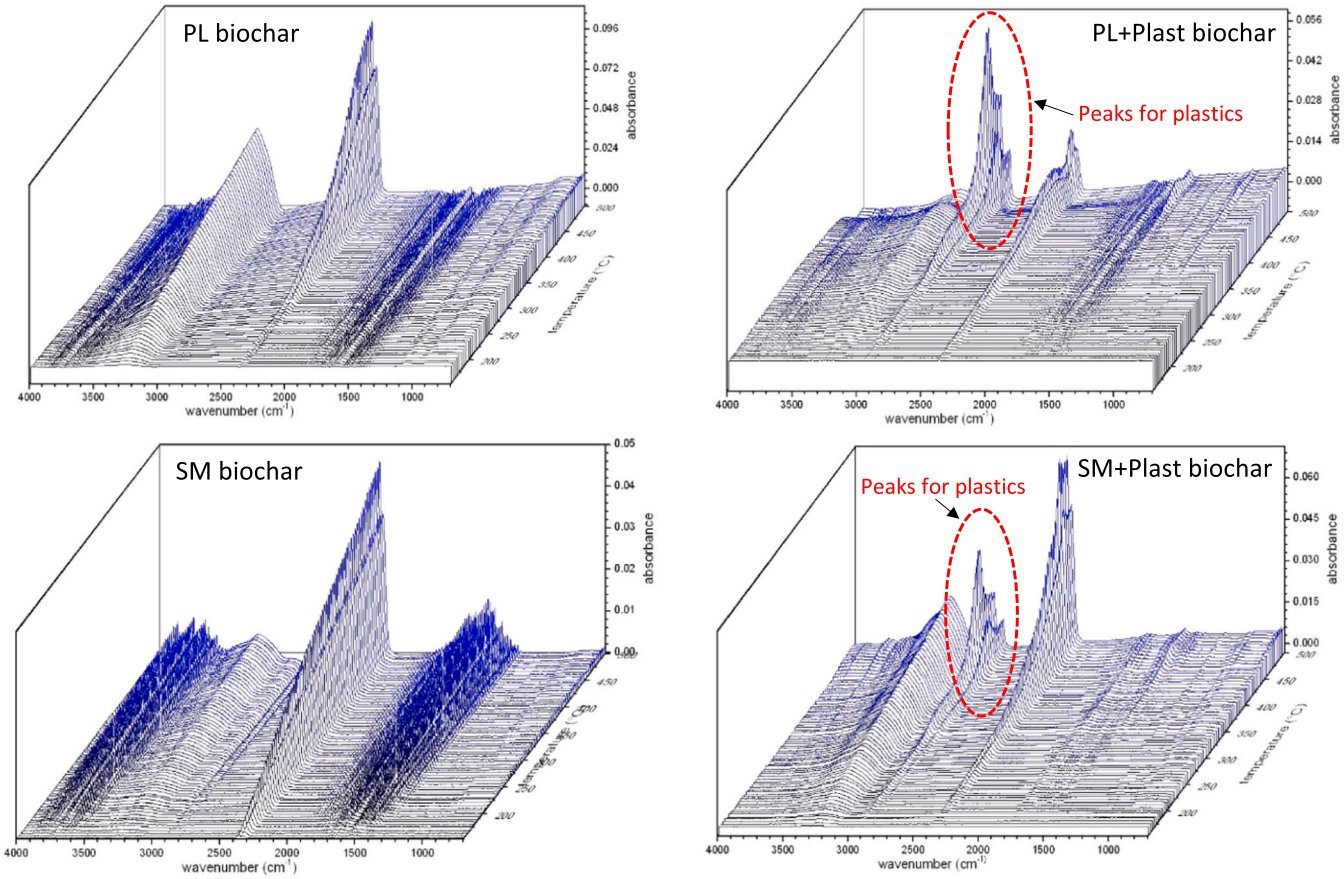

Fig. 6. Absorbance spectra stack plots (3D TGA-FTIR) from the pyrolysis of poultry litter (PL) biochar, swine manure (SM) biochar, and biochars produced from blended poultry litter and plastics (PL+Plast) and swine manure and plastics (SM+Plast).

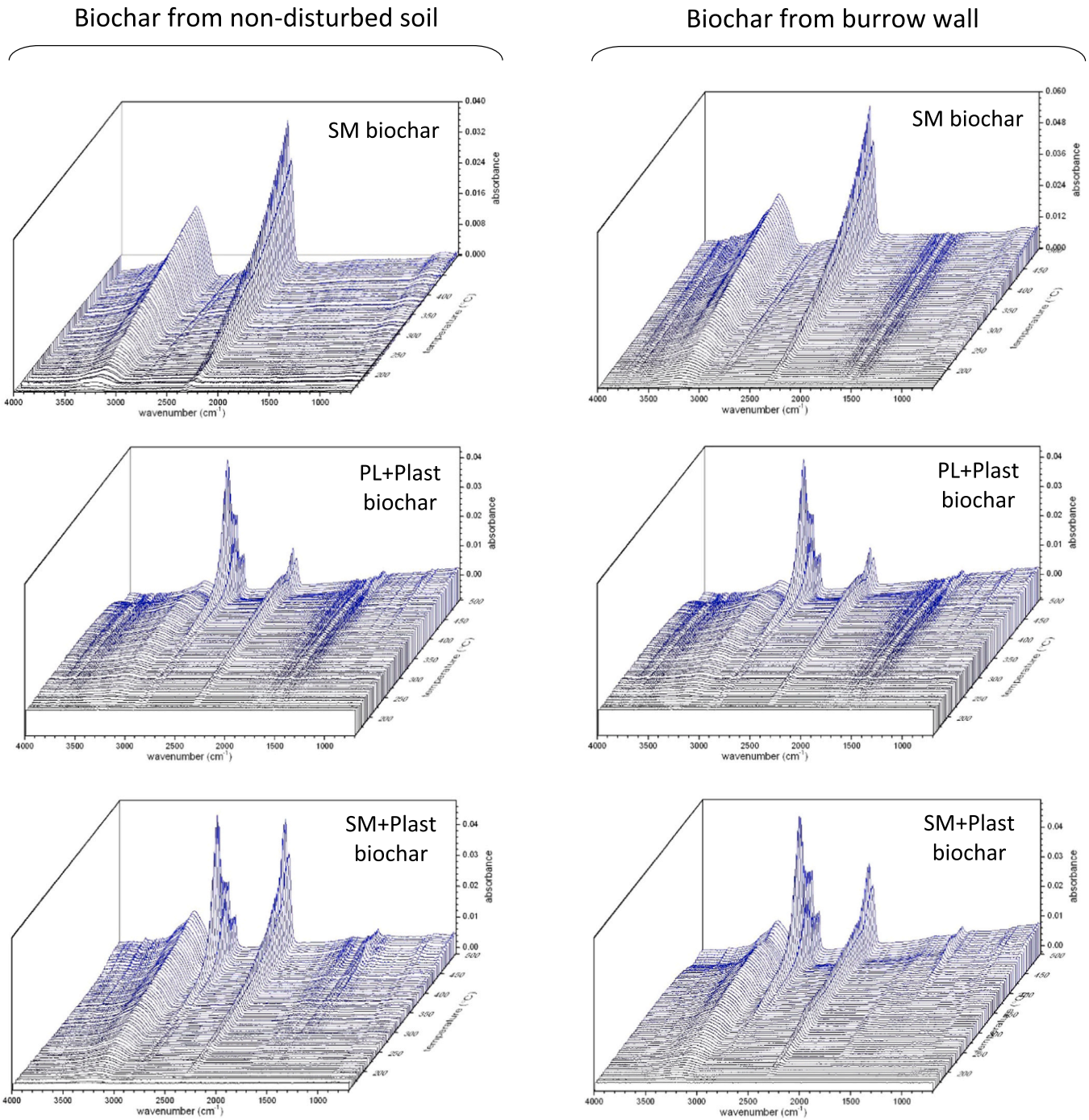

Fig. 7. Absorbance spectra stack plots (3D TGA-FTIR) from the pyrolysis of biochars collected from non-disturbed soil and burrow walls after 30 d of incubation. Biochar abbreviations as in Fig. 2.

fresh plastichars (Fig. 5). However, when biochars were incubated in soil for 30 d, the esterase activity of plastichars increased markedly compared to that of fresh co-pyrolyzed biochars. Moreover, such an increase was higher for plastichars recovered from the burrow walls. These findings suggest that earthworm intervention was essential to improve the enzymatic activation of biochars produced from blended mulch films and manure.

Structural and physicochemical properties of biochar play an important role in the adsorption of extracellular enzymes. Particularly, high open porosity and pore size as well as the abundance of surface functional groups (carboxylic, carbonyl, etc.) favor the retention of enzymes in biochar (Pandey et al., 2020). Additionally, hydrophobicity of biochar surface could favor adsorption of enzymes depending on the soil pH and isoelectric point of the enzyme (Sanchez-Hernandez, 2018; Elzobair et al., 2016). In our study, carboxylesterase activity was higher in plastichars than in SM biochar. The most plausible explanation for this observation is the physicochemical and structural alterations of biochar induced by the addition of plastic films in the co-pyrolysis. Plastichars had a higher abundance of surface functional groups (carbonyl) than biochars produced from manure alone, thus suggesting an improved affinity of biochar for carboxylesterase through these chemical ligands. Moreover, alterations of plastichar surface respect to that of manure-derived biochar could have favored the adsorption of carboxylesterase. For example, Chen et al. (2017) showed that co-

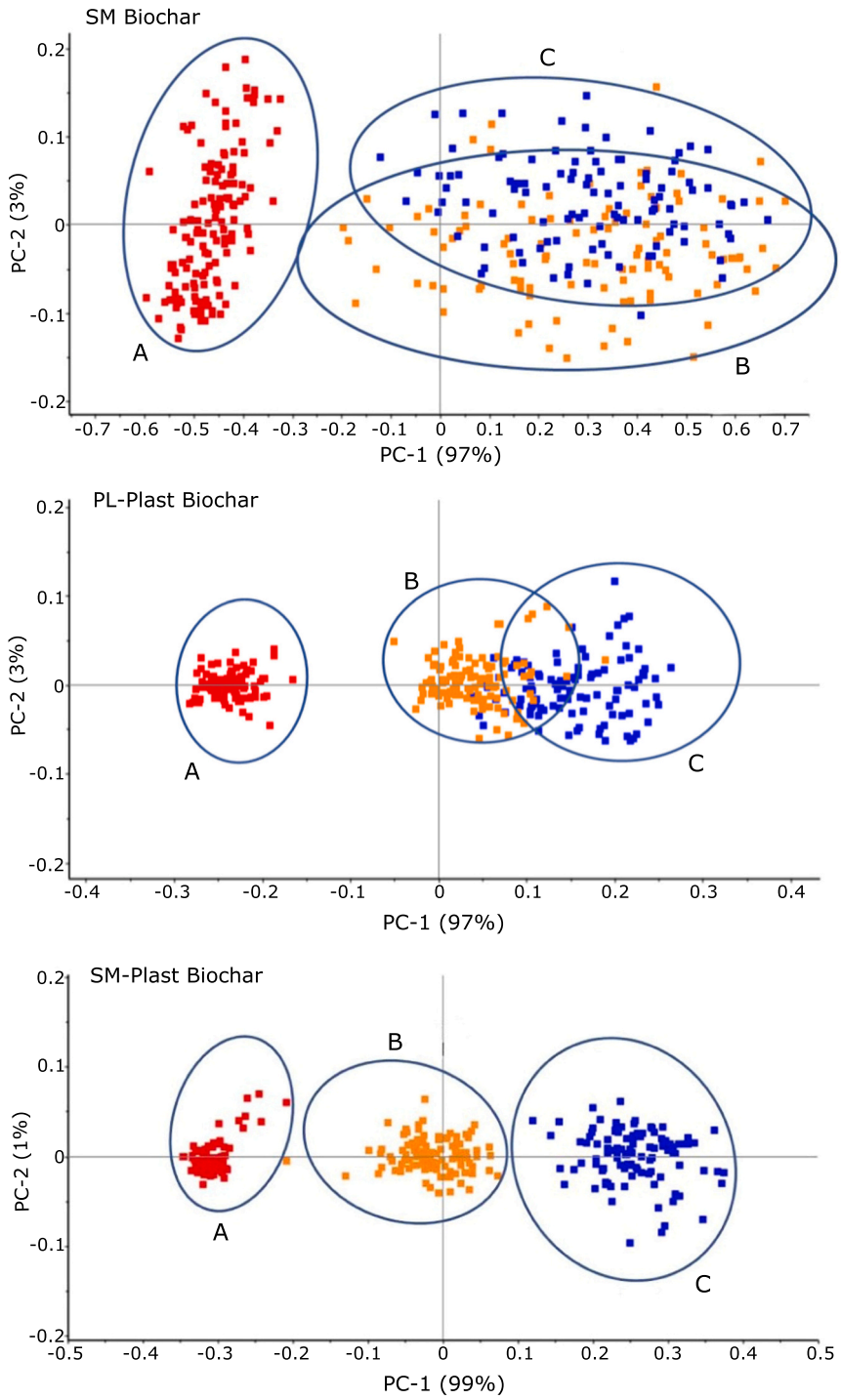

Fig. 8. PCA score plots from the hyperspectral reflectance images for clustering biochars sample group. A=fresh biochar, B=biochar collected from undisturbed soil, and C=biochar collected from the burrows (wall) created by the earthworms.

pyrolysis of paulownia wood with different types of plastics markedly altered the biochar morphology.

3.5. Characteristics of TGA-FTIR and hyperspectral images of biochars

Characteristics of different biochars especially the volatile compounds evolved from pyrolysis were compared using TGA-FTIR. The volatile compounds evolved from pyrolyzing chicken litter, swine manure and their blended with mulch films, and the 3D TGA-FTIR spectra are depicted in Fig. 6. The spectra indicated the evolution of gas products during sample pyrolysis as functions of wave number and temperature. From 25–200 °C, IR spectra of gas products were similar with low intensities. But, above 200 °C, the absorption intensities of the IR spectral peaks begin to intense and reach maximum near 460–490 °C, indicating the largest gas releases in this temperature range. In Fig. 6, PL, PL+Plast, SM, and SM+Plast all displayed absorbance peaks in the region 3600–3200 cm^{-1} and 2400–2250 cm^{-1}. Characteristic peaks at 3600–3200 cm^{-1} region is representative N–H and O–H bonds, whereas strong absorbance peaks in the 2400–2250 cm^{-1} is indicative of the existence of CO_2 due to asymmetric stretching of the carbonyl group (C=O). In both plastichars, the absorbance spectra showed similar feature. The strong absorbance peaks near 2950–2850 cm^{-1} and

1500 cm^{-1} are assigned alkanes and alkenes such as the bands of stretching of CH_3–, =CH– and –C=C–, bending of –C=CH_2 and –CH_2–. This result indicated that the main reaction is depolymerization of plastic polymer. The 3D TGA-FTIR spectra for undisturbed soil, and burrow walls of the treatments PL+Plast, SM+Plast, and SM biochar are displayed in Fig. 7. All biochars showed similar spectra regardless of their location in the microcosm, i.e., non-disturbed soil and burrow walls.

A principal component analysis of the hyperspectral reflectance images allowed us to cluster the biochars according to their location in the microcosm as well (Fig. 8). Fresh biochars and those collected from non-disturbed soils and the burrow walls after 30d of incubation were clearly separated, and such clustering was more evident for the plastichars. These results suggest structural and chemical modifications of biochar after incubation in soils inoculated with earthworms, which would explain the higher adsorption capacity of plastichars to retain enzymes than manure-derived biochar.

4. Conclusions

Our study showed that co-application of the earthworm *L. terrestris* and plastichar caused an increase in the production of carboxylesterase activity along the burrow walls and in the casts that were deployed on topsoil by the earthworms. High esterase activity was also observed in the subsoil burrow walls probably because of crushed-cast accumulation mixed with biochar. The enhanced soil esterase activity was attributed to adsorption of extracellular enzymes onto biochar surface. Interestingly, biochars produced from co-pyrolyzing blended plastic mulch film wastes and animal manures promoted higher enzyme activity; a significant finding that was corroborated by *in vitro* treatment of biochars with purified carboxylesterase. Results in this study significantly advanced our understanding of biochar enzymatic activation in soil, and provided a basis for further development of an *in situ* soil bioremediation method using extracellular enzymes. The use of earthworms as biological vectors in biochar activation defines this potential bioremediation strategy as environmentally friendly and cost-effective.

CRediT authorship contribution statement

JCS-H designed experiments, collected and analyzed data, and wrote draft manuscript. KSR conceived the idea, coordinated project, analyzed and interpreted data, participated in writing and revising manuscript, and final approved the version to be published. AAS performed chemical analyses of biochar, analyzed and interpreted data, and participated in writing and revising manuscript. SC performed FTIR analyses of biochar, interpreted data, and participated in writing and revising manuscript. BP performed hyperspectral analyses of biochar, interpreted data, and participated in writing and revising manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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