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Building a risk matrix for the safety assessment of wood derived biochars

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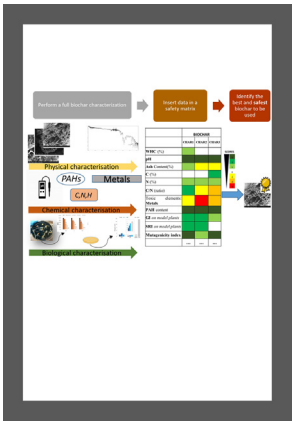
HIGHLIGHTS

- International normative require biochar characterization and quality certification.
- Specific tools are necessary to characterize and give a"go" to biochar application.
- Genotoxicity assessment can be performed as part of biochar characterization.
- Physico-chemical and biological methods of characterization complement each other.
- Altogether, this helps to generate a quality and safety assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Biochar is recognized as an efficient amendment and soil improver. However, environmental and quality assessments are needed to ensure the sustainability of its use in agriculture. This work considers the biochar's chemical-physical characterization and its potential phyto- and geno-toxicity, assessed with germination and Ames tests, obtaining valuable information for a safe field application. Three biochar types, obtained from gasification at different temperatures of green biomasses from the Tuscan-Emilian Apennines (in Italy), were compared through a broad chemical, physical and biological evaluation. The results obtained showed the relevance of temperature in determining the chemical and morphological properties of biochar, which was shown with several analytical techniques such as the elemental composition, water holding capacity, ash content, but also with FTIR and X-ray spectroscopies. These techniques showed the presence of different relevant surface aliphatic and aromatic groups. The procedures for evaluating the potential toxicity using seeds germination and Ames genotoxicity assay highlights that biochar does not cause detrimental effects when it enters in contact with soil, micro- and macro-organisms, and plants. The genotoxicity test provided a new highlight in evaluating biochar environmental safety.

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1. Introduction

Biochar is a carbon-based solid product produced from agricultural and food processing waste, manure, or sewage sludge (Yargicoglu et al., 2015; Domingues et al., 2017a; Lee et al., 2019; Qin et al., 2019; Kumar et al., 2021; Marinos et al., 2022; Rangabhashiyam et al., 2022) and it is derived from the heat-treatment of these biomasses under limited or absent oxygen supply. Biochar is known to affect atmospheric carbon sequestration by working as a CO_2 sink (Domingues et al., 2017b), to favour water and nutrient retention and for improving soil quality (Igalavithana et al., 2015),and fertility (Ding et al., 2016) and health also for stimulating soil microbiota (Gujre et al., 2021; He et al., 2021a). Moreover, biochar has the capacity to trap important toxic elements (i.e. As, Cd, Cu, Pb), alleviating human health hazards connected with human consumption of possible contaminated foods (Natasha et al., 2021). Production and use of biochar in agriculture has increased worldwide in the last 10 years (An et al., 2021), but its important properties have been known for centuries (Foss, 2005). As part of a circular economy, biochar is an ecological and eco-friendly material as it helps to improve the recovery and reuse of agricultural or food processing wastes and achieves the "*end of waste*" goal. At the European level, biochar is no longer considered a waste (European Parliament and Council, 2008) but, after an appropriate scientific assessment of its safety, it entered the European markets, form this also the definition of "soil improver". The current European (European Union Regulation, 2019a, 2019b) regulatory system establishes that biochar can be legally used as soil amendment for agronomic purposes, and biochar is legally accepted as fertilizer (Ndoung et al., 2021) in organic agriculture but only if specific requirements (i.e. maximum of 6 mg kg^{-1} of dry matter considering the sum of EPA-PAH) are met (European Biochar Foundation (EBC), 2021). In USA, the Association of American Plant Food Control Officials is engaged in the administration of fertilizer laws and regulations, but in this country, rules and accepted claims may differ from State to State. In USA a certification program coordinated by the International Biochar Initiative (IBI) allows buyers to acquire IBI Certified™ char that is characterized and considered safe (https://biochar-international.org/certification-program/). The use of biochar is allowed also in China and with many different applications (Zhang et al., 2015b). In this country, the "China Biochar Network" or CBN is actively involved in its management and further developmental strategies.

Mandatory physico-chemical analyses and biological tests need to be completed before biochar could be employed in agriculture. At the European level, biochar must be characterized following the European Biochar Certificate (European Biochar Foundation (EBC), 2021) and at international level following the IBI directory (International Biochar Initiative, 2015). All the intrinsic physical, chemical and biological properties of biochar depend upon the feedstock material and productions technique (Igalavithana et al., 2015; Xie et al., 2015; Yargicoglu et al., 2015; Marmiroli et al., 2018; Lu et al., 2020). This study aims to provide a valuable investigation of correlation between the properties of biochar and feedstock material/production conditions, focusing on gasification temperatures which can influence biochar structure and quality (Titiladunayo et al., 2012; Song et al., 2014; Ippolito et al., 2020; Natasha et al., 2021): a deeper understanding of biochar's properties can help organic agriculture towards increasing sustainability and smarter improvement (Phillips et al., 2020).

Biochar retains in its ultrastructure many of the by-products derived from the processing technology, the latter may differ substantially depending on the production process and by the operating settings used. Hence, for a safe use of biochar in soil, all the properties of biochar must be evaluated to provide a complete characterization. In particular, the biological properties of biochar can provide information about its impact on the physiological responses of plants. In addition, to ensure a safe use of biochar in soil with no potential safety issue for animals, plants, micro- and macro-organisms, and humans, we performed a short-term bacterial reverse mutagenic assay (the Ames test) on biochar extract in an organic solvent. The Ames test has provided information on the genotoxicity of a wide range of compounds which could potentially damage bacterial DNA, causing gene mutations (Mortelmans and Rupa, 2004). For many substances, the Ames mutagenic test is currently used to investigate their carcinogenicity and teratogenicity (Rainer et al., 2018). Since biochar can come into direct contact with many organisms and in all phases of its life-cycle, from production to manipulation, storage, transport and spreading it in the soil, it is paramount to investigate its mutagenicity. There are very few studies on the mutagenicity and carcinogenicity of biochar (Piterina et al., 2017), and the relationship between the feedstock material and production conditions with Ames test results has been poorly investigated. Although the release of chemical compounds from biochar can be limited and gradual, it must also be tested to consider the release of chemicals from biochar into the environment (El Sharkawi et al., 2018; Chen et al., 2019; An et al., 2021). Indeed, the high pH, salinity and the presence of contaminants such as polycyclic aromatic hydrocarbons (PAH) or heavy metals can have toxic effects, although it was demonstrated that the same is not true when biochar is mixed in soil. This effect varies depending on several aspects such as the type of biochar, application rate, soil characteristics, and presence of contaminants (Stefaniuk et al., 2016; Kończak et al., 2020; Godlewska et al., 2021; Sun et al., 2021). Therefore, the Ames test is going to become a new promising tool for a more complete investigation of biochar safety, as the latter may enter human food chain and accumulate permanently in soils and in the environment. Hence, only a complete characterization of biochar features allows to choose the more eco-friendly, sustainable, and efficient amendment with also the best ratio between original biomass production parameters and safety properties of the final product. In addition, it is worth mentioning the economic value which rises strong interest within agri-food industries and in the energy field. The aim of this work was to investigate how settings in the production of biochar, especially the temperature of production, influence its characteristics, including biochar genotoxicity, and to create a risk matrix that can guide for the safety evaluation of biochar. An approach, the latter, that can be transferred and applied for the safety definition of other type of biochars.

2. Material and methods

2.1. Feedstocks and biochar production

To promote sustainability and circular economy, the feedstocks used in this study were mixed broadleaf woods as they are widely, and locally, available. Wood derived biochar has various environmental application and to obtain one fit-for-purpose, appropriate conditions for its production need to be carefully selected (Rangabhashiyam and Balasubramanian, 2019; He et al., 2021b; Hu et al., 2021). It has been reported that the temperature of pyrolysis is one of the factors that has the highest impact on the characteristics of the final product (Zhang et al., 2015a; Hassan et al., 2020). Wood has a typical composition of about 18–35% lignin, 65–75% cellulosic carbohydrates, with approximately 25–29% cellulose, of which 49–50% carbon, 5.4–6% hydrogen, 33.5–44% oxygen, 2.3% nitrogen, 0.3% sulphur and traces of several metal ions (Pettersen, 1984; Ghysels et al., 2019). The feedstocks used in this study derived from broadleaf woods (both hard and soft) started with an average composition of 18–20% lignin, 70–72% cellulosic carbohydrates, with approximately 19–25% cellulose. The composition of C and H in different wood plants changes only few percent and similarly the lignin content of wood is little variable, often changing only a few percent (Lamlom and Savidge, 2003; Novaes et al., 2010). The feedstocks were collected from different areas of the Tuscan-Emilian Apennines (Italy) to obtain: Borgotaro Grigio (BG), Correggio (CG), and Modena Tomaselli (MT), but all from the same type of broadleaf woods allowing to focus the research primarily on the influence of the two different industrials set up and two different temperatures employed for biochar production. Biochar yield from wood is close to 10%. Specifically, BG was produced at 500–600 °C low constant temperature by an industrial fixed bed, downdraft system, open core, compact gasifier where the wood and the gas move in the same direction (produced by Advanced Gasification Technology (AGT), Italy). and MT was produced with the same technology but at 900–1000 °C high constant temperature AGT

uses two co-generation implants, 250kWe each (details on gasification plant can be found in (Lugato et al., 2013) CG was produced by the electric company EVA (managed by EN.COR, Correggio, Italy) at 900–1000 °C using a downdraft system (250kWe power). Briefly, an automatic belt brings the wood residues to a drying system connected to a gasifier which consists of a reactor (high temperature vessel where the feedstock is conveyed, broken down and converted to syngas), a scrubber system (liquid spray cooling system that allows to wash out dust and tar), electrostatic filters (filters necessary to further clean the gas that can be used to fuel electrical generation engines) and a control system cabinet. During the reaction process, the biochar produced is collected at the bottom of the reactor and passed in a pelletizer to obtain biochar pellets (biochar produced ~27 kg/h). For all processes, the residence time was two hours. Diagrams of the different production schemes are reported in Supplementary materials (Fig. S1A, B). The starting point was to acquire information on three different types of biochar produced with the same feedstock (broadleaf woods), with the same industrial plant, but at two different temperatures (BG and MT), and using the same temperature, but in two different industrial plants (MT and CG). These data (chemical, physical and biological) were integrated within the final tool of this work: to establish a risk assessment matrix and to identify ranking criteria to guide the safe use of biochar in agriculture. The workflow of the investigation is reported in Fig. S2.

2.2. Samples preparation

All samples were prepared according to International Organization for Standardization (ISO) 13,909–4 (ISO13909-4, 2016). After homogenization, biochar samples were divided into representative portions. All air-dried samples were sieved in a vibratory mill with progressive 4 mm, 2 mm, 0.71 mm, 0.06 mm sieves before analysis according to UNI EN 15428 (EN European Standards, 2007), with minor modifications. Sieved samples were divided into different particle-sizes, but only the intermediate ones (0.71–2 mm) were studied as they represent the most widely used in agriculture (Gah, 2016).

2.3. Physical-chemical analyses for biochar characterization

2.3.1. pH and electrical conductivity (EC)

The pH and electrical conductivity (EC) values of each sample were measured with a glass electrode (SevenCompact Duo, Mettler-Toledo, Columbus, OH, United States) in a 1:5 (*v*/v) biochar/deionized water mixture after 1 h shaking and stabilization, according to ISO 10390:2005 and UNI EN 13038:1999 respectively (European Committee for Standardization (CEN), 1999; ISO 10390, 2005).

2.3.2. Bulk density

The bulk density was evaluated according to ISO 23499:2008 protocol (ISO23499, 2008) with minor modifications. The samples were filled into a graduated cylinder with a capacity of 100 mL and the mass determined by weighting. Results were expressed in g L^{-1}.

2.3.3. Water holding capacity (WHC)

The WHC was measured according to Yu (Yu et al., 2013), with minor modifications. Samples of known weight were oven-dried at 105 °C for a standard drying time of 24 h. Then, water was added to each sample until excess was observed. Samples were then allowed to sit for 24 h to assure homogeneity of water content throughout the sample. After that, samples were drained by gravity for another 24 h through a Whatman filter paper n. 41 and weighed to determine wet mass. Results yielded the amount of water being held by each mixture and were expressed as a percentage of retained water according to the protocols.

2.3.4. Dry matter content (DMC)

Dry matter content (DMC) was calculated according to UNI EN 13040:2008 protocol (European Committee for Standardization (CEN), 2008). At least 50 g of each sample were put in a convection oven at 103 °C for 24 h to eliminate water and volatile molecules and then the samples were re-weighted.

2.3.5. Organic matter content (OMC) and ash content

Organic matter content (OMC) and ash content were evaluated according to UNI EN 13039:2011 (European Committee for Standardization (CEN), 2011). Biochar samples were oven-dried (M710 Thermostatic Oven, F.lli Galli, Milan, Italy) at 103 °C to a known constant weight, and then incinerated at 450 °C in a muffle furnace (Model A022, Matest S.p.A, Bergamo, Italy) for 15 h. After incineration, residues were weighted, and OMC and ash content were calculated as the difference between the fresh and final incinerated weights. Results were expressed as a percentage of the initial total dry weight.

2.3.6. Elemental composition (C, H, N, O)

Elemental analysis was conducted for each sample to determine C, H, N, O contents of the biochars according to UNI EN 13654–2 (EN European Standards, 2001). Dried biochar samples were milled through a 1-mm sieve (Cutting mill SM 300, Retsch® mbH, Haan, Germany). To determine the total C, H, and N content, ground samples of 0.15 g were loaded into tin foil cups and analyzed. The analysis was performed with a LECO Truspec® CHN Analyzer (LECO Corporation, Saint Joseph, Michigan, USA). In addition to the elementary composition, the associated elementary ratio was considered. The H/C and O/C ratio provides important information on the structure of biomass. The O fraction and H/C, O/C, C/N molar ratio were calculated as previously described (Wijitkosum and Jiwnok, 2019). The organic material within biochar (5 g) was mineralized in boiling sulfuric acid and Kjeldahl solution. In this process the organic nitrogen is converted to ammonium sulfate, alkalizing the solution liberates ammonia which is quantitatively steam-distilled in 2% boric acid and determined by titration with 0.1 N sulfuric acid (Rorison et al., 1976). To determine nitrite and nitrate, the protocol EPA 300.0 protocol was used (Edgell et al., 1994). Briefly, char (4 g) was mixed with water (40 mL) for 10 min, the mix was filtered in a 0.45 μm mesh and analyzed by HPLC with a conductivity detector (ICS6000 DP, Thermo Fisher Scientific).

2.3.7. Mg and P concentration

Mg and P were determined following the protocols UNI EN ISO 13657:2004 and UNI EN ISO 17294-2:2018 (ISO13657, 2004; ISO17294-2, 2018). Briefly, 0.5 g of biochar were microwave-assisted digested with aqua regia (8 mL), filtered and brought to a volume of 50 mL with distilled water before being analyzed by ICP-MS (ICAP Q, Thermo Fisher Scientific) and the concentration derived with calibration samples.

2.3.8. Metal concentration

The concentration of metals was determined by Atomic Absorption Spectrometry (AAS) method according to the EBC (Schmidt et al., 2015). Biochar samples were incinerated in ceramic crucibles in a muffle furnace at 500 °C for 16 h. Then, all the ash samples were retrieved from the crucibles and digested with a three-step method with nitric acid 65% (Carlo Erba, Milan, Italy) at 165 °C for 30 min, 200 °C for 30 min, and finally 230 °C for 30 min in a heated digester thermoblock (DK20, Velp Scientifica, Usmate Velate, MB, Italy). Digested solutions were diluted with deionized water to 30% (*v*/v) acid concentration. Elements' concentration was recorded using AAS (AA240FS, Agilent Technologies, Santa Clara, CA, United States). Calibration curves for each metal were prepared using 1000 ppm certified standard solutions (Agilent Technologies, Santa Clara). Three technical and biological replicates were performed for each sample. The metal concentrations were expressed in mg kg^{-1} biochar.

2.3.9. Zeta potential evaluation

The zeta potential of biochar surfaces was determined to characterize their chemical environment, using a protocol previously described (Batista et al., 2018). A 0.05 g sample of biochar, previously sieved through a 0.5 mm mesh was weighed into 250 mL polyethylene bottles, into which 200 mL of 0.15 mmol L^{-1} $NaNO_3$ was added. The suspensions were

dispersed ultrasonically for 1 h and then divided into six parts, which were then individually poured into 100 mL plastic bottles. The pH of the suspensions was adjusted within the range from 3 to 10 using NaOH or HNO_3. After the pH had stabilized, the suspensions were let to stand overnight. Then the zeta potential was measured using a Zetasizer Nano Series ZS90 (Malvern Instruments, Malvern, UK).

2.3.10. Environmental Scanning Electron Microscopy (ESEM) evaluation and EDX analysis

An Environmental Scanning Electron Microscope ESEM FEG2500 FEI (FEI Europe, Eindhoven, Netherlands) with energy dispersive X-ray spectroscopy (EDX) was used to investigate the surface morphology: all the macro, micro, and nano pores of biochar structures. Samples of biochar of about $1cm^2$ were positioned on a microscope stub attached with double adhesive tape. No preparation of the sample was necessary for the analyses. Analysis during acquisition were in point analysis mode. The working parameters were as follows: the working distance was approximately 10 mm, and the scanning time 1–3 μs, environmental low-vacuum (60 Pa) with a large field detector to allowed optimal secondary electron (SE) imaging, performed at 5 kV and 10 kV with a beam size of 2.5 μm and 20 kV with beam size of 4 μm for the EDX analysis. (Marmiroli et al., 2018). EDX analysis was carried out with a Bruker XFlash®6 | 30 X-ray detector, the acquired spectra (for Mg, Ca, K, P, Mn, Si, Ti, Al, Fe, Cu, Zn, Co, Cd) were fully deconvoluted and standard-less quantification was performed using the P/B-ZAF (Peak/Background evaluation matrix with atomic number (Z), absorption (A), and secondary fluorescence (F) correction) interactive method supported by Esprit 1.9 "Quantify Method Editor" option software.

2.3.11. Other analytical methods

For details on the methods used for: porosity of char, PAH Content, X-Ray Diffraction, and Fourier Transform InfraRed Spectroscopy with Attenuated Total Reflection (FTIR) refer to Supplementary Materials.

2.4. Phytotoxicity tests for biochar characterization: germination test and root elongation assay

The germination test and root elongation assay were carried out following the ISO 11269-1 (ISO11269-1, 2012) and ISO 11269-2 (International Organization for Standardization, 2012) protocols with minor modifications. Seeds of *Pisum sativum* L. and *Hordeum vulgare* L. were used. Four doses of each biochar (0.5, 1, 3, and 5% *w/v*) were used and added to MS agar media plates (0,5% Murashige and Skoog basal medium, 2% sucrose). Seeds were surface sterilized using 70% (*v*/v) ethanol for 3 min and 50% (v/v) sodium hypochlorite (Sigma Aldrich, St. Louis, USA) for 30 min, rinsed with deionized water and air dried. All the seeds were chosen after a previous screening to control germination rate (>80%). Nine seeds were placed on each MS + biochar petri dishes. Sealed plates were incubated in a growth chamber at 25 °C for 72 h in darkness.

Root length was observed and germination index (GI%) and root/shoot index (SRI) were derived, as below.

$$GI\% = (Gt * Lt/Gc * Lc) * 100.$$

where Gc = germinated seeds in the control; Gt = germinated seeds in the treatments; Lc = main root length in the control (measured in mm); Lt = main root length in the treatments (measured in mm).

$$SRI\% = (Ls/Lr)^* 100$$

where Ls = average shoot length (measured in mm) and Lr = average root length (measured in mm).

2.5. Genotoxic analysis

2.5.1. Biochar liquor extraction with DMSO

Five g of each air-dried biochar sample (size 710-2000 μm) were added to 300 mL of 1:1 acetone:hexane mixture for ten hours with Soxhlet instrument to extract soluble analytes, including mutagens and pro-mutagens compounds. The solvent was evaporated by rotavapor and the resulting fraction was weighed, recovered by means of 2 mL of 1:1 acetone:hexane mixture and finally dried. The extract was resuspended in 5 mL dimethylsulfoxide (DMSO) (Fisher Scientific Italia, Milan, Italy) to a final concentration of 1 g mL^{-1}.

2.5.2. Bacterial reverse mutation assay (Ames test)

Before each set of experiments, bacterial cultures were prepared fresh in Oxoid n.2 specific rich medium (Oxoid Ltd., Basingstoke, Hants, UK) and the genotype of each *Salmonella typhimurium* strain was checked (Maron and Ames, 1984). For the assay *S. typhimurium* TA98 and TA100 were used to test the potential genotoxicity of biochar extract in DMSO with and without the rat liver extract (S9 mix) (Trinova Biochem (Giessen, GmbH, Germany), which activates any pro-active or inactive compounds, simulating the liver metabolism in higher organisms. The Ames test was carried out with the preincubation procedure (Maron and Ames, 1984; Tejs, 2008; de Mello Silva Oliveira et al., 2016) with minor modifications. All the extracts were placed in a solution with bacteria, sterile buffer or rat liver extract (S9 mix) and left to react for a period between 20 and 30 min in an incubator at 28 °C while shaking. Experiments were conducted separately for each bacterial strain and three biological replicates were assessed. The pre-incubation mixture was prepared as follows: 0.1 mL of fresh bacterial culture (final concentration of 10^6–10^7 cells mL^{-1}), 0.5 mL of sterile buffer or S9 mix and 0.1 mL of 100-times diluted test samples or 0.1 mL of control sample. After the incubation time, the samples were added to the freshly prepared "top agar" containing histidine and biotin which allow for a few cell divisions during the initial phase of the incubation period, and then the samples were poured into Petri dishes. The top agar was left to dry and then test plates were turned upside down and incubated at 30 °C for 48 h. This procedure has been shown to be more sensitive to mutagens than standard incorporation methodology (Tejs, 2008). Revertant colonies were counted and compared to the number of spontaneous revertant colonies on solvent control plates. The test samples and the control substances were dissolved in organic solvent. Positive control was 2-aminoanthracene (2-AA); negative control was DMSO, both purchased from Sigma Aldrich (USA). They were diluted prior to treatment and the final concentration used was 1 μg mL^{-1}. Positive and negative controls were used fresh to avoid problems due to chemical instability. The S9 mixture was prepared according to Maron and Ames (1984).

Data from the Ames test were reported as mutagenic index expressed as the ratio of number of reverted colonies in tested plates versus the number of reverted colonies in the negative control plates as indicated previously (Vargas et al., 1995; Piterina et al., 2017). In this way, inaccurate measurements due to the presence of spontaneous reverting colonies were reduced.

2.6. Statistical analyses

Observations were made at least in triplicates for each analysis. The analysis of variance (ANOVA) followed by Tukey's post hoc test (Tables S1 and S2), and the PCA analysis were performed with IBM SPSS v.27.

3. Results and discussion

3.1. The porous structure of biochar

One of the fundamental properties of biochar is linked to its porous structure and is its great ability to retain water molecules in the macro-, micro-, and nano-pores and to return it to the soil at appropriate times, in unfavorable environmental conditions. The nano-sized cavities are those

that gave biochar the characteristic of being considered like a nano fertilizer (Marmiroli et al., 2018). Bulk density and porosity play an important role in determining biochar behavior once in the soil and in modifying the soil properties such as decreasing the bulk density (Blanco-Canqui, 2017) thus influencing the ecosystem services provided from the soil. In our case, bulk densities were statistically different in the three samples in relation to temperature with values ranging from 0.14 g L^{-1} (MT) to 0.25 g L^{-1}(C) (Table 1). The porosity of biochar was clearly observed by ESEM analysis (Fig. 1A-C), but there were no major structural differences among the three samples. All cavities sized from few μm to few mm for the larger ones.

The gasification process is often linked to increasing porous structure formation of biochar (Oni et al., 2019) and this interconnected porous network has a role in soil aeration (Aslam et al., 2014; Barnes et al., 2014) influencing the soil texture and bulk density, and in the absorptive caption capacity of biochar for water and nutrients. Pores can be classified in three groups according to the

standard of the International Union of Pure and Applied Chemistry

(IUPAC): macropores (>50 nm), mesopores (2–50 nm), and micropores (<2 nm) (https://iupac.org/). The porosity of the three biochar has been measured and data are reported in Table S3. Total pore volume was similar and high for MT and BG (values ranged between 0.27 and 0.34 cm^3/g), while the pore volume of CG was low (around 0.07 cm^3/g) and CG has also the lowest Specific Superficial Area (SSA) (Multi-point BET, Brunauer–Emmett–Teller theory, (m^2g^{-1})) (Table S3). In addition, moderate temperatures (400–700 °C) are suitable for the development of the pore structure (Leng et al., 2021).

Biochar can bind nutrients and microorganisms making them available to plant roots caption during their soil expansion (Aslam et al., 2014). The hydro-physical properties of biochar are among the most important beneficial characteristics of biochar, as water is a key factor influencing agricultural production (Singh et al., 2019). Sometimes the plant water demand is not well balanced to water availability (i.e. marginal lands, spoiled lands deriving from excessive industrial agriculture, arid lands etc.). In these negative environmental circumstances, biochar has been proposed as the tool to capture water from the soil and let it be available to plant roots as much as needed (Basso et al., 2013; Batista et al., 2018), an effect observed when biochar was applied in coarse-textured soils (Schmidt et al., 2021). The effect is observed with very high application rates, unless the addition of biochar would be done only around the plant roots, a strategy that would allow to achieve a high local concentration and, consequently a high water retention in the areas where this is mostly needed (Jeffery et al., 2011; Nelissen et al., 2015; Schmidt et al., 2021). The most explanatory and indicative hydro-dynamic property of biochar is the Water Holding Capacity (WHC). The WHC depends on the porosity of biochar's bulk volume: at lower gasification temperatures, the hydrophilicity of biochar increased and in fact the WHC value of biochar BG was about 80% whereas for CG was 45% and 70% for MT (Table 1). An increase in the gasification temperature leads to an increase in the hydrophobicity of biochar, which also depends on the composition of the feedstock material.

Table 1
Physical and chemical characterization of the three biochars.

Properties	Units	BG	CG	MT
Particle-size	mm	0.71–2	0.71–2	0.71–2
pH	pH	10.17 ± 0.11 b	9.28 ± 0.13 a	10.38 ± 0.04 c
EC	μS/cm	4581.5 ± 2.12 c	476.8 ± 0.99 a	792.05 ± 4.6 b
Bulk density	g/cm^3	0.17 ± 0.002 b	0.25 ± 0.01 c	0.14 ± 0.008 a
WHC	%	80.16 ± 17.7 b	45.37 ± 10.5 a	70.87 ± 12.9 b
DMC	%	97.39 ± 1.59 b	93.30 ± 0.23 a	94.41 ± 3.69 b
OMC	% d.w.	64.16 ± 19.62 a	86.45 ± 2.34 b	90.86 ± 4.54 b
Ash content	% d.w.	35.84 ± 19.62 b	13.55 ± 2.34 a	9.14 ± 4.54 a

Means of three biological replicates are reported in Table 1 with standard deviations. Letters means significantly differences at $p < 0.05$ (Tukey HSD test) (see also Tables S1 and S2). In Table 1 are illustrated pH, electrical conductivity (EC), bulk density, water holding capacity (WHC), dry matter content (DMC), organic matter content (OMC), and ash content values for BG, CG, and MT biochar samples.

3.2. pH and EC of different biochar

Chemical and physical characterization of the biochar has been provided both to better know its microstructure and internal organization (see Fig. 1) and to correlate the quality of the biochar obtained to the production parameters, especially temperature.

Biochar is known to be alkaline as pyrolysis induces the separation of acidic functional groups early in the carbonization process (Zhang et al., 2015a; Zhou et al., 2021). Indeed, in this work the pH values were greater than 9 but there were some differences among the three biochar (Table 1). The process temperature evidently affected pH values of biochar. Hence, although the pH value of biochar produced at the lower temperature may not seem much different from the others, it is in the composition of the side groups and of their charges that rests the reactivity of biochar.

The EC is an intrinsic property of biochar and was different between the three samples which can be related to the presence of minerals in the biochar structure (Table 1). The EC value of BG was much higher than MT and CG indicating again that the function of the internal structure was dependent on the production temperature. This effect is opposite to what reported previously, and it could be related to the not observed decrease of salt concentration and elements during the loss of volatile materials and due to the possible decomposition of some salts (Cantrell et al., 2012; Huang et al., 2017; Kończak et al., 2019).

Dry Matter Content (DMC) marks the quality of biochar in terms of both organic matter and ash contents: higher DMC values normally reflect higher value biochar, according to EBC guidelines (2015). Organic matter and ash content showed significant differences between BG, CG and MT (Table 1). All values of organic matter, however, were high and suggestive of good quality of biochar. According to EBC and IBI indications, a biochar of quality should contain organic matter up to at least 50% of its dry weight. The quantity of ashes is an indicator often linked to the pH values: higher amounts of ashes lead to higher pH values because ashes contain a higher amount of minerals and inorganic substances which are strictly dependent on feedstock biomass (Nartey and Zhao, 2014), therefore its application is particularly favored in acidic soils. On the other hand, biochars with low ash content are easier to transport and to incorporate into soil (Tomczyk et al., 2020).

The alkaline state of biochar, has been linked to its capacity to increase the pH of acidic or neutral soils (Jeffery et al., 2011), an increase in soil pH showed a positive outcome on crop productivity (Jeffery et al., 2011). Indeed, this modification could change the form of some toxic element such as aluminum (Paz-Ferreiro et al., 2020). This could result in an improvement of soil fertility and an easier absorption of nutrients at the root level of a crop of interest (Cheng et al., 2006; Laird et al., 2010; Wang et al., 2014; Ding et al., 2016).

3.3. Chemical composition

3.3.1. C, H, O and N composition

Table 2a reports the percentages of different elements and their ratios which provide information about the composition and the structure of biochar and its relationship with feedstock biomass and production process. All the three samples exhibited a large amount of C (%), based on the IBI and the EBC standards, this is a key characteristic as biochar used as an amendment can enhance soil carbon concentration and therefore improve soil quality (Dong et al., 2019). The H/C, O/C and C/N ratios also depend on feedstock and process temperature and are indicative of biochar sorption properties. A decrease of H/C and O/C results from dehydration and decarboxylation reactions. The O/C ratio reflects the polarity and the abundance of surface functional groups containing polar oxygen in biochar; a higher ratio indicates that more polar functional groups are present. These groups actively take part in adsorption of heavy metals and polar organics (Xiao et al., 2018). The H/C ratio reflects aromaticity and stability of

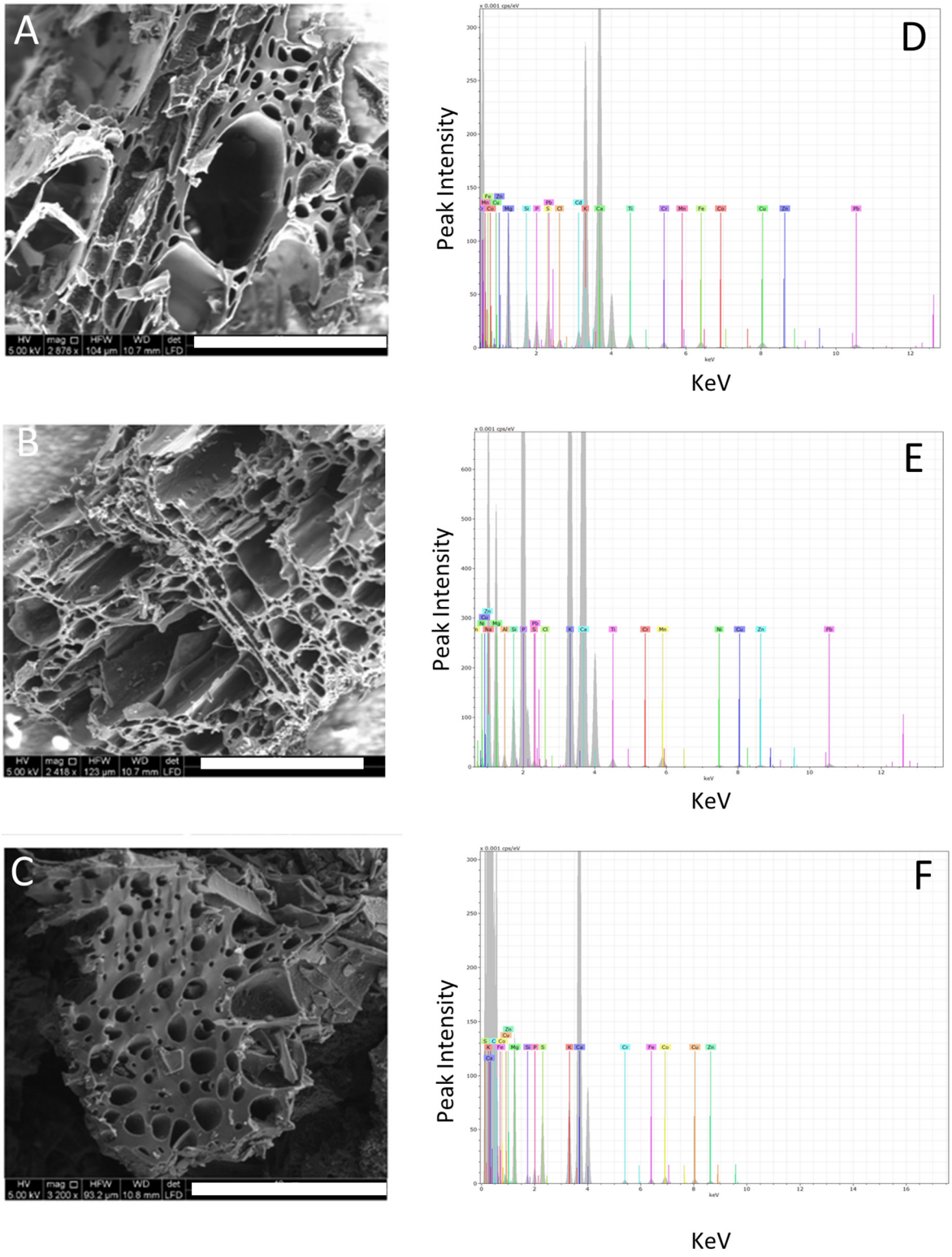


Fig. 1. Structures and EDX spectra of biochar. Left panel: images of internal structure and composition of BG (A), MT (B), and CG (C) biochars by ESEM with evidence of microsized cavities (scale bar is 50 μm in A and B and 40 μm in C). The pores have also different shape and distribution. In the second panel on the right: deconvoluted X-ray emission spectra acquired from biochars BG (D), CG (E), and MT (F). The electron beam energy was set at 20 KV and the acquisition live time at 60 s. In each spectrum, the X axis represents the X-ray energy in keV, and the Y axis represents the elemental peak intensity equivalent to the percentage of the element among all the others. A standard-less quantification was performed using the P/B-ZAF (Peak/Background evaluation matrix with atomic number (Z), absorption (A), and secondary fluorescence (F) correction) interactive method supported by Esprit 1.9 "Quantify Method Editor" option software.

biochar and generally decreases with increasing heating temperature (Xiao et al., 2016) as seen for C and MT compared to BG. A higher H/C ratio refers to the predominance of surface chemical bonds while lower values to pore-filling (Wei et al., 2020). H/C ratio values of our biochar are relatively low considering the existing literatures and correlated with the temperature of the production process. The use of high C/N ratio biochar is not

Table 2
Chemical properties of biochars Borgotaro Grigio (BG), Correggio (CG) and Modena Tomaselli (MT).

A

	C-H-N-O % compositions and ratios								
	C (%)	H (%)	N (%)	O (%)	H/C	O/C	C/N	O/C*	H/C*
BG	58.29	3.75	3.40	34.56	0.06	0.59	17.14	0.44	0.72
CG	54.75	1.54	0.48	43.23	0.03	0.79	114.06	0.59	0.36
MT	72.42	1.05	0.01	26.52	0.01	0.37	7242.00	0.28	0.12

B

	Chemical composition (II) (mg kg^{-1})														
	NO_3^-	NO_2^-	N tot	P	Mg	Ca	K	Mn	Cd	Cr	Fe	Ni	Pb	Cu	Zn
BG	16	12	848	1500	5200	883.7	93.4	1664	**2.3**	0.39	1086	**55.2**	23.3	52.0	313.7
CG	8	10	5118	1300	5840	247.5	55.3	232	0.05	<LOD	4104	12.1	22.7	38.7	134.1
MT	10	11	3421	950	3790	290.0	859.1	263	<LOD	<LOD	1155	1.7	42.5	19.4	22.1

A: shows the chemical composition of biochars in %, in table the following elements were investigated: C (carbon), H (hydrogen), N (nitrogen), O (oxygen). H/C, O/C, and C/N ratio were calculated or their atomic ratio (see *). Data are reported as percentage of element on dry weight basis.
B: shows the chemical composition of biochars in terms of main elements, data were expressed as mg kg^{-1}. Interestingly is high concentration of N in CG, and the amount of P in BG and CG. All the other elements tested (i.e. As, Co, Hg) were below the detection limits. Bold type referred to values above the EBC§ guideline upper limit threshold. <LOD: below the limit of detection.
* Atomic ratio.
§ European Biochar Certificate guideline: Cd <1.5 mg kg^{-1} and Ni < 50 mg.

recommended in nutrient-depleted soils as in this case N is too low to mobilize microbes (Clough et al., 2013; Farkas et al., 2021). Therefore, MT biochar should be used with caution, maybe in parallel with the addition of N fertilizers. Moreover, the total concentration of N was highest in CG, followed by MT and BG, confirming that increasing the temperature of production increases N content as the latter is trapped along with C into aromatic or heterocyclic rings (Almendros et al., 2003; Wang et al., 2012). NO_3^- and NO_2^- concentrations were very low in all samples. Considering the atomic ratios of O/C and H/C and their distribution in a van Krevelen diagram (see Fig. S3) we can see that BG char holds a position close to the one expected for lignin. The increase in the temperature used for the production of MT and CG moved the two biochars in a region with poor hydrogen structures consisting of condensed aromatic rings and hydrocarbons, and especially for MT which is found in a region typical of soot biochar (Kim et al., 2003; Hammes et al., 2006).

3.3.2. Metal and pollutants content

Biochar has been recognized as a carrier for many elements, which can improve soil mineral composition (Sashidhar et al., 2020), important to assure good plant health, but high metal concentrations can be of concern. The elements of more concern for agriculture in biochar samples are reported in Table 2b. Metal concentrations were below the IBI and EBC guideline thresholds in all three biochar samples, although cadmium (Cd) and nickel (Ni) were close to the upper limit of the EBC requirements (Cd <1.5 mg kg^{-1} and Ni <50 mg kg^{-1} for EBC and Cd 1.4–39 mg kg^{-1} and Ni 47–600 mg kg^{-1} for IBI) in BG sample.

The three biochar types were analyzed for 16 PAHs and in all the samples the values were below the threshold established by International Guidelines (< 4 mg kg^{-1} for EBC and 6–300 mg kg^{-1} for IBI Biochar Standards V2.0). As shown in Table S4, samples BG and MT had 0.25 and 0.15 mg kg^{-1} phenanthrene respectively, which are both far below the allowed value. All the other PAHs were under the detection limit and, consequently, the permitted amounts.

3.3.3. Elemental composition

The total elemental composition was determined via EDX analysis (Fig. 1D-E), revealing the presence of trace elements. In Fig. S.4 are reported examples of the percentage of each element detected in some of the different samples of biochar, since the spectra are fully deconvoluted the tables report the percentage of each element in every sample. In addition to the metals detected by AAS, EDX indicated that Ca, K, Mn are often present in high concentrations as they are common in natural feedstocks (see Table 2B). P was highest in MG and MT with values in the range for biochar derived from mixed hardwoods, pure maple, pine, and much lower than biochar derived from poultry litter (Freitas et al., 2020). The presence of Ti, Mn, and Al are due both to the raw material composition and to their release from the friction of the material constituting the gasifier used to convert biomass to biochar and which comes into direct contact with biomasses or from the material of which are made the shearers and harvesters.

3.3.4. Characterization of functional groups present

The FTIR spectra of the three biochars are shown in Fig. S5. These spectra suggest that there was a variety of functional groups typical of oxygenated hydrocarbons on a graphene sheet type surface (Tomczyk et al., 2020), aromatic groups that have different resistance to the temperature and therefore, will undergo different modifications (Stefaniuk and Oleszczuk, 2015). H, N, O, P, S all were incorporated into the aromatic rings as heteroatoms (Brennan et al., 2001). The presence of heteroatoms creates a certain heterogeneity in biochar surface chemistry, caused mainly by the differences in electronegativity of heteroatoms as compared to that of carbon (Xiao et al., 2018). As highlighted in Fig. S5, there were differences in the biochar surface composition between the three biochars. The FTIR spectra of the three samples showed a broadband from 4000 to 3500 cm^{-1} which can be attributed to –OH from H_2O or to phenolic and alcoholic groups (Pretsch et al., 2009; Tomczyk et al., 2020), an absorption in the region 2900–2800 cm^{-1} attributed to alkyl -CH stretching from aliphatic functional groups, and another broadband from 2500 to 1800 cm^{-1} linked to carboxyl, carbonyl acids and carbon monosubstituted alkynes stretching. The presence of carboxyl and carbonyl groups conjugated ketones and quinones sulfones, and azo compounds (Abdullah et al., 2015; Tomczyk et al., 2020) is described by the bands occurring around 1600 cm^{-1}, while ketones, aldehydes and esters occur around 1735 cm^{-1} (Uchimiya et al., 2011). It is especially in these last ranges that many of the differences between the different biochars are detected. Indeed, BG biochar showed larger peaks of C═O, C═C, C═N, S═O, N═N stretching compared with the other two samples (Fig. S5A). As expected, an increase in process temperature during the production of biochar leads to an increased percentage of aromaticity (Lian and Xing, 2017), an increase in well-organized carbon layers (Uchimiya et al., 2011), but with lower content of functional groups. This has been reported to have a negative effect on the cation exchange capacity (CEC) (Mukherjee et al., 2011), and therefore also on nutrient absorption capacity of biochar.

The XRD analysis was used to evaluate the existence of a crystalline structure with the aim to detect all mineral phases present in biochars. The XRD patterns (Fig. S6) clearly showed three crystal phases for all biochar samples. In the BG sample, the lower production temperature corresponded to a more disordered structure linked to the many peaks of carbonate groups. In contrast, CG and MT biochars had a more ordered structures and fewer carbonate groups peaks. Indeed, the production temperature is one of the factors that strongly determines the structure and the composition of biochars. In a theoretical biochar structure development (Lehmann and Joseph, 2012) a lower production temperatures correlates with a higher proportion of aromatic C while higher temperatures increase the presence of sheets of conjugated aromatic C, becoming graphene like structures (Lehmann and Joseph, 2012; Tomczyk et al., 2020). $CaCO_3$ abundance as well as high pH values of biochar have been recently rediscovered as essential properties related to availability of nutrients in biochar and enhanced crop responses (Phillips et al., 2020). The quality of crystalline substances varied among samples: the three crystal phases observed in BG were fairchildite, calcite, and calcium carbide. It is worth noticing the presence of two other peaks at 2 Θ angles of 35° and 37° probably linked to the hydrogen (H_2) diffraction pattern. The presence of hydrogen in the inner structure of biochar may be due to a thermal decomposition of hemicellulose and cellulose (Collard and Blin, 2014). The diffraction spectra of biochar CG and MT showed a strong similarity in composition of different crystal substances: calcite, barbosalite,calcium silicate,and other minor peaks with a very weak intensity. The dominant materials found in BG were calcite and fairchildite while in both CG and MT samples calcite was found. Calcite can help in adsorbing toxic elements such as Pb (Ramola et al., 2020).

The zeta potential of the biochar surfaces was analyzed to understand their potential electrostatic interactions, which can play a role in absorption mechanisms. A greater zeta potential often coincides with a lower content of acidic groups on the biochar's surface that might result from the presence of $CaCO_3$, as revealed by the XRD analysis (Fig. S6). Biochar's surface charge becomes predominantly negative because of the deprotonation of oxygen-containing surface groups (i.e., -COOH and -OH groups), thus favouring the absorption of cations from solution through electrostatic attraction. Interestingly, CG and MT samples showed a similar zeta potential trend suggesting they might share an identical absorption mechanism (Table 3), especially at pH 7 and 8, which are more common in general environmental soils and are at the same time the mean pH values of soils used in this study.

The properties mostly affected were the composition of the chemical environment of both the internal and external surfaces of biochar, and consequently its biological properties. A lower temperature of production contributes to create a structurally disordered biochar with a reduced presence of ordered graphite layers. This is linked with the greater availability of functional groups inside the porous structure as shown by the FTIR spectrum. According to the FTIR spectrum of BG there was a starkly different chemical structure when compared to the other two biochar samples. Surface reactive groups were also found by zeta potential analysis and in this case the differences between biochar samples produced at different temperatures were evident. The ability to bind water molecules is closely influenced by surface chemistry (Batista et al., 2018; Xiao et al., 2018). In fact, BG showed the highest water retention capacity as compared to the other samples. In addition, the quality of the biochar depends on factors of production. BG showed the highest amount of DMC that can be divided into organic and inorganic contents, defining biochar structure, quality, and BG had the highest value among the three.

3.4. Impact of biochar on seeds germination and root elongation

Crucial to biochar analysis was the biological response of plants to biochar treatment. Both germination index (GI) and the shoot/root index(SRI) were semi-linear, dose dependent. They showed a similar response in both *Pisum sativum* and *Hordeum vulgare* cultivars (Fig. 2). The germination index decreased with increasing doses of biochar (Fig. 2, A and B), an effect observed for both the utilized cultivars. The increasing toxic effect is correlated to the higher concentration of micronutrients and salts. A relationship between the length of the coleoptile and the root could be appreciated (Fig. 2C-D). These values decreased as biochar concentrations increased. This meant that a greater presence of biochar stimulated the growth and elongation of roots but decreased shoot elongation. The result was also visually confirmed by the presence of many secondary radical hairs (data not shown). A similar effect was observed also in the field. Here, plant root biomass increased on average 32% while the root length by 52%, upon adding biochar into the soil. This effect is probably caused by the positive uptake of the nutrient and water, improving the overall plant growth (Bruun et al., 2014; Schmidt et al., 2021; Zhang et al., 2021). Moreover, from Fig. 2 it seems that a supplement of 1% *w/v* of biochar to the soil can be considered a threshold limit for toxicity in the field, corresponding to a maximum application of 20 ton hectare^{-1} in agreement with that already found by Major et al. (Major et al., 2010) and indicated by IBI guidelines (Major, 2010).

3.5. Assessing genotoxicity of biochar

Many works report that biochar has the capacity to influence microbial population in soil (Zhou et al., 2017; Brtnicky et al., 2021; Gujre et al., 2021; He et al., 2021a). The effects are various and sometimes even contradictory. Studies report that application of biochar influences activity of the microbial community or its biomass, changes the bacterial or fungal diversity, increases N_2 fixing bacteria (Dempster et al., 2012; Ding et al., 2016; Chen et al., 2017; Anyanwu et al., 2018; Andrés et al., 2019; Brtnicky et al., 2021; Zhang et al., 2021). Even if the reported effects are different, it is undoubtful that biochar interacts with the microbiome present in soil. These effects have been described at the population level, but not at the mechanistic level. Previously, it was shown that wheat straw biochar, when applied on artificial soil decreases earthworms growth and induces DNA damages (Domene et al., 2015). This is interesting, although the concentrations used where high and far from what is usually used in the fields

Table 3
Physical properties of biochar: Zeta potential values of biochars Borgotaro Grigio (BG), Correggio (CG) and Modena Tomaselli (MT) at pH range from 3 to 10.

	BG		CG		MT	
pH	AV	s.e.	AV	s.e.	AV	s.e.
3	−16.75	0.93	−14.67	1.10	5.98	0.62
4	−15.99	0.47	−32.59	1.43	−5.94	0.89
5	−11.63	0.84	−35.14	1.10	−31.83	0.82
6	−16.97	0.27	−33.90	1.01	−34.26	2.11
7	−18.29	0.52	−35.16	1.47	−31.03	2.42
8	−17.31	0.20	−28.30	1.22	−33.54	1.40
9	−27.05	1.05	−33.86	0.53	−37.80	0.43
10	−21.72	1.64	−39.12	2.04	−35.91	0.84

Averaged data (AV) were expressed in mV ± standard error (technical $n = 3$) of solutions with dissolved biochar adjusted at different pH values with a focus for pH values of 7 and 8 (grayed-out).

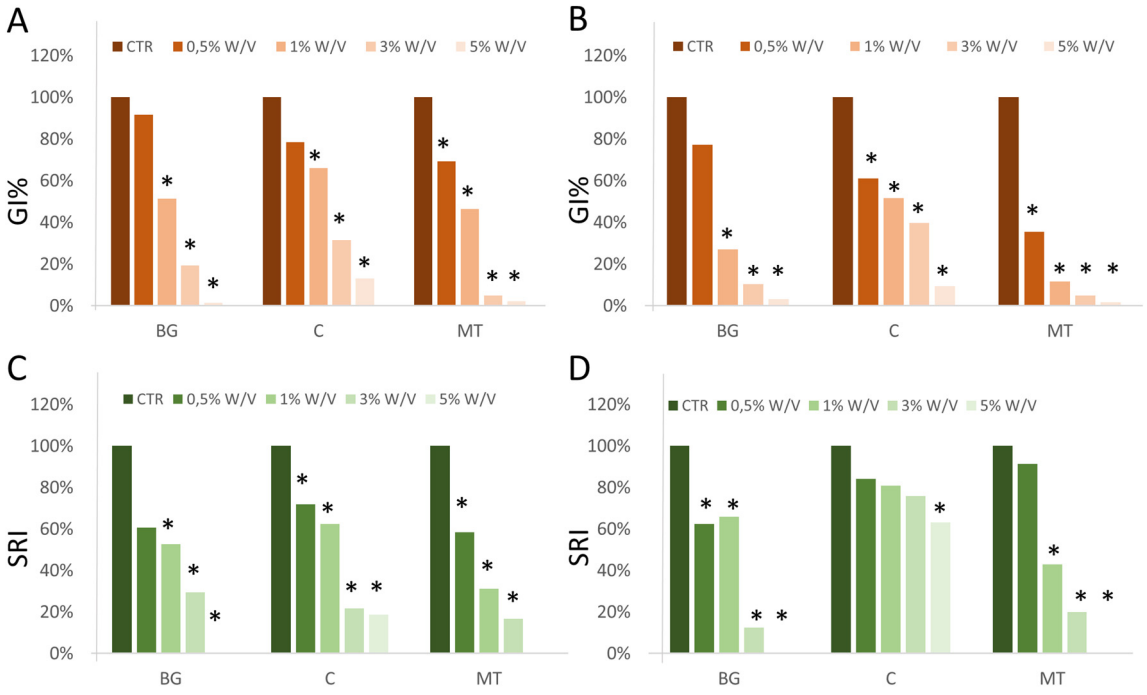


Fig. 2. Effects on Germination Index (GI) expressed as percentage in pea (*Pisum sativum* L.) (A) and in barley (*Hordeum vulgare L.*) (B) treated with different doses of biochar samples. Shoot/Root Index (SRI) expressed as percentage of pea (*Pisum sativum L.*) (C) and barley (*Hordeum vulgare L.*) (D) treated with different doses of biochar samples. 0, 0.5, 1, 3, and 5% *w/v* biochar concentration shown on x axis. *corresponds to statistically different values compared with the control (one-way ANOVA, Tukey's test, $p < 0.05$).

(Brtnicky et al., 2021). In our work the potential genotoxicity of biochar was evaluated for each sample considering organic extracts in DMSO. Biochar was tested at concentrations close to field conditions. The results showed two slightly different situations when comparing the mutation results obtained with the two tested bacterial strains. This was due to the different sensitivity of each strain towards the chemical compounds in the extracts analyzed. Fig. 3 illustrates the mutagenicity index of biochar extracts in DMSO for each tested sample and each microbial strain, in the absence (—S9) or presence (+S9) of rat liver microsomal fraction.

The effect of biochar extracts on *Salmonella typhimurium* TA98 strain is shown on the in Fig. 3A. Here, there is a great difference in mutagenic index between positive and negative controls. The data were then separated by S9 activation (+S9) and absence of activation (—S9). Monitoring the effect on *Salmonella typhimurium* TA98, all biochar extracts showed a mutagenic index below that of the positive control. In the case of BG and MT, activated extracts had indices comparable to the negative control, while biochar CG extracts, that had been activated with +S9, acted as promutagen since the mutagenic index was almost twice that of the negative control. The behavior of biochar extract samples without activation is shown in the middle of Fig. 3A and B.

Although all biochars had mutagenic potentials below the negative control limit, there were also differences between the samples. BG showed the lowest index as compared to CG and MT. BG was the biochar produced at lowest temperature. Fig. 3Bshows the effects of biochar extracts on *Salmonella typhimurium* TA100. In general, none of the samples showed a strong mutagenic activity as their indices were below the positive control maximum and were not at least twice as high as the negative control. In the case of BG, even when DMSO extracts were activated by S9, this biochar had a mutagenic index lower than the accepted threshold limit of non-mutagenicity. Instead, in the case of CG and MT, when these samples were activated by the S9 mix, their mutagenicity indices became higher than the threshold level. But the difference was small and not sufficient to indicate a mutagenicity issue for these types of biochar. In the case where the samples were not activated by +S9 (Fig. 3), all mutagenicity indices were below the allowed threshold level, but with differences between samples. Biochar CG showed the highest mutagenic index, followed by MT and BG. The tested dose was a 100-fold dilution of the extract in DMSO (which had concentration 1 mg mL $^{-1}$) which corresponds to the upper limit concentration for phytotoxicity found in the biological germination tests. Therefore, although there is a different sensitivity of each bacterial strain to biochar samples, none exhibited a mutagenic character at the concentrations tested. In all cases, BG biochar had the lowest mutagenicity index of all, both in the presence of the metabolic liver extract and in its absence. In accordance with what we have found with the Ames assay on plant derived-biochar, Piterina and colleagues (Piterina et al., 2017) found that temperature and times of pyrolysis are important. For example, biochar pyrolysed at 400 °C for 10 min, from a lignocellulose precursor was mutagenic, but not when formed at 800 °C for 60 min, or at 600 °C for 30 min, the latter are conditions close to the one used in this work. Biochars from poultry litter, and manures of calves fed on grass had low mutagenicity; biochar from pig manure had high mutagenicity; biochars from cow manures and biochars from solid industrial waste had intermediate mutagenicity.

3.6. Biochar risk matrix

Overall, from the PCA analysis (Fig. S7) it emerged that the main factors contributing to the biochar characteristics were: zeta potential, water holding capacity, dry matter content, electric conductivity, and the ash content. At the end of all the required analyses, the risk of adding biochar to the soil is assessed to determine if any safety issue arises and, as in any other environmental risk assessment procedure, a risk score matrix is defined (Table 4). To do this, we applied the scoring-ranking system described by Farkas et al. (2021) and corrected considering the important factors as previously described (Lehmann et al., 2020). In Farkas et al., the authors described the development of a "Multi-Criteria Decision Support System"(MCDSS) associated with a scoring system, ranging from +5 to −5, for each of major parameters necessary to describe biochar characteristics as suggested by IBI and EBC. Specifically, the same scoring systems was applied to: WHC, pH, Ash content, C and N concentration, C/N ratio, and presence of toxic elements, also in this work, whenever appropriate, the type of soil to which the biochar could be applied, was also considered (acid or neutral). For GI and SRI, biochar gave growth inhibition at each of

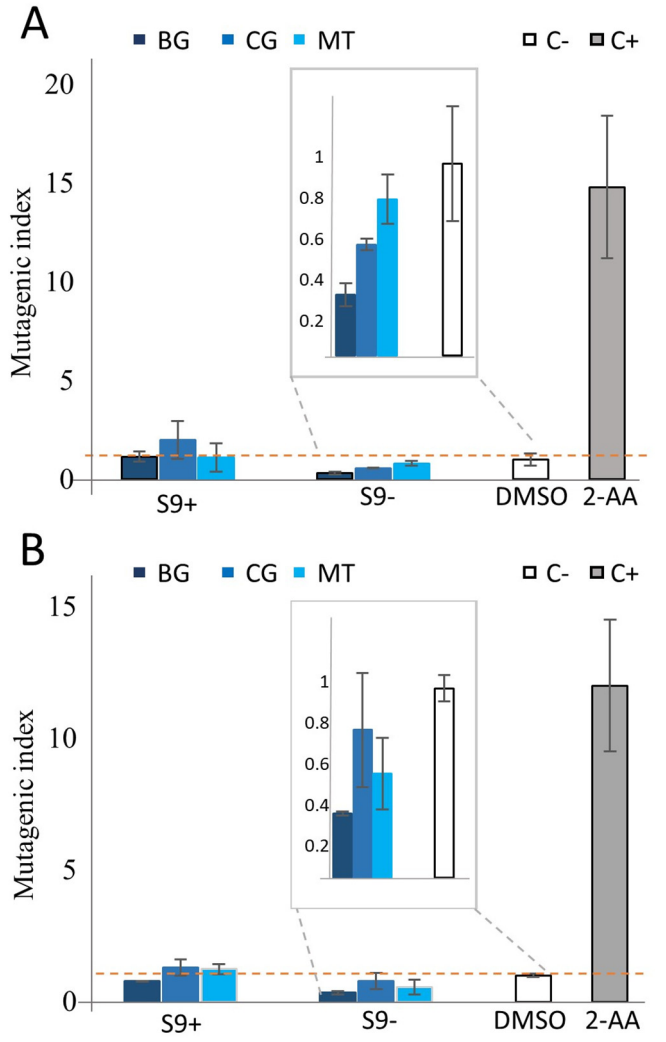
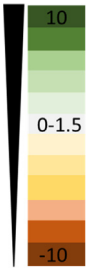


Fig. 3. Mutagenic index of *S. typhimurium* strains TA98 (A) and TA100 (B) exposed to 1:100 diluted organic biochar extracts with and without metabolic activation. The negative control (C-) is DMSO, while the positive control (C+) is 2-aminoanthracene (2-AA). The inlay graph reports an enlarged view of mutagenic index of *S. typhimurium* strains TA98 and TA100 exposed to 1:100 diluted organic biochar extracts without metabolic activation, no S9.

the concentration tested. For simplicity, scores were given only at the lowest concentration used: 0.5% (*w*/w) (the maximal score of 5 was given when the inhibition was not statistically different from the control, +3 was assigned when inhibition was between 20 and 30%, +1 when values reached 31–40% of inhibition, 0 for 41–50% inhibition, −1 when inhibition was between 51 and 70%, −3 when inhibition was between 71 and 90%, −5 when inhibition was between 91 and 100%). Finally, the mutagenicity test was considered, in this case +5 was given when the mutagenicity index did not exceed the values of the negative control for both strains (TA98 and TA100), only in one case the value for CG was 1, as the mutagenicity index was slightly above the negative control for TA98 and TA100 when the S9 mix was present. Next, each score was considered according to its importance with respect to soil health (Lehmann et al., 2020). Following the factors listed as most important for soil health, a 50% higher score was assigned to those variables that would have the highest impact on soil. Other characteristics such as the presence of heavy metals are, according to Lehmann et al. (2020), negative features, therefore the presence of heavy metals in the biochar received a lower score of 50% to highlight their toxicity, when present in the biochar.

Considering all the data, the three biochars (MT, BG, and CG) result overall safe and at the standard working concentration in the fields (not exceeding

Table 4
Biochar risk matrix.
Most of the scores were given following the MCDSS (Multi-Criteria Decision Support System) described by Farkas et al. (2021), while scores for GI, SRI and mutagenicity index were defined within this work. Sums are reported considering the possible amelioration of acidic or neutral soils using biochar.



	BIOCHAR		
	BG	**C**	**MT**
WHC (%)	1	0	0
pH[1]*	7.5	7.5	7.5
pH[2]*	-7.5	-7.5	-7.5
Ash Content[1](%)	1	-1	-1
Ash Content[1](%)	-1	1	1
Pore Volume	5	0	5
C (%)	0	0	3
N (%)	1	1	1
C/N (ratio)	3	-1	-3
Toxic element – Cd**	-1.5	5	5
Toxic element – Cr	5	5	5
Toxic element – Cu	5	5	5
Toxic element – Fe	5	5	5
Toxic element – Ni**	-1.5	5	5
Toxic element – Pb	5	5	5
Toxic element – Zn	5	5	5
Toxic element- As	5	5	5
Toxic element- Co	5	5	5
Toxic element- Hg	5	5	5
PAH content	5	5	5
GI[3] *Pisum sativum*	3	3	1
GI[3] *Hordeum vulgare*	3	0	-1
SRI[3] *Pisum sativum*	3	1	0
SRI[3] *Hordeum vulgare*	0	3	3
Mutagenicity index (+S9)	5	1	5
Mutagenicity index (-S9)	5	5	5
SUM score (acidic soil)	***78.5***	***75.5***	***81.5***
SUM score (neutral soil)	***63.5***	***60.5***	***66.5***

1: scores were given considering the aim of improving acidic soils (see Farkas et al., 2021),
2: scores were given considering the aim of improving neutral soils (see Farkas et al., 2021),
3: scores were given considering only the 0.5% (w/w) concentration.
*Considering the important factors to improve and or maintain soil health, the main positive factors identified in Lehmann et al. (2020) received an additional 50% score to underline their importance.
**Considering the important factors to improve and or maintain soil health, the main negative factors identified in Lehmann et al. (2020) received an additional 50% score reduction to underline their negative impact.

~20 ton hectare^{-1} or below) no concern are reported. Moreover, considering the sum of the score, and particularly their pH, the three biochars would perform better in acidic soils. Although the matrix predict that BG and MT would perform slightly better than CG in acidic soils, while MT would be the best in neutral soils, no huge differences exist among them.

4. Conclusions

This study evidences the correlation between feedstock properties, industrial production settings and biochar quality. The assessment of the mutagenic property of different biochars with *Salmonella typhimurium* strains TA98 and TA100 represents a novelty in toxicity testing when considering application and use of biochar in the environment. The Ames test was easy to apply and provided a correct risk assessment strategy. Slightly increased events of base substitutions in TA100 exposed to CG and MT with liver microsomal fraction (S9) were recorded. Frameshifts in TA98 exposed to activated CG were observed but were not significant. None of the biochars

showed mutagenicity with TA98 or TA100 in the absence of S9. BG biochar had fewer reversion events than the other biochars. This study also aimed to suggest the inclusion of the Ames test within specific routine investigations of risk in the environmental application of new biochar samples. Overall, at the end of the study a "safety" table based on a biochar risk matrix was generated. From this analysis it emerges that all biochars tested can be safely used as amendments in fields and that their employment will surely generate a positive outcome within the soil environment, both biological and chemical.

CRediT authorship contribution statement

Marta Marmiroli: Conceptualization, Investigation, Supervision, Writing – review & editing. **Marina Caldara:** Investigation, Visualization, Supervision, Writing – review & editing. **Serena Pantalone:** Investigation, Writing – original draft. **Alessio Malcevschi:** Supervision. **Elena Maestri:** Supervision, Writing – review & editing. **Arturo A. Keller:** Supervision, Writing – review & editing, Funding acquisition. **Nelson Marmiroli:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nelson Marmiroli reports financial support was provided by European Commission. Nelson Marmiroli reports financial support was provided by Emilia-Romagna Region. Elena Maestri reports financial support was provided by European Commission.

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Appendix A. Supplementary data

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