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Earthworm-biochar interactions: A laboratory trial using *Pontoscolex corethrurus*

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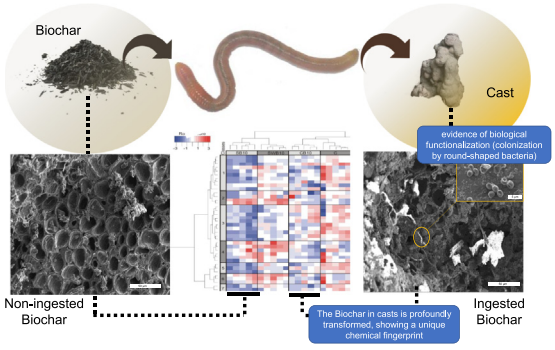
HIGHLIGHTS

- Biochar did not negatively affect *P. corethrurus* even at the highest dose (10% w/w).
- Py-GC–MS detected various pyrolysis-produced biomarker compounds in the biochar.
- Biochar in casts shows novel chemical fingerprints and a structural breakdown.
- Passage through earthworm guts promotes biochar functionalization.
- Earthworm biological roles should be considered in field biochar application.

GRAPHICAL ABSTRACT



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ABSTRACT

Biochar application can improve soil quality, but at high rates may cause negative effects on soil organisms such as earthworms. Conversely, as ecosystem engineers and catalysts of microbial activity, earthworms can also affect soil quality and interact with biochar, though little is known concerning endogeic earthworm species interactions with biochar. Hence, a standard ecotoxicological laboratory trial was undertaken to assess the impact of Brazil-nut husk biochar application (0, 2.5, 5 and 10% w/w) on the endogeic cosmopolitan species *Pontoscolex corethrurus*, and the effects of bioturbation and gut passage on biochar chemical characteristics (assessed using pyrolysis coupled with gas chromatography and mass spectrometry) and morphology (evaluated using scanning electron microscopy). Biochar addition up to 10% w/w had no negative effects on *P. corethrurus*, and ingested biochar particles showed signs of physical degradation and bacterial colonization. Nine pyrolysis-produced chemical biomarkers (phenolic and aromatic compounds) were detected that can be used as indicators of biochar presence in soils and earthworm castings. Earthworm casts showed distinct macromolecular chemical signatures compared with uningested artificial soil with and without biochar. Earthworm bioturbation and biochar ingestion may have important effects on functionalization and biochar chemical and physical properties that should be considered in field applications, especially where endogeic biochar-consuming species are present.

1. Introduction

Biochar-type substances have been intimately associated with the high amounts of organic carbon and the sustained fertility of Amazonian

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Dark Earths (ADEs), anthropogenic soils frequently hailed as an example of human-enhanced soil improvement with high potential for sustainable agricultural production and development (Glaser et al., 2001b; Lehmann and Joseph, 2009; Schmidt, 2013). ADEs were formed by the continuous deposition of pyrolysed organic residues (plant and animal remains) and inorganic materials (ceramic sherds) by indigenous Amazonian communities over long time periods (Glaser and Birk, 2012; Kern et al., 2017). The organic matter of ADEs is a mixture of highly aromatic substances (e.g., black carbon), as well as bonded carboxylic groups (Linhares et al., 2012), resulting from the incorporation and oxidation of incomplete combustion residues that contribute to the chemical recalcitrance of these soils (Glaser et al., 2001a; Novotny et al., 2009).

Much of the encouragement for the biochar movement, which amply promoted the use of biochars in agriculture, came from research on ADEs (Benites et al., 2009; Novotny et al., 2009). In fact, biochars are soil amendments with high capacity to improve soil quality (Lehmann, 2007), by positively affecting its physical and chemical characteristics, such as cation exchange capacity, stable carbon pool, nutrient retention and water holding capacity (Glaser et al., 2002; Lehmann and Joseph, 2009; Liang et al., 2010). Once applied to soils, biochar can be incorporated via bioturbation (Ponge et al., 2006) and may even be "functionalized" by chemical reactions and biological activity (Novotny et al., 2009; Sun et al., 2016). In these processes, soil microbiota and fauna (especially earthworms) may play an important role as catalysts, by moving soil and organic particles within and between horizons, mixing organic and inorganic fractions, changing porosity, aeration and water infiltration, and affecting chemical, physical and biological properties and processes (Ponge et al., 2006; Cunha et al., 2016; Sanchez-Hernandez et al., 2019b).

Nevertheless, most studies have used the standard litter-dwelling and feeding (epigeic) species - mainly *Eisenia andrei* or *E. fetida*, while the potential ecotoxicity of biochar to soil-dwelling geophagous (endogeic) earthworms has not been adequately addressed, especially in the tropics. Only a few studies have used endogeics like the common tropical species *Pontoscolex corethrurus* (Paz-Ferreiro et al., 2014, 2015; Kamau et al., 2020) or the temperate species *Aporrectodea caliginosa* (Tammeorg et al., 2014; Sanchez-Hernandez, 2018). The limited number of studies including only a few species and biochar types still limits our understanding of the potential ecotoxicological effects of biochar applications to soil-dwelling earthworms, although progress on this is expected, particularly from longer-term field experiments (e.g., Kamau et al., 2019; Briones et al., 2020).

Earthworms add mucus and secrete enzymes in their intestines (Brown et al., 2000), and the gut microbes are involved in numerous biochemical processes that can affect soil nutrient availability in casts and overall soil fertility (Van Groenigen et al., 2019). Furthermore, earthworms are known to ingest small charcoal particles together with mineral soil and later deposit their dark casts at different depths in the soil (Topoliantz and Ponge, 2003; Eckmeier et al., 2007), potentially affecting soil microbial communities, soil fertility and plant production (Paz-Ferreiro et al., 2014, 2015). This enhanced soil enzyme and microbial activity can have important impacts on biochar properties in worm-worked soils (Sanchez-Hernandez, 2018; Sanchez-Hernandez et al., 2019a). However, few studies have evaluated the characteristics of biochar-enriched casts, and their possible physical, biological and chemical transformations. Furthermore, understanding the chemical and physical changes in biochar-enriched soils and on its earthworm populations (e.g., Kamau et al., 2019), may help to predict how they affect biochar functionalization, potential C sequestration and greenhouse gas emissions (e.g., Namoi et al., 2019; Sanchez-Hernandez et al., 2019b).

Hence, the present study was undertaken to assess the impact of earthworms on biochar physical and chemical properties, and the effect of different biochar doses on earthworm survival. The endogeic species *P. corethrurus* was chosen as it represents one of the commonest earthworms in the tropics and subtropics (Taheri et al., 2018a), and because it is well-associated with modern and Pre-Columbian settlements, including ADEs (Brown et al., 2006; Cunha et al., 2016). Furthermore, we used biochar of a common tree species (Brazil-nut) associated with Amerindian communities and ADEs throughout the Amazon (Thomas et al., 2015). To evaluate chemical changes to the biochar, the organic macromolecular structure of artificial soil and earthworm casts were studied using pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC–MS), while physical changes due to biochar passage through earthworm guts were studied using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Preparation of biochar and artificial soil

The biochar was produced from the epicarp of Brazil nuts (*Bertholletia excelsa*), in a stainless steel pyrolyser reactor at 350 °C at heating rate of 10 °C min^{-1}, with the isotherm at final temperature for 4 h and 30 min. Basic elemental characteristics (CNHS/O; Vario EL III) of the biochar revealed CHNO – 66% C, 4.2% H, 2.3% N and 27.2% O, respectively. The pH in water (6.45) and the electrical conductivity (11.99 μs/cm) were determined according to standard methods for soils (Teixeira et al., 2017), with the modification that electrical conductivity was measured in the same solution used for the pH measurements (Thermo Scientific Orion Star A329, USA).

The experiment was conducted at the Embrapa Forestry Research Center, in Colombo, Brazil, using artificial soil with a formulation based on the international protocol (OECD, 1984), consisting of 70% fine sand, 20% white clay (kaolin), and 10% *Sphagnum* sp. moss. The materials were mixed until completely homogenized, and pH adjusted to 6.0 ± 0.5 using calcium carbonate, and the moisture level corrected to 60% water holding capacity. Artificial soil was used in order to control/reduce the high variability of organic compounds generally found in natural soils. Furthermore, *Sphagnum* moss was used as this is the standard organic substrate used in OECD tests and because it contains organic compounds very different from those of biochar and natural soils, which could be detected and identified using Py-GC–MS (see below).

2.2. Earthworms

All earthworms used in the toxicological tests were adult (clitellate) individuals of the species *Pontoscolex (Pontoscolex) corethrurus* (Müller, 1857), collected at the Embrapa Forestry experimental station in Colombo, Southern Brazil (25°19′18.4″S, 49°09′34.6″W), where they are locally abundant in *Pinus* sp. plantations (Silva et al., 2019). The earthworms from this particular site had been previously subjected to genetic analysis (barcoding using CO1; Luis Cunha et al., unpublished data), and were shown to all belong to the Lineage 1 of *P. corethrurus*, the most widespread worldwide (Taheri et al., 2018b), and conforming to the neotype of the species (James et al., 2019). The animals were kept in artificial soil under laboratory conditions at 22 °C for acclimatization, for at least one week before the experimental trials.

2.3. Acute toxicity test

The acute toxicity test was based on the Organization for Economic Co-operation and Development - Guide to chemical tests n° 207 (OECD, 1984). In the test, mortality and biomass of earthworms exposed to different doses of biochar (0%, 2.5%, 5% and 10% w/w) were evaluated in the artificial soil using an entirely randomized design, consisting of four treatments and five replicates (experimental units = boxes). The test was carried out using 250 g soil mixed with different doses of biochar in plastic containers (11 × 11 × 3.5 cm), adjusting moisture substrate to around 60% water holding capacity using a liquid nutrient solution containing 2.5% fructose and glucose and 0.24% MS medium (Murashige and Skoog, 1962), which was added to all treatments. The MS medium is universally used in tissue culture and plant regeneration "in vitro", due to its high nutrient concentration,

particularly N, P, K, Ca, Mg, S, Na, Mn, Fe, Cl, Co, Bo. These sugars and nutrients were used instead of the standard earthworm food (manure) of the OECD test, in order to avoid interference in the biochemical analysis of the biochar and the *Sphagnum*. Preliminary tests by our group using this medium had already shown that it was useful for feeding earthworms. Five adult earthworms (previously voided of their gut contents for 24 h in moist tissue paper) were placed in each box and incubated for 28 days at 20 °C at Embrapa Forestry. Earthworms were counted and weighed (fresh mass, including gut contents) at 0, 7, 14, 21 and 28 days after the beginning of the experiment.

Earthworm casts were collected every two days in the different treatments: the control treatment without biochar, and those with 2.5%, 5% and 10% biochar (CB2.5, CB5 and CB10, respectively). The first two cast collections (at 2 and 4 days) were discarded in order to avoid any possible residues of the original soil. All casts were stored in the freezer at −20 °C until further analysis. Soil samples were collected from all treatments (n = 5): artificial soil, artificial soil with 2.5, 5 and 10% biochar (SB2.5, SB5 and SB10, respectively), artificial soil with earthworms (SW), and artificial soil with 2.5, 5 and 10% biochar and earthworms (SWB2.5, SWB5 and SWB10, respectively).

The earthworm biomass data were subjected to a repeated measures analysis of variance (ANOVA) using biochar concentration (0, 2.5, 5 and 10%) and time (days) as factors considering significance at $p < 0.05$. The analyses were performed using r (R Core Team, 2016).

2.4. Macromolecular organic matter analysis

The macromolecular analysis was divided in two stages: (i) initially, the characterization of the different substrates (n = 5) was carried out, such as *Sphagnum*, biochar, artificial soil and artificial soil with 10% biochar (SB10); (ii) subsequently, casts and soils with earthworms (worm-worked soils) were characterized to assess the earthworm effects on the transformation of these substrates. The samples analyzed were: casts with (CB10) and without biochar addition, artificial soil with (SW) and without worms (S), and artificial soil with 10% biochar without (SB10) and with worms (SWB10).

All lyophilized samples (0.1 g) were extracted with dichloromethane: methanol in ultrasonic bath (3×, 2:1, 2 ml, 15 min) to remove free compounds. Then, ca. 0.75 mg of solid material was characterized by Py-GC-MS at Embrapa Forestry. The pyrolysis was carried out at 700 °C for 20s, with oven temperature and transfer line at 280 °C. The pyrolysed products were inserted on-line in a gas chromatograph with the injector at 230 °C in a split mode 1:50. The pyrolysed products were separated using a capillary column DB-5 ms (30 m × 0.25 mm, 0.25 μm film thickness) with the following oven program: 40 °C (5 min), heating rate at 7 °C min^{-1} to 280 °C (5 min). Helium was used as the carrier gas (1.0 ml min^{-1}). The GC–MS interface and the ion source temperatures were 250 °C and 200 °C, respectively. The mass spectrometer was operated in the positive electronic mode at 70 eV, the total scan time was 0.58 s for the range of 50–650 Da, and current emission 250 mA.

The total ion currents pyrograms were deconvolved using the AMDIS software (National Institute of Standards and Technology, NIST). Linear alkanes polyethylene pyrolysis products were used to calibrate the retention index (RI; Melo et al., 2017). The compounds were identified from the deconvolved mass spectra using a mass spectrum target library built in the AMDIS software from previous pyrolysed biomasses (Melo et al., 2017; Matos et al., 2020). The products obtained from Py-GC–MS were semi-quantified, using the software Xcalibur (Thermo), by the ratio of the characteristic mass fragment peak height (m/z) of each compound to the total sum of peak heights, according to the formula:

$$Qij = \left(xij / \sum xi\right) * 100$$

where xij is the integrated m/z height for compound j in sample i, Σxi is the sum of all integrated m/z heights for all the compounds detected in sample i, and Qij is the relative quantification of compound j in sample i.

The compounds below 0.1% of total were marked as non-detected, and their values (including zeros) were used to calculate the minimum amount detected (Melo et al., 2020). The minimum amount value divided by the square root was used to replace the values of all compounds below 0.1% total (Reimann et al., 2008). Subsequently, the modified 80% rule was applied to exclude/maintain the variables (Yang et al., 2015), and in case of missing values across the treatments in the maintained variables, the Wei et al. (2018) protocol was used. The multivariate analyses (i.e., principal component analysis and heatmap) were run after data transformation to centred log ratio (CLR), i.e., dividing each value of a variable for an individual by the geometric mean of all the variables for that individual and then calculating the logarithms (CoDaPack® software) in the Unscramble® and R softwares (R packages: gplots, heatmap.plus and, RColorBrewer). The univariate statistical analyses of the pyrolysis products (n = 5) were tested using R software with the following packages: stats, openxlsx, multcomp, lattice, agricolae, hnp and effects (R Core Team, 2016). The response variables (compounds) were initially classified in terms of their data distribution, following the assumptions of normality and homogeneity of variances, then specific statistical tests were used accordingly, i.e., ANOVA, linear model (LM) and generalized linear model (GLM) followed by the post-hoc Tukey test for the parametric data (LM and GLM) and Fisher exact test for the non-parametric data, adopting the critical probability value of 5% for statistical significance. These procedures followed a standard protocol proposed for GC–MS fingerprint analysis results by Melo et al. (2020).

2.5. Scanning electron microscopy (SEM)

Biochar samples were manually removed from surface of the containers with artificial soil and no earthworms and from surface-deposited earthworm castings, both of these only from the highest biochar dose (10% w/w), in order to evaluate the effect of passage through *P. corethrurus* intestinal tracts. Biochar fragments were removed with the aid of tweezers and a light microscope. The casts were broken with the tweezers in order to reveal fragments inside the casts. The biochar samples (n = 5) from control soil and casts were mounted on stubs and covered with gold (exposure time 180 s, current 50 mA) in a spraying system (Balzers MED010). The stubs were analyzed in a scanning electron microscope (LEO Evo40) with Secondary Electron Detectors (SE), at the Embrapa Instrumentation research center in São Carlos, Brazil.

3. Results

3.1. Acute toxicity test

There was no mortality or significant reduction in the mean weights of the *P. corethrurus* worms in the different treatments (Fig. 1) in the different time-periods of measurement (7, 14, 21 and 28 days), showing that even the highest dose of biochar (10% w/w) did not affect their weight over this time-scale of measurement.

3.2. Macromolecular analysis of organic matter sources and artificial soils

Characterization of the biochar, *Sphagnum*, soil and SB10 samples yielded 102 pyrolysis products, that could be classified into nine chemical classes: (1) anhydrous sugars, (2) aromatic substances, (3) cyclic hydrocarbons, (4) cyclopentanones, (5) furans, (6) nitrogen compounds, (7) phenols, (8) pyrans and (9) small molecules (Fig. 2). The samples showed different distributions in relation to the classes. In *Sphagnum* and Soil, the main class observed was phenol, with corresponding values of 46.8% and 33.4%, respectively (Fig. 2). In the biochar and SB10 samples, phenol contents were lower (around 30%), and the aromatics were more abundant, particularly in the biochar. The major pyrolysis products obtained from *Sphagnum* were furfural (Q28),

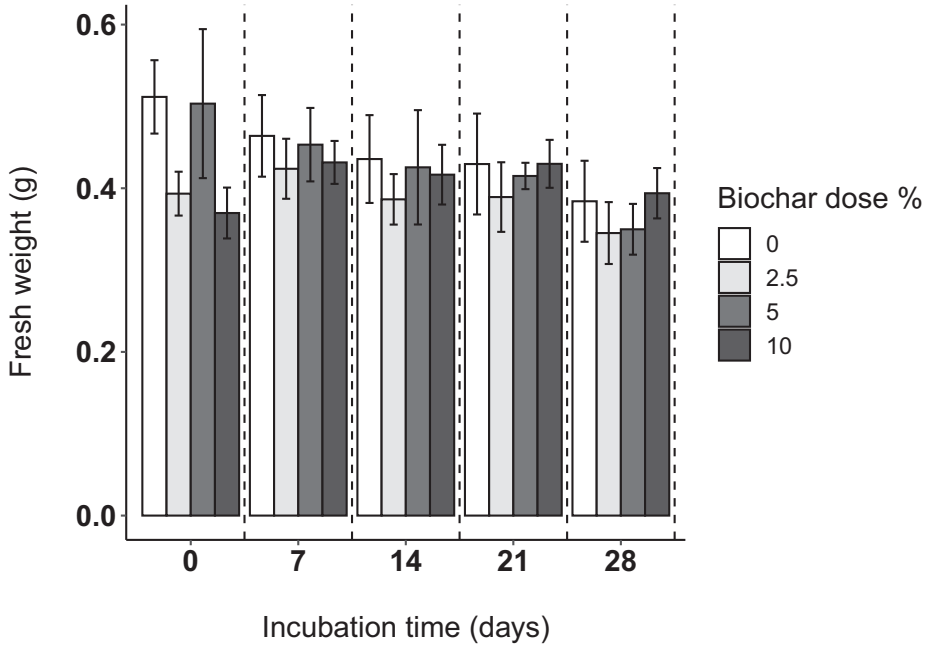


Fig. 1. Changes in fresh weight (g) of *Pontoscolex corethrurus* individuals kept in artificial tropical soil exposed to different doses of biochar (0, 2.5, 5 and 10% w/w) over 28 days. The figure displays bars with means ± standard errors per treatment at each measured time (0, 7, 14, 21, 28) calculated from five replicates. Differences between dose treatments were not statistically significant.

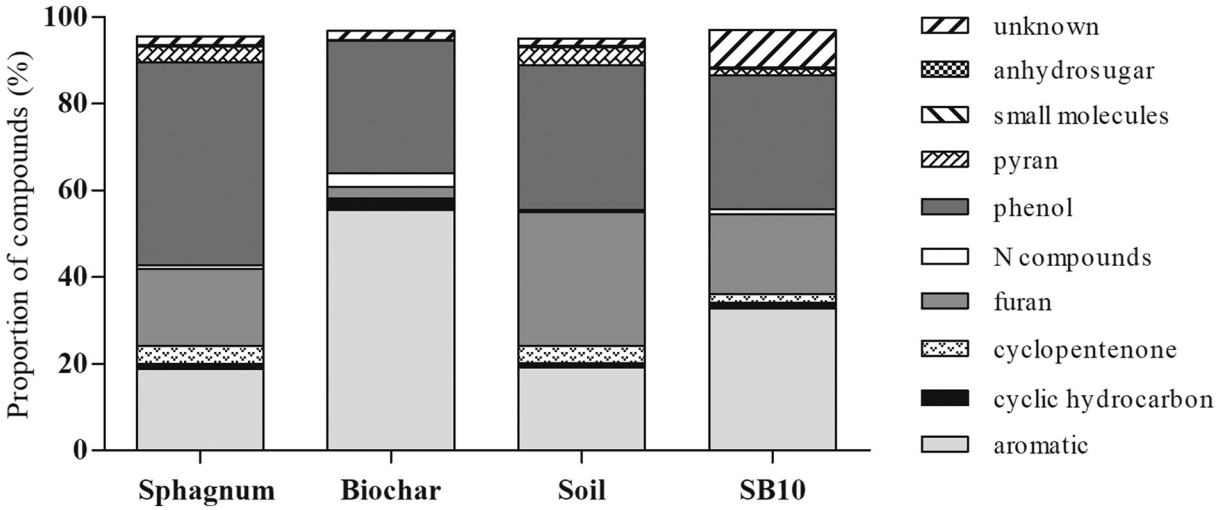
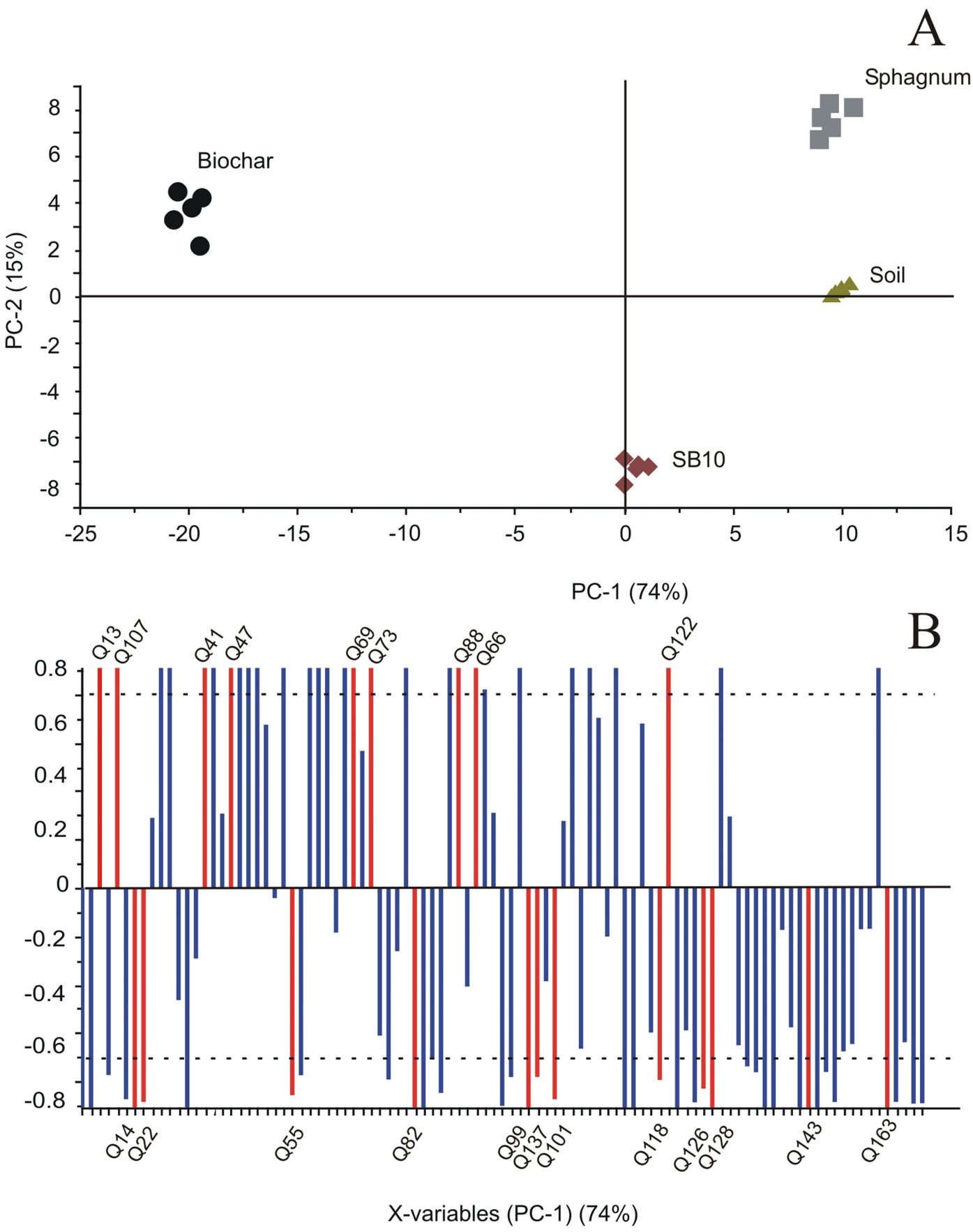


Fig. 2. Proportion of the major pyrolysis-produced chemical classes in *Sphagnum*, biochar, artificial soil (Soil), and artificial soil with 10% (w/w) biochar (SB10).

phenol (Q53), C1-phenol (Q78), 4-isopropenylphenol (Q122) and 4-vinylphenol (Q107). In the biochar, the most abundant pyrolysis compounds were aromatic like benzene (Q14), toluene (Q22) and naphthalene (Q99). The soil and SB10 samples showed furfural, phenol and 5-methyl-2-furaldehyde as the most abundant pyrolysis products. The chemical class distribution indicates that the soil and SB10 were more similar to *Sphagnum* than to the biochar (Fig. 2).

The pyrolysis-produced phenolic products of macromolecular structure of *Sphagnum* appeared as the major monomers C2-phenols, 4-vinyl-phenol and 4-isopropenylphenol, with a smaller contribution of guaiacol. Conversely, in the biochar the main units identified were C1-guaiacol, C1-syringol and C2-guaiacol. Charcoal formation in the biochar was detected by the major increase in aromatic compounds, and these pyrolysis products were also found in higher degree in SB10 (32.8%). The same was observed for the N-compounds class, in which the major pyrolysis product detected was benzonitrile with a lower abundance of (*E*)-cinnamonitrile, indole and methyl-indol isomer. Regarding the pyrolysis products from carbohydrates (i.e., cellulose and hemicellulose), the main

Fig. 3. Score-correlation loading biplots (A and B) of pyrolysis products of biochar, *Sphagnum*, artificial soil (Soil) and artificial soil with 10% biochar (SB10). The pyrolysis-product distribution was transformed to CLR (centred log ratio). The highlighted pyrolysis products (red bars) represent the compounds that were significantly different in the treatments and were assigned as Q13 (cyclohexadiene), Q14 (benzene), Q22 (toluene), Q41 (3-methyl-2-cyclopentan-1-one), Q47 (2,3-dihydro-5-methyl-furan-2-one), Q55 (benzonitrile), Q66 (2-hydroxy-3-methyl-2-cyclopentan-1-one, Q69 (C3-furan), Q73 (D#1), Q82 (guaiacol), Q88 (C2-phenol_I2), Q99 (naphthalene), Q101 (C1-guaiacol), Q107 (vinylphenol), Q118 (C2-guaiacol), Q122 (4-isopropenylphenol), Q126 (syringol), Q128 (biphenyl), Q137 (C1-syringol), Q143 (dibenzofuran), Q163 (9 (H) - Fluoren-9-one). Details; C1 – methyl, C2 – dimethyl or ethyl, C3 – trimethyl, methyl – ethyl or propyl.


A
Sphagnum
Biochar
Soil
SB10
PC-2 (15%)
PC-1 (74%)
8
6
4
2
0
-2
-4
-6
-8
-25
-20
-15
-10
-5
0
5
10
15
B
Q13
Q107
Q41
Q47
Q69
Q73
Q88
Q66
Q122
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Q14
Q22
Q55
Q82
Q99
Q137
Q101
Q118
Q126
Q128
Q143
Q163
X-variables (PC-1) (74%)

representative classes were cyclopentanone and furan. Highest values of furan were found in the soil (30.9%), SB10 (18.4%) and *Sphagnum* (17.9%) samples, while cyclopentanones were more prevalent in *Sphagnum* and soil samples (4%), followed by SB10 (1.9%). Lowest abundance of carbohydrate pyrolysis products was detected in the biochar samples.

In the principal component analysis (PCA) using the proportions of these products, the first two axes (PC1 and PC2) explained 89% of the total variation of the dataset. The PC1 explained 74% of the variation and separated the biochar from *Sphagnum* and the artificial soil samples (soil and SB10), while the PC2 explained 15% of the total variation, and separated the SB10 (Fig. 3A). The pyrolysis-produced compounds with correlation values above 0.7 were considered important, and are shown in Fig. 3B. The positive values in the y-axis indicate the pyrolysis products more associated with *Sphagnum* and soil, and revealed eight compounds belonging to the classes of phenol, furan and cyclopentanone that were good indicators of these samples: cyclohexadiene (Q13), 4-vinyl-phenol (Q107), 3-methyl-2-cyclopentan-1-one (Q41), C3-furan (Q69), D#1 (unidentified compound) (Q73), C2-phenol_2 (Q88), 2-hydroxy-3-methyl-2-cyclopentan-1-one (Q66) and 4-isopropenylphenol (Q122) (Fig. 3B). The negative values in the y-axis indicate the aromatic and phenol class compounds more associated with biochar samples: benzene (Q14), toluene (Q22), benzonitrile (Q55), guaiacol (Q82), naphthalene (Q99), C1-syringol (Q137), C1-guaiacol (Q101), C2-guaiacol (Q118), syringol (Q126), biphenyl (Q128), dibenzofuran (Q143) and 9 (H) -fluoren-9-one (Q163) (Fig. 3B).

Using this data from the PCA, the pyrolysis-produced compounds were studied individually, in order to identify biomarkers for the organic matter sources (i.e., *Sphagnum* or biochar) provided to the earthworms during the experiment, with the potential to discriminate between soils with and without biochar addition. These results are presented in Table 1. The pyrolysis-produced compounds toluene (Q22), guaiacol (Q82) and syringol (Q126) are not individually good indicators of the biochar, and also cyclohexadiene (Q13) cannot be used as a *Sphagnum* biomarker. Nine biochar biomarkers were identified: 9 (H)-fluoren-9-one, benzene, benzonitrile, biphenyl, C1-guaiacol, C1-syringol, C2-guaiacol, dibenzofuran and naphthalene. Conversely, pyrolysis-produced compounds of the classes of phenols and furans, such as 2-hydroxy-3-methyl-2-cyclopentan-1-one, 2,3-dihdro-5-methyl-furan-2-one, 3-methyl-2-cyclopentan-1-one, C2-phenol_I2, C3-furan, were indicative of *Sphagnum*. In fact, the relative amount of 4-isopropenylphenol was an excellent biomarker for the presence of *Sphagnum* in the samples.

3.3. Macromolecular analysis of earthworm casts and artificial soils

Not all the biochar indicator compounds (Table 1) are relevant individually in the differentiation between earthworm casts with (CB10) and without biochar, and only C1-guaiacol and C2-guaiacol were significantly different ($p < 0.05$, Fig. 4). Biphenyl was present in greater relative abundance in SB10 and SWB10, followed by CB10. The presence of this biomarker in the treatments is indicative of the addition of biochar in SB10 and SWB10 and also proof of biochar ingestion by the earthworms in the CB10 samples. Naphthalene distribution was similar to that of biphenyl: treatments SB10 and SWB10 showed a higher relative abundance (Fig. 4D), corroborating the detection of the biochar in the samples. Again, the presence of naphthalene in CB10 is indicative of biochar ingestion by earthworms during the experiment.

Regarding the pyrolysis-produced phenol biomarkers for biochar, the compounds C1-guaiacol showed a greater relative abundance in the SWB10 samples, followed by SB10 and CB10 (Fig. 4B). In the samples of soil, casts and SW this substance was not detected, and interestingly it was present in greater relative abundance in the soils with earthworms (SWB10) than without (SB10). The other phenol compound from biochar, C2-guaiacol, had highest relative abundance in CB10, which may indicate a concentration process of this compound in earthworm casts with the addition of biochar.

A heatmap of the pyrolysis-products of the different treatments (Fig. 5) showed two clusters on the horizontal axis: the first one included samples SB10 and SWB10, and the second, samples CB10, casts (C) and SW + S (Soil). The first cluster indicated high similarity, especially due to the relative abundance of pyrolysis-produced polycyclic aromatic hydrocarbons (PAHs), such as dibenzofuran and phenanthrene (vertical axis, class 2), biphenyl, fluorene, C1- naphthalene (vertical axis, class 4), naphthalene, benzene, toluene (vertical axis, class 6) and C1-dibenzofuran (vertical axis, class 12). Other compounds that were found in greater relative abundance in SB10 and SWB10 treatments belonged to the phenol class (i.e. C1-guaiacol and C1-syringol), and were grouped in class 13 in the heatmap (vertical axis).

The second cluster (C, CB, SW + S), still showed clear separation of soil (S and SW) and cast (C and CB) samples, and the latter was also subdivided in two groups, i.e., casts from treatments without biochar (C) and casts from those with biochar (CB). This second cluster is formed mainly by the concentration of *Sphagnum* biomarkers, represented by 2-hydroxy-3-methyl-2-cyclopenten-1-one, 2,3-dihydro-5-methyl-furan-2-one, vinyl-phenol (vertical axis, class 3), 4-

Table 1
Relative abundance of various biomarkers of *Sphagnum*, Brazil nut biochar, artificial soil (Soil) and artificial soil with 10% biochar (SB10) obtained from Py-GC–MS data. *m/z* = characteristic mass fragments. Values shown in parentheses are standard deviations, and mean values followed by different uppercase letters differ statistically at $p < 0.05$.

	RI	Substance name	Biomarker		*m/z*		*Sphagnum* (%)		Biochar (%)		Soil (%)		SB10 (%)	
Q99	1189,4	Naphthalene	Biochar	128	102	–	1,64^{c}	(0,20)	13,43^{a}	(1,92)	1,99^{c}	(0,20)	4,81^{b}	(0,99)
Q14	688,2	Benzene	Biochar	78	63	50	2,22^{c}	(0,49)	9,55^{a}	(2,50)	1,82^{c}	(0,31)	3,81^{b}	(1,44)
Q143	1529,1	Dibenzofuran	Biochar	168	139	113	0,36^{d}	(0,08)	6,00^{a}	(1,30)	0,69^{c}	(0,03)	2,47^{b}	(0,53)
Q55	985,2	Benzonitrile	Biochar	103	76	51	0,11^{c}	(0,01)	2,39^{a}	(1,71)	0,01^{d}	(0,00)	0,50^{b}	(0,12)
Q137	1443,2	C1-syringol	Biochar	168	153	125	0,01^{c}	(0,00)	1,89^{a}	(1,53)	0,01^{c}	(0,00)	0,34^{b}	(0,25)
Q128	1387,0	Biphenyl	Biochar	154	–	–	0,49^{c}	(0,14)	1,87^{a}	(0,26)	0,46^{c}	(0,02)	1,00^{b}	(0,27)
Q101	1179,5	C1-guaiacol	Biochar	138	123	95	0,22^{b}	(0,14)	1,40^{a}	(1,01)	0,01^{c}	(0,00)	0,30^{b}	(0,18)
Q163	1753,7	9 (H)-Fluorene-9-one	Biochar	180	152	126	0,01^{c}	(0,00)	0,78^{a}	(0,15)	0,01^{c}	(0,00)	0,34^{b}	(0,10)
Q118	1275,6	C2-guaiacol	Biochar	152	137	122	0,20^{b}	(0,05)	0,52^{a}	(0,25)	0,01^{c}	(0,00)	0,01^{c}	(0,00)
Q122	1301,7	4-Isopropenylphenol	*Sphagnum*	134	119	91	9,06^{a}	(1,40)	0,01^{d}	(0,00)	4,99^{b}	(0,61)	3,15^{c}	(0,76)
Q66	1024,7	2-Hydroxy-3-methyl-2-cyclopenten-1-one	*Sphagnum*	112	84	55	1,92^{a}	(0,31)	0,01^{d}	(0,00)	1,25^{b}	(0,19)	0,55^{c}	(0,21)
Q73	1046,3	RI = 1190.1 (unknown compound)	*Sphagnum*	128	113	82	1,62^{a}	(0,48)	0,01^{c}	(0,00)	1,11^{b}	(0,13)	0,39^{c}	(0,15)
Q107	1216,7	Vinyl-phenol	*Sphagnum*	65	91	120	1,56^{a}	(0,19)	0,01^{d}	(0,00)	0,68^{b}	(0,05)	0,38^{c}	(0,05)
Q47	938,4	2,3-Dihydro-5-methyl-furan-2-one	*Sphagnum*	98	70	41	1,43^{a}	(0,29)	0,01^{d}	(0,00)	1,11^{b}	(0,09)	0,47^{c}	(0,18)
Q69	1035,6	C3-furan	*Sphagnum*	110	95	67	0,34^{a}	(0,03)	0,01^{d}	(0,00)	0,25^{b}	(0,03)	0,21^{c}	(0,03)
Q88	1136,1	C2-phenol_I2	*Sphagnum*	122	107	77	0,29^{a}	(0,04)	0,01^{d}	(0,00)	0,17^{b}	(0,02)	0,13^{c}	(0,01)
Q41	919,8	3-Methyl-2-cyclopentene-1-one	*Sphagnum*	95	67	41	0,17^{a}	(0,02)	0,01^{d}	(0,00)	0,15^{b}	(0,01)	0,12^{c}	(0,01)
Q22	762,6	toluene	–	91	65	–	4,99^{b}	(0,65)	9,34^{a}	(2,35)	3,03^{c}	(0,32)	4,81^{b}	(1,34)
Q13	674,2	Cyclohexadiene	–	77	51	–	0,60^{bc}	(0,10)	2,36^{a}	(0,88)	0,43^{c}	(0,06)	0,90^{b}	(0,38)
Q82	1098,7	Guaiacol	–	124	109	81	0,36^{b}	(0,21)	2,33^{a}	(1,13)	0,13^{c}	(0,02)	0,45^{b}	(0,17)
Q126	1348,7	Syringol	–	154	139	93	0,17^{b}	(0,11)	2,87^{a}	(2,20)	0,01^{c}	(0,00)	0,40^{b}	(0,31)

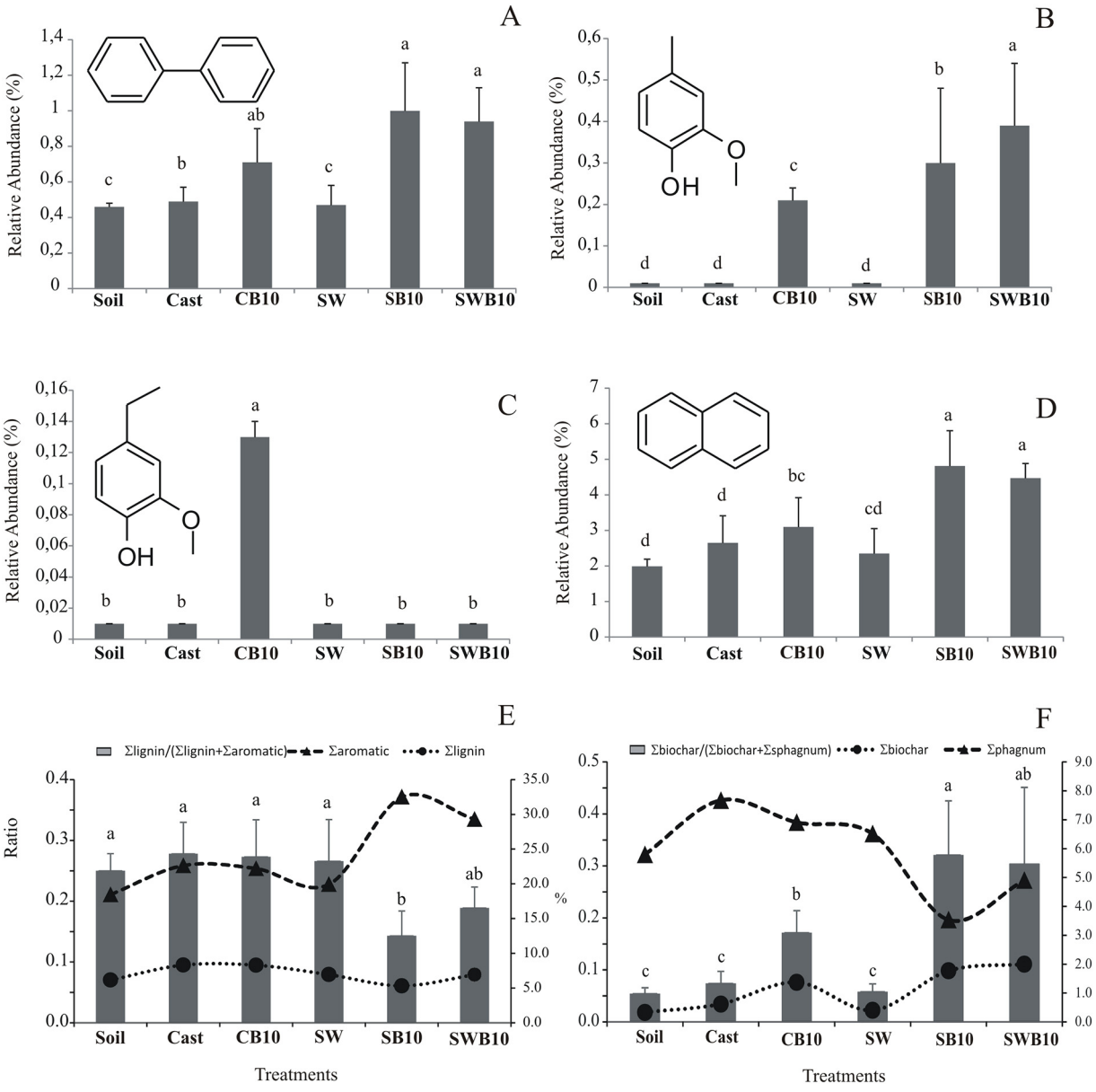


Fig. 4. Relative abundance of the pyrolysis-produced compounds biphenyl (A), C1-guaiacol (B), C2-guaiacol (C), and naphthalene (D) in the different treatments; (E) Sum and ratio of the aromatic and phenolic pyrolysis-produced compounds in the different treatments; (F) sum and ratio of the pyrolysis-produced phenolic biochar and *Sphagnum* biomarkers in the different treatments. Artificial soil (Soil), earthworm casts, CB10 (earthworm casts from the treatment with 10% w/w biochar), SW (artificial soil with earthworms), SB10 (artificial soil with 10% w/w biochar), SWB10 (artificial soil with 10% w/w biochar and earthworms). Different lowercase letters above the bars mean significant differences between treatments at $p < 0.05$.

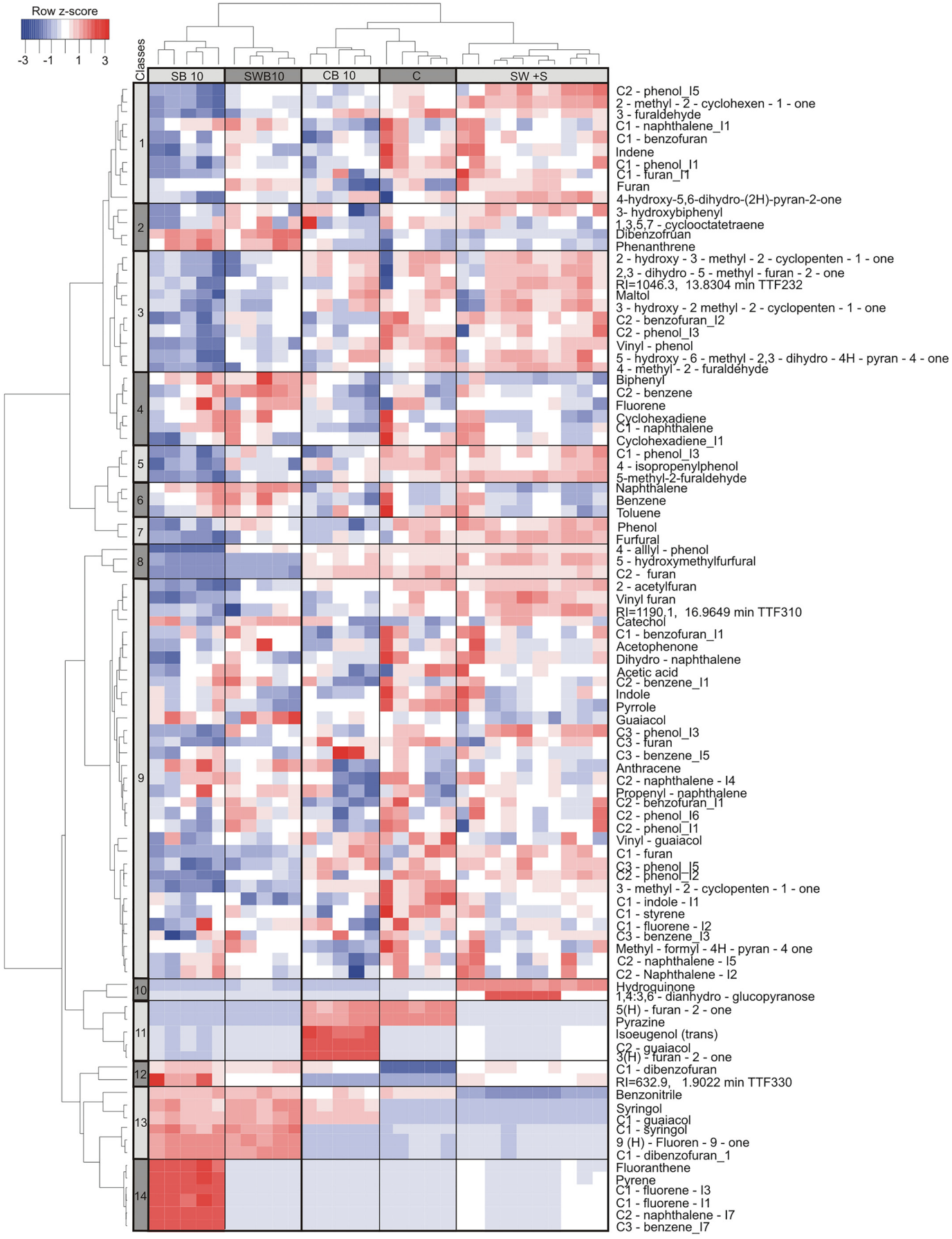
isopropenylphenol (class 5), 3-methyl-2-cyclopenten-1-one, C3-furan and C2-phenol_I2 (class 9) (Fig. 5). Once again, pyrolysis-produced phenolic compounds (i.e., C2-guaiacol and isoeugenol (trans), class 11) were clearly abundant in CB.

The sum of aromatic pyrolysis products was greater in the SB10 and SWB10 treatments when compared to the other treatments (CB, C, S + SW), although the sum of lignin/tannin-like polyphenolics was practically constant in all treatments (Fig. 4E). However, the ratio of these pyrolysis products clearly separated SB10 samples from the soils without biochar (S and SW) and earthworm casts (C and CB). Interestingly, no differences were detected between SWB10 and the other treatments. These results corroborate the presence of biochar in SB10 and SWB10, but the ratio involving the total sum aromatic compounds did not provide any clue for the presence of biochar in CB.

On the other hand, the presence of phenolic compounds can be used as a proxy to identify the presence of biochar or *Sphagnum* in samples (Fig. 3). The sum of sphagnum phenolic markers was higher in Soil, cast (C), CB10 and SW treatments, while the sum of the phenolic biochar markers was higher in SWB10 and SB10 (Fig. 4F). Interestingly, CB10 showed a similar ratio of phenolic markers as that seen in soils with earthworms and biochar (SWB10), indicating the major abundance of phenolic markers of biochar in CB compared to the treatments without biochar. Hence, phenolic biochar markers are useful in showing the ingestion of biochar by earthworms in the treatment with biochar (CB10), despite its greater similarity to soil, SW and cast samples due to the high amount of *Sphagnum* biomarkers (Fig. 4F).

3.4. Biochar morphology before and after earthworm gut passage

The scanning electron microscope images revealed the morphology of the biochar before (pre-ingestion) and after (ingested) passing through earthworm intestinal tracts (Fig. 6). In Fig. 6A, the pre- ingested biochar shows a vascular bundle of fibers and plant vascular cells, forming a lignified spiral structure. These fibers are elongated cells


Row z-score
-3
-1
1
3
Classes
SB 10
SWB10
CB 10
C
SW +S
1
2
3
4
5
6
7
8
9
10
11
12
13
14
C2 - phenol_I5
2 - methyl - 2 - cyclohexen - 1 - one
3 - furaldehyde
C1 - naphthalene_I1
C1 - benzofuran
Indene
C1 - phenol_I1
C1 - furan_I1
Furan
4-hydroxy-5,6-dihydro-(2H)-pyran-2-one
3- hydroxybiphenyl
1,3,5,7 - cyclooctatetraene
Dibenzofuran
Phenanthrene
2 - hydroxy - 3 - methyl - 2 - cyclopenten - 1 - one
2,3 - dihydro - 5 - methyl - furan - 2 - one
RI=1046.3, 13.8304 min TTF232
Maltol
3 - hydroxy - 2 methyl - 2 - cyclopenten - 1 - one
C2 - benzofuran_I2
C2 - phenol_I3
Vinyl - phenol
5 - hydroxy - 6 - methyl - 2,3 - dihydro - 4H - pyran - 4 - one
4 - methyl - 2 - furaldehyde
Biphenyl
C2 - benzene
Fluorene
Cyclohexadiene
C1 - naphthalene
Cyclohexadiene_I1
C1 - phenol_I3
4 - isopropenylphenol
5-methyl-2-furaldehyde
Naphthalene
Benzene
Toluene
Phenol
Furfural
4 - alllyl - phenol
5 - hydroxymethylfurfural
C2 - furan
2 - acetylfuran
Vinyl furan
RI=1190.1, 16.9649 min TTF310
Catechol
C1 - benzofuran_I1
Acetophenone
Dihydro - naphthalene
Acetic acid
C2 - benzene_I1
Indole
Pyrrole
Guaiacol
C3 - phenol_I3
C3 - furan
C3 - benzene_I5
Anthracene
C2 - naphthalene - I4
Propenyl - naphthalene
C2 - benzofuran_I1
C2 - phenol_I6
C2 - phenol_I1
Vinyl - guaiacol
C1 - furan
C3 - phenol_I5
C2 - phenol_I2
3 - methyl - 2 - cyclopenten - 1 - one
C1 - indole - I1
C1 - styrene
C1 - fluorene - I2
C3 - benzene_I3
Methyl - formyl - 4H - pyran - 4 one
C2 - naphthalene - I5
C2 - Naphthalene - I2
Hydroquinone
1,4:3,6 - dianhydro - glucopyranose
5(H) - furan - 2 - one
Pyrazine
Isoeugenol (trans)
C2 - guaiacol
3(H) - furan - 2 - one
C1 - dibenzofuran
RI=632.9, 1.9022 min TTF330
Benzonitrile
Syringol
C1 - guaiacol
C1 - syringol
9 (H) - Fluoren - 9 - one
C1 - dibenzofuran_1
Fluoranthene
Pyrene
C1 - fluorene - I3
C1 - fluorene - I1
C2 - naphthalene - I7
C3 - benzene_I7

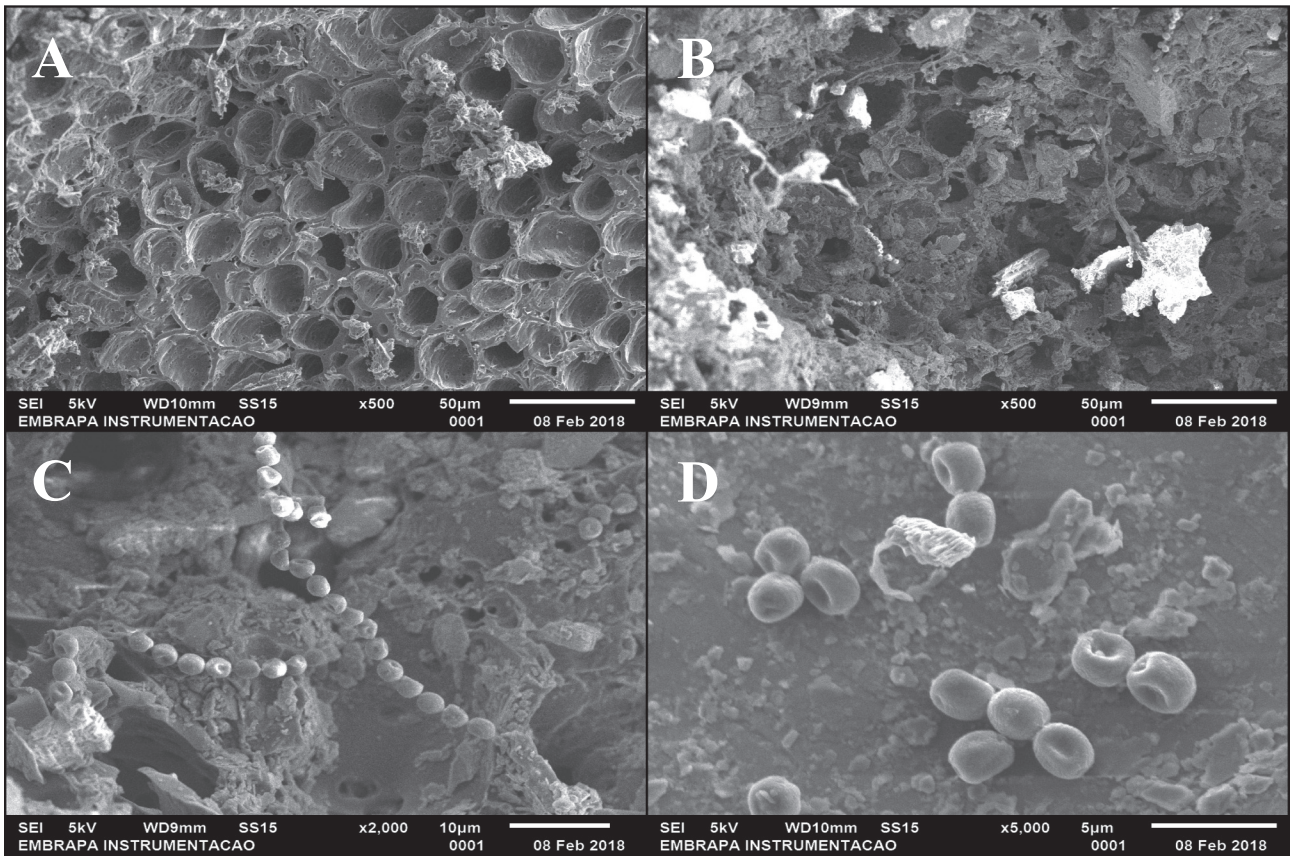


Fig. 6. Scanning electron microscopy of biochar of the Brazil nut epicarp; A) biochar before passage through the intestinal tract of *P. corethrurus*, 500× increase. B) biochar after passing through the earthworm intestinal tract, 500× increase. C and D) Presence of microorganisms on the biochar surface after earthworm gut passage, 2000 and 5000× increase.

with a thick, lignified cell wall, usually organized as a bundle. They are elastic and provide strength and elasticity to plant tissue, in addition to distributing water and nutrients through the pericarp in living cells (Sonego et al., 2019). Around the bundles of fibers, the scleral cells that fill the gaps between these bundles are also visible. This highly porous structure is formed by parallel microtubules in the shape of hives, with some of the pores being blocked by fine particles.

In the biochar that passed through earthworm intestinal tracts, changes to the porous surface are visible (Fig. 6B), demonstrating structural breakdown and the formation of an irregular surface. The parallel microtubules are hardly detected, indicating that biochar in the cast underwent great morphological change. Round-shaped bacteria were also detected on the surface of the biochar after gut passage (Fig. 6C, D).

4. Discussion

4.1. Detection of chemical biomarkers in the organic materials, soils and casts

Using Py-GC-MS we found eight *Sphagnum* and nine biochar-specific biomarkers. Two of them (4-vinyl-phenol and 4-isopropenylphenol) were good *Sphagnum* biomarkers, and their origin is probably linked to a tannin-like polyphenolic biopolymer (Van der Heijden et al., 1997) present in the moss. The pyrolysis products C1-guaiacol, C2-guaiacol, and C1-syringol were good biochar biomarkers and indicate the original lignin present in the epicarp of the Brazil nut (Del Río et al., 2005; Rowell et al., 2005). These may resist the pyrolysis process due to the relatively lower temperature (350 °C) used for the biochar production (Mohan et al., 2006; Huber et al., 2006; Kaal et al., 2012; Li et al., 2020). In fact, the biochar obtained in the present experiment could be classified as amorphous char (Xiao and Chen, 2017), where heat-altered molecules and aromatic polycondensates are randomly mixed (Keiluweit et al., 2010).

The importance of polysaccharides in *Sphagnum* structures was evidenced by the abundance of pyrolysis products from the classes pyran, furan, cyclopentanones and anhydrous sugars. The low abundance of these classes in the biochar are likely related to the greater susceptibility of the Brazil nut epicarp fibers to degradation during the biochar production process (Cordella et al., 2012; Poletto et al., 2012).

4.2. Earthworm (P. corethrurus) interaction with biochar

Biochars often contain potentially toxic elements for animals, such as polycyclic aromatic hydrocarbons (PAH) (Spokas et al., 2012; Oleszczuk et al., 2013; Malev et al., 2016), highly toxic compounds with mutagenic and carcinogenic effects (Liang et al., 2010). The presence of extractable PAH in biochars could represent a potential risk to earthworms in soils with biochar application (Malev et al., 2016), though some species appear to be more resistant (e.g., *E. fetida*) than others (Achazi et al., 1998). In the present experiment extractable PAH were not measured, but their occurrence cannot be excluded. In case they were present, these compounds were not toxic (100%

Fig. 5. Heatmap of the relative abundance of pyrolysis products in the samples of artificial soil with 10% (w/w) biochar (SB10), soil with 10% biochar and earthworms (SWB10), casts from soils with 10% biochar (CB10), earthworm casts (C), artificial soil with earthworms (SW) and artificial soil (S). The pyrolysis-product distribution was transformed to CLR (centred log ratio; see Materials and methods section). The letter l followed by a number at the end of the compound name refers to different isomers. C1 = methyl, C2 = dimethyl or ethyl, C3 = trimethyl, methyl–ethyl or propyl.

survival) to *P. corethrurus* over the 28-day experimental time-frame, but further research is needed to assess this for *P. corethrurus*. Longer experiments (e.g., 90 days) may also be needed to evaluate potential chronic effects (e.g., Buch et al., 2017), which were not studied here but cannot be ruled out, given that oxidative stress has been detected in cellular components of some species like the anecic earthworm *Lumbricus terrestris* exposed to 5% biochar at time-intervals similar to the present study (Sanchez-Hernandez et al., 2019a). Nonetheless, *P. corethrurus* survived for almost two years (104 weeks) in greenhouse mesocosms with 40 kg soil and an unknown amount of biochar (likely 3, 6 or 9%; Rondon et al., 2007), planted with the pasture grass *Brachiaria humidicola*, although survival was negatively affected (60% less) by biochar in the fertilized treatment and unaffected in the unfertilized soil (Noguera, 2009).

Long term survival, and even increase in earthworm abundance in long-term trials have been observed by other authors, despite avoidance or chronic effects in the lab (Weyers and Spokas, 2011; Tammeorg et al., 2014; Kamau et al., 2019). However, Briones et al. (2020) reported a decrease in temperate endogeic earthworm abundance (several species) with increasing biochar doses (10, 25 and 50 T ha^{-1}) after 4 and 17 months of application. Clearly, further work is warranted on the potential long-term ecotoxicological impacts of various biochars and doses on endogeic earthworms, considering the little available information, and the concentration of trials using *P. corethrurus* in tropical conditions and only a few species in temperate soils (Weyers and Spokas, 2011; Sanchez-Hernandez et al., 2019b). In addition, biochar of various feedstocks should be tested, as effects are not always similar (e.g., Kamau et al., 2020).

Interestingly, earthworm casts from soil with biochar showed lower abundance of pyrolysis-produced aromatic compounds, like naphthalene and biphenyl. This reduction may be indicative of the selection by earthworms of biochar fractions with fewer amounts of these compounds. Earthworms are selective in their choice of food (Curry and Schmidt, 2007), and this choice is based on, among various factors, the biochemical properties of the ingested soil (Neilson and Boag, 2003), size and age of the earthworm, as well as the chemical characteristics and weathering of the litter (Satchell and Lowe, 1967; Lowe and Butt, 2003), and the microbial population present in the soil (Gilot-Villenave, 1994). In laboratory studies carried out by Liebeke et al. (2015), there was an increase in certain active metabolites called "drilodefensins" in earthworm intestines, that protected them from the effects of food with high polyphenol contents. In our study, the greater abundance of polyphenols in casts (e.g., indicated by pyrolysis-produced C1-guaiacol and C2-guaiacol) and the possible selective ingestion of biochar, may be an indication that when ingested, the macromolecular phenolic compounds of biochar are expelled in the casts. Furthermore, after gut passage the biochar had different chemical characteristics than the uningested biochar: lignin biomarkers of biochar were concentrated in the casts, since the pyrolysis-produced phenolic compounds were prevalent over the aromatic biochar biomarkers.

Earthworms also have the ability to influence the availability of nutrients and heavy metals through their direct and indirect effects on microbial activity and nutrient cycling i.e., via the gut-associated (GAPs) and the nutrient-enrichment processes in worm-worked soil, particularly castings and burrows (Devliegher and Verstraete, 1996; Medina-Sauza et al., 2019). In the present case, we found differences in the chemistry of soils with and without earthworms, with lower abundance of pyrolysis-produce PAH (class 14) in SWB10 than SB10, but the role of these earthworm-induced processes in affecting macromolecular PAH are still not well known, and deserve further attention.

Only a small proportion of biochar was ingested by earthworms, considering the low percentage (10%) of biochar in the substrate, and casts from soils with biochar (CB10) were chemically more similar to those without biochar, than to the soils containing biochar but no earthworms. However, this ingested biochar was considerably chemically (i.e., increased amount of lignin biomarkers) and morphologically (i.e., presence of round-shaped bacteria, evidence of structural breakdown) modified during the gut passage. In fact, earthworms tend to ingest much more soil than biochar (Topoliantz and Ponge, 2003), and although *P. corethrurus* can ingest measurable amounts of charcoal (Topoliantz and Ponge, 2005), detection of preferential ingestion is not an easy task, and merits further investigation (Domene, 2016). Earthworms may selectively ingest larger soil particles (e.g., coarse sand) or specific organic matter sources (e.g., biochar), to augment their diet or to help in the grinding process in the gizzard and intestines (Darwin, 1881; Schulmann and Tiunov, 1999). In this regard, biochar ingestion may have several benefits: it may be useful to raise the pH of ingested soil and the gut contents, as well as in catalysing gut microbial activities (Topoliantz and Ponge, 2005). It also has a porous structure that can be a refuge for microorganisms and microbial metabolites (Warnock et al., 2007), has detoxifying properties (Pietikäinen et al., 2000), adsorbs to pesticides (Liu et al., 2018; Varjani et al., 2019), and has some recalcitrant carbon fractions that may be used by intestinal symbionts (Marks et al., 2014).

The morphological structure of biochar includes pores of different diameters created in the pyrolysis process during biochar formation from biomass. Biochars can have an extremely complex network of pores, channels and fibrous surfaces (Dehkhoda and Ellis, 2013), formed from resistant biomass structures like cell walls and conducting vessels (Lee et al., 2013), and from released volatile materials (Downie et al., 2009). This structure is an ideal physical refuge for microorganisms, and the addition of biochar can change both the activity and structure of the microbial communities (Atkinson et al., 2010; Steinbeiss et al., 2009; Gómez et al., 2014). It can also affect water retention (Méndez et al., 2012). However, the passage of the biochar through earthworm guts changes the morphological structure of biochar, probably due to the abrasion with soil in the earthworms' gizzard and intestine (Barois et al., 1993). Hence, gut passage may affect not only the biochar's ability to host microorganisms, but also the biochar's water retention ability.

Therefore, the soil mixing, biochar ingestion, gut-associated and cast-associated processes induced by *P. corethrurus* activities mean that this species is not only an important agent in the incorporation of charcoal into soils (Topoliantz and Ponge, 2003;), it is also an important promoter of physical, chemical and microbial changes to biochar, which may play an important role in the functionalization of biochars in soils, their durability and impacts on soil nutrient availability and water retention. These processes were already implied in the assumption that these worms may have an important and as-of-yet unmeasured role in the formation of ADEs (Ponge et al., 2006; Cunha et al., 2016). However, further work on these topics is warranted, particularly considering the cosmopolitan nature of this species, and the increasing interest in biochar application for soil quality improvement worldwide.

5. Conclusions

Biochar application did not affect the weight and survival of *P. corethrurus* earthworms even at the highest dose used (10% w/w) in the artificial soil. However, the experimental conditions (short time-frame, artificial soil, laboratory microcosms) do not allow speculation on the possible long-term effects of biochar application in tropical soils to endogeic earthworm species. Further work on this topic is warranted, particularly in sites with species other than the cosmopolitan *P. corethrurus*, known to be relatively tolerant to biochar in soils, even at high proportions. As biochar application becomes more common-place, efforts are needed in order to adequately assess the potential impacts of different feedstock biochars on the soil fauna and their activities, particularly those that can affect ecosystem services in soils.

The Py-GC–MS proved to be an efficient tool in the detection of various pyrolysis-produced biomarker compounds in the substrates and the biochar, and in showing the impact of earthworms on the relative abundance of different components in the casts, due to the ingestion

of lignin-rich biochar. However, although the number of detected compounds was quite large (over 100), this is just the "tip of the iceberg" of the proverbial diversity of chemical compounds in soil organic matter. The present study confirms that in artificial soil conditions, biochar ingestion and passage through earthworm guts can aid in the physical breakdown of biochar, as well as chemical changes, which may enhance functionalization of the biochars. Further work is warranted on this topic, in order to better understand the implications of these results for field studies of biochar application and soil fertility improvement.

CRediT authorship contribution statement

Talita Ferreira: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Data curation, Visualization. **Fabricio Augusto Hansel:** Methodology, Investigation, Formal analysis, Writing – original draft, Data curation, Visualization. **Claudia M.B.F. Maia:** Investigation, Writing – review & editing. **Marcela Guiotoku:** Investigation, Writing – review & editing. **Luís Cunha:** Conceptualization, Supervision, Resources, Visualization, Writing – review & editing. **George G. Brown:** Conceptualization, Supervision, Resources, Visualization, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146147.

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