Soil & Environmental Health 1 (2023) 100050

Contents lists available at ScienceDirect

Soil & Environmental Health

journal homepage: www.sciencedirect.com/journal/soil-and-environmental-health

Research Paper

Multi-analytical techniques to study changes in carbon and nitrogen forms in a tomato-cultivated soil treated with biochar and biostimulants

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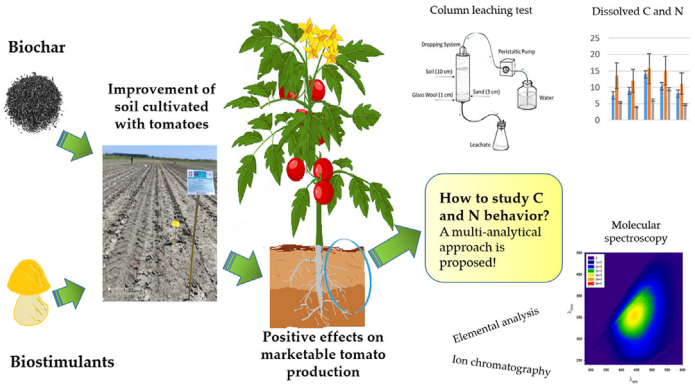
HIGHLIGHTS

- Different amendments affect DOC and dissolved nitrogen in soils.
- Leaching column test can be designed for realistic rainfall scenarios.
- Biochar when combined with biostimulants improves soil quality and tomato growth.
- Tomato production was increased due to amendments.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Lena Q. Ma
Technical Editor: Mary Beth Kirkham

Keywords:
Soil carbon sequestration
Microorganisms
Leaching column
Molecular spectroscopy
Biofertilizer
Dissolved organic carbon
AMF

ABSTRACT

Agro-environmental applications of biochar and biochar in combination with biostimulants require a full understanding of the mobility and fate of the carbon and nitrogen fractions in soils. The effects of biochar and biostimulants on forms of nitrogen and carbon in soil during a field-scale incubation were investigated by a multi-analytical approach. This study was conducted on a tomato-cultivated agricultural land treated with low doses of biochar (about 0.1%) and different biostimulants: Micosat F®, arbuscular mycorrhiza fungi (AMF), or a consortium of *Pseudomonas fluorescens*, *Bacillus* sp., and a nitrogen-fixing bacteria (Consortium B). Forms of carbon and nitrogen and their mobility before, during, and after tomato growth, were studied with different techniques including elemental analysis, adsorption and molecular fluorescence spectroscopy, ion chromatography, and a column leaching test. Due to the low load of biochar and the short study time, elemental analyses might not be sensitive enough to determine C and N variation in the soil. Based on the dissolved organic carbon (DOC) and

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https://doi.org/10.1016/j.seh.2023.100050
Received 13 June 2023; Received in revised form 10 November 2023; Accepted 10 November 2023
Available online 17 November 2023
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dissolved nitrogen forms, the treatments with biochar and biostimulants affected the mobility of these elements with an overall decrease at the end of tomato growth. The organic carbon is mainly ascribable to humic and fulvic acids, as indicated by spectroscopic analysis. The leaching column test demonstrated that cumulative leached C is about one order of magnitude lower than the DOC. In addition, simulated rain cycles profoundly affected their leaching, so it is important to design leaching tests based on local and seasonal weather conditions. In short, positive effects were observed in the marketable production of tomato when soil was treated with biochar combined with a mixture of biostimulants.

1. Introduction

Many studies have demonstrated the value of agricultural and environmental applications of biochar. They contribute to CO_2 sequestration, as biochar can positively modify some physical, chemical, and biological properties of the soil. This leads to an increase crop production and reduction in emission of greenhouse gases, such as nitrous oxide, in croplands (Paustian et al., 2016, Zomer et al., 2017; Woolf et al., 2010; Bola et al., 2022; Joseph et al., 2021; Zhang et al., 2023). Carbon-based materials, in particular nano-structured materials such as biochar with large adsorption capacity (Marmiroli et al., 2018), can decrease nitrate levels (Hagemann et al., 2017), support the colonization of microorganisms, and modify soil microbial habitats. Biochar also change carbon and nitrogen biofixation (Clough and Cordon, 2010), resulting in the provision of relevant ecosystem services (Lehmann et al., 2011; Zhu et al., 2017; Joseph et al., 2021).

Even though biochar leads to positive agronomic and environmental outcomes, its use is still limited due to high production and transportation costs (Robb et al., 2020; Zilberman et al., 2023). Although biochar market prices have substantially decreased from \$2850 t^{-1} in 2013 to \$600–\$1300 t^{-1} in 2021, with great potential for further cost reductions (Jirka and Tomlinson, 2015; Thengane et al., 2021), the future economic feasibility of biochar use need to not only meet the net agronomic benefits but also the carbon sequestration output (Zilberman et al., 2023).

While many studies have been focused on the effects on soil chemistry of biochar as an amendment at rather high loads (1%), agronomic strategies that apply low amounts of biochar (<0.1%) can be more sustainable. In addition to economic considerations, the use of low amounts of biochar, when used in marginal soils, could increase available plant nutrients and stimulate yield (Knoblauch et al., 2021). Furthermore, the use of biochar is of particular interest both as a carrier of nutrients (fertilizer) and as a support for the development of biostimulants, which include fungi and bacteria (Joseph et al., 2015; Rasse et al., 2022). Field studies are needed to scale-up from controlled environments to field trials in order to consider the dynamics of the system (geographical location and timespan of the experiments), potential sampling disturbances, and plant/soil/microbial feedback in realistic scenario of exposure (Chen et al., 2023).

Unlike conventional soil amendments, biostimulants that include fungi and bacteria can promote plant metabolic processes, thus improving nutrient use efficiency, tolerance to abiotic stresses, qualitative and quantitative characteristics of crops, and nutrient availability in the soil and rhizosphere (du Jardin, 2015; Berruti et al., 2016; Mosa et al., 2015). The mycorrhizal fungi *Azotobacter, Rhizobium,* and *Azospirillum* are used for this purpose. Their positive effects are countered by some critical aspects such as persistence in the crops following seed coatings and mixing with substrates that contain organic or inorganic compounds, which include peat moss, perlite, vermiculite, clays, and biochar (Marmiroli et al., 2018).

The combination of the physico-chemical properties of a soil amendment with the biological ones of microorganisms is the philosophy behind a "multi-purpose" fertilizing material (Schröder et al., 2018; Savarese et al., 2022). Recent legislation on fertilizers defines categories of materials to obtain fertilizing products, including "recycled waste", and it considers some microorganisms as fertilizers (Official Journal of the European Union, 2019). In this study, agricultural soils cultivated with tomato for commercial production were amended with low amounts of biochar (2 t ha^{-1}) alone or combined with commercial biostimulants, including Micosat F® commercial consortium from C.C.S. (Centro Colture Sperimentali, Aosta, Italy), arbuscular mycorrhizal fungi (MycAgro Lab - limited liability company - Dijon, France), and an innovative microbial consortium composed of different microorganisms including *Pseudomonas fluorescens* and *Bacillus* sp. They were added to the soil to improve not only crop productivity, but also mobility and storage of organic carbon and nitrogen.

To assess the effects of the amendments, it is important to characterize the forms of organic carbon and nitrogen before and after addition. The study describes how various analytical techniques, applied to soil before, during, and after supplementation and cultivation, allowed us to gather information on the forms of organic carbon and the mobility of carbon and nitrogen when the level of amendment loading was low. The study included a leaching-column experiment that simulated high rainfall conditions. The area is characterized by diversified agriculture with both grasses and trees. The cultivation of tomato was chosen, because it belongs to a typical crop rotation used in the Po Valley (alfalfa/cereals/tomato). Moreover, tomato represents, among the three species mentioned, the crop that requires the most fertilizer and water.

2. Materials and methods

2.1. Field experiment and soil sampling

The field experiment was conducted over a four-month period in the "Ganazzoli Filippo" farm (Parma, Emilia-Romagna, Italy). The texture of the soil is silty clay. NPK fertilization adopted is typical of tomato cultivation in this area: i) a pre-transplant fertilization in April with an NPK 9-13.5-13 dose, with an equivalent of 90 kg ha^{-1} of N, 135 kg ha^{-1} of P, and 130 kg ha^{-1} of K; ii) a top-dressing fertilization in June with calcium nitrate at 15.5%, with an equivalent of 46.5 kg ha^{-1} of N.

The agricultural land was supplemented with biochar (a commercial product derived from wood waste carbonization) in combination with microbial biofertilizers before tomato cultivation. Biochar was obtained from the low-temperature pyrolysis of forest biomass, whose characterization has been described in Rombolà et al. (2022). Being a commercial product, its characteristics comply with European regulations (EU regulation 2019/1009). The three biofertilizer formulations used were: i) Micosat F®, a community of mycorrhizal fungi made and distributed by C.C.S. (Centro Colture Sperimentali, Aosta, Italy) (*Trichoderma harzianum, Funneliformis mosseae* GP11, *Septoglomus viscosum* GC41, *Funneliformis coronatum* GU53, *Funneliformis caledonium* GM24, *Komagataella pastoris* -formerly *Pichia pastoris* PP59, *Streptomyces* sp. SA55, *Bacillus amyloliquefaciens* BA41, *Pseudomonas fluorescens* PA29, and *Agrobacterium radiobacter* AR39); ii) arbuscular mycorrhizae fungi (AMF), a granular inoculum based on *Rhizoglomus intraradices*, acquired from MycAgro (MycAgro Lab - limited liability company - Dijon, France; http://www.mycagrolab.com); iii) the consortium B specifically developed within the Horizon 2020 SIMBA project (Sustainable Innovation of Microbiome Applications in the Food System), which includes a consortium of *Pseudomonas fluorescens, Bacillus* sp., and nitrogen-fixing bacteria (Tabacchioni et al., 2021).

The experimental design for the field consisted of a randomized complete block divided into 3 plots of 14 m^2 (10 × 1.4 m), which gave rise to five treatments (GA, from the name of the experimental field utilized): GA1 – Control sample; GA2 – Soil treated only with biochar: about 0.1%; GA3 – Soil treated with biochar and Micosat F®; GA4 – Soil treated with biochar and AMF; GA5 – Soil treated with biochar, AMF, and Consortium B.

Biochar was dispensed and incorporated into the top 20 cm of soil immediately, using a hand hoe, to ensure a uniform distribution. Biochar was added to soil at about 0.1% (about 2 t ha^{-1}). Tomato plants were transplanted after 20 days from germination in a greenhouse. Transplantation was mechanical, and plant density was about 3100 plants ha^{-1}. Micosat F® and mycorrhizae were added (2 g) for each plant twice: after transplanting and during the growing season (20 days after transplant). Consortium B was added (4 g) for each plant with the same timing. Inoculation was carried out with a microbial suspension in water, and it was precisely poured at the base of each plant. Soil was sampled three times during tomato growth in 2021: F0 (before treatment, March 31, 2021), F1 (during treatment, May 07, 2021), and F2 (after treatment, August 25, 2021). Five replicates of soil were withdrawn from each site at randomly chosen points utilizing a soil core sampler that sampled at the 0–30 cm depth. The five replicates from each sampling site were mixed uniformly to obtain a composite sample of about 1 kg. An approximately 50 g subsample was dried at room temperature for four days and then sieved (mesh size: 2 mm) to obtain homogeneous subsamples free of stones, large roots, wood sticks, and other coarse fragments.

2.2. Analytical measurements

2.2.1. Total carbon and elemental analysis

A sieved sample was ground in a mortar prior to analysis. Total carbon (TC) and inorganic carbon (IC) were analyzed with a total organic carbon (TOC) analyzer (Model SSM 5000A, Shimadzu Corp., Kyoto, Japan). TOC content was calculated as the difference between TC and IC.

2.2.2. Characterization of dissolved carbon and nitrogen forms

Dissolved carbon and nitrogen forms were measured according to Ghidotti et al., (2017). Soil samples were extracted with ultrapure water from a Millipore Direct-Q 5 UV system water (18.2 Ω cm, Merck KGaA, Darmstadt, Germany) at a ratio of 1:10 w/v. One g of soil was suspended in 10 mL of ultrapure water. The test tubes were shaken for 24 h at 10 rpm at room temperature and then centrifuged for 10 min at 3500 rpm. The supernatants were filtered under vacuum through a 0.45 μm membrane filter (Merck, Darmstadt, Germany).

Elemental analysis: Dissolved organic carbon (DOC) of the water-soluble organic matter was measured with a Shimadzu TOC-L analyser (Shimadzu Corp., Kyoto, Japan). Quantification of each analysis is presented here as a mean of two to three injections of 100 μL, where the coefficient of variance for the replicate injections was <2 %.

Dissolved nitrogen (DN) concentrations were measured with a TN-module coupled with a TOC-L analyzer (Shimadzu Corp., Kyoto, Japan).

Ion chromatography: Nitrate, nitrite, and ammonium were determined by ion chromatography (Eco-IC Metrohm, Herisau, Switzerland) equipped with an IC SI-90 4E Shodex™ column and a Metrosep C4-150 © Metrohm for anion and cation separation, respectively.

2.2.3. Molecular spectroscopy

The absorbance of dissolved organic matter (DOM) was determined within a spectrum of 200–800 nm using a Cary 300 UV–Visible Spectrophotometer (Agilent Technologies, Santa Clara, United States); quartz cuvettes (l = 1 cm) were used for this purpose and properly cleaned before each use. Ultrapure water (18.2 Ω cm) was used as a reference. The values of specific ultraviolet absorbance at 254 nm, $SUVA_{254}$, is expressed in L mg^{-1} m^{-1} (Liters · $milligrams^{-1}$ · $meters^{-1}$) were measured using Equation (1), where a_{254} is the absorption coefficient at wavelength 254 nm. The a_{254} indexes for the DOM aromaticity were calculated using Equation (2).

$$SUVA_{254} = \frac{a_{254}}{DOC(mg/L)} \quad (1)$$

$$a_{254} = \frac{A_{254}}{l(m)} \quad (2)$$

In the above equations, A_{254} is the absorbance at a wavelength of 254 nm, do and l is the cell path length in meters.

The excitation-emission (EEM) fluorescence spectrum of each sample was determined using a FluoroMax-4 system (Horiba Scientific; Kyoto, Japan), in quartz cuvettes (1 cm). Wavelength emission and excitation wavelength were scanned in a range of 280–500 nm and 280–600 nm, respectively, through sequential 5 nm steps. After background correction, EEMs were analyzed with multivariate statistics using the PARAFAC (parallel factor analysis) model, which used the N-way model algorithm of Matlab software useful to decompose N-dimensional data arrays, such as EEMs. PARAFAC components were determined using unimodality (resulting spectra needed to have only one peak) and non-negativity constraints (each value should have been equal or greater than 0).

The method considered also Stokes' shift (the shift between excitation and emission spectra for each component), core consistency, analysis of residuals, and leverage values (Murphy et al., 2013; Stedmon and Bro, 2008; Ghidotti et al., 2017): these are all methods to assess the reliability of the number of components and the shape of the resulted spectra found by the model.

2.3. Leaching in soil columns

Leaching tests were conducted using two columns (made of PVC) with a diameter of 4.5 cm and a height of 20 cm. At the bottom of each column was 1 cm of glass wool and 3 cm of washed sand, which had the function of filtering the soil leachate. The leachate flowed from below the columns due to a central hole connected to a collection pipe. The column was filled with about 210 g of air-dried soil (10 cm height). The setup for a leaching column is shown in Fig. 1. Two conditions were considered: one column was filled with soil mixed with biochar and mycorrhizae (GA4, F1) as a reference condition for biochar amended treatments; a second column was used as control and then filled with the soil without any treatment (GA1, F1).

During the experiment, rain was simulated by dropping distilled water in the column from four capillary tubes. The intensity of the rainfall and its duration simulated semi-real conditions, in which the surface soil has alternate periods of saturation with water drainage. In Table 1, all the obtained data are reported. Every day the leachate was collected and analyzed. Daily collected leachates were analyzed for DOC, TN, $N–NO_3^-$, $N–NO_2^-$, and $N–NH_4^+$ concentrations.

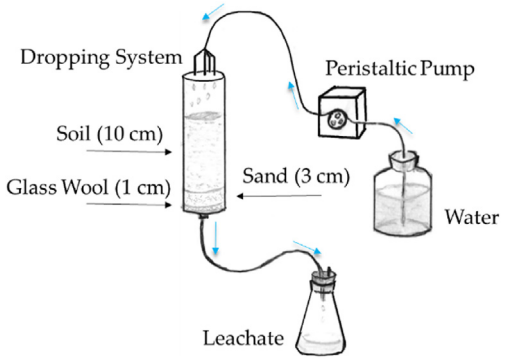

Fig. 1. Diagram of the column leaching system.

Table 1
Parameters of the leaching experiment in soil columns. GA1 and GA4 represent the two conditions GA1 - Control sample; GA4 - Soil treated with biochar and AMF, respectively.

Period	Rainfall rate	Rainfall duration	Volume of collected leachate	
			Control (GA1)	*Test (GA4)*
	mm/h	*Minute*	*ml*	*ml*
Day 1	150	40	39.7	46.8
Day 2	33	180	148	151
Day 3	33	180	153	150
Day 4	33	180	145	144
Day 5	–	–	0	0
Day 6	–	–	0	0
Day 7	150	40	145	145
Day 8	150	40	126	123

2.4. Productivity, refractive index, and total refractive production

Tomato productivity (kg/ha) was determined by manual harvesting and weighing of tomatoes from each plot.

Refractive index (°Brix) was measured utilizing a digital handheld refractometer (HI 98613 model, Hanna Instruments IT, Verona, Italy) on mashed tomato samples (one sample for each plot was mixed to obtain a suspension for analyses).

Total refractive production (kg °Brix ha^{-1}) was then calculated by multiplying tomato productivity by refractive index and reported on a standard proportion of 100 t ha^{-1}.

2.5. Statistical analysis

The mean and standard deviation of three replicates were used to compare the results of soils and soils treated with biochar and various biostimulants. The analysis of variance (ANOVA) test was followed by Dunn's post hoc test ($p < 0.05$) to compare all soils, and they were conducted with PAST software, ver 4.13 (PAlaeontology STatistic is a freeware data analyzer app and calculator developed by Professor Øyvind Hammer, Natural History Museum – University of Oslo).

3. Results and discussions

3.1. Soil and biochar characterization

Biochar was tested in co-exposure with different biostimulants. The amount used per hectare was kept low, as compared the amount used in other experiments reported in the literature, were biochar was used as a sole soil amendment (Liu et al., 2022). The biochar had a TC % of 47.14 ± 0.06 and a DOC of 1.3 ± 0.15 mg g^{-1}. These values are consistent with a biochar obtained at low temperature (Liu et al., 2022). Considering the biochar load in the soil, its contribution to the total carbon was about 0.05%. This value is often below the analytical error, and the direct effect of biochar on the content of C cannot be tracked (Table 2). The effects of the GA2 and GA3 treatments, which were biochar alone and with Mycosat F®, respectively, were not significantly different from the control (GA1). When mycorrhizae and mycorrhizae + Consortium B were added (GA4 and GA5), at the end of the treatment (F2) the TOC was superior to the control by approximately 20 and 24%, respectively (Table 3).

The water-soluble fraction of soil organic carbon (Fig. 2a) was similar for untreated and treated soils with an average value of 18.4 ± 0.7 mg DOC/100g soil (GA1-5, F0). The small quantity of added biochar did not influence the release of soil carbon, in contrast to other studies that showed that higher levels of biochar caused an increase in DOC (Fan et al., 2020). At the end of tomato growth (F2), DOC decreased significantly in all the soils. During the tomato growth phase (F1), two important deviations from the reduction trend of DOC were recorded.

Table 2
Concentration in the soil of Total Carbon (TC); Inorganic Carbon (IC); and Total Organic Carbon (TOC). For each soil, the Specific UV Absorbance ($SUVA_{254}$) values measured for the dissolved organic carbon fraction are reported. Mean values ± sd (n = 3). F0 = before tomato growth; F1: during tomato growth; F2: after tomato growth. GA1-5 represents the five conditions utilized in the study. (GA1) Control sample, (GA2) Soil treated only with biochar: about 0.1%, (GA3) Soil treated with biochar and Micosat F®, (GA4) Soil treated with biochar and AMF, and (GA5) Soil treated with biochar, AMF and Consortium B, respectively.

Soil treatment		F0	F1	F2
		%	%	%
GA1	**TC**	3.07 ± 0.02	2.97 ± 0.02[a]	2.80 ± 0.02[a]
	IC	1.49 ± 0.05	1.33 ± 0.05[a]	1.21 ± 0.05[a]
	TOC	1.58 ± 0.07	1.64 ± 0.07	1.59 ± 0.07
	$SUVA_{254}$	1.2 ± 0.9	1.1 ± 0.2	1.0 ± 0.1
GA2	**TC**	3.1 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
	IC	1.36 ± 0.02	1.16 ± 0.02[a]	1.15 ± 0.02[a]
	TOC	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1
	$SUVA_{254}$	1.0 ± 0.9	0.8 ± 0.2	1.0 ± 0.1
GA3	**TC**	2.9 ± 0.1	3.0 ± 0.1	2.9 ± 0.1
	IC	1.19 ± 0.04	1.33 ± 0.04[a]	1.27 ± 0.04[a]
	TOC	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
	$SUVA_{254}$	0.9 ± 0.9	1.1 ± 0.2	0.9 ± 0.1
GA4	**TC**	3.09 ± 0.04	2.96 ± 0.04	3.15 ± 0.04
	IC	1.43 ± 0.05	1.14 ± 0.05[a]	1.16 ± 0.05[a]
	TOC	1.66 ± 0.09	1.82 ± 0.09	2.00 ± 0.09[a]
	$SUVA_{254}$	0.9 ± 0.9	0.6 ± 0.2	1.0 ± 0.2
GA5	**TC**	3.01 ± 0.08	2.93 ± 0.08	3.19 ± 0.08[a]
	IC	1.3 ± 0.02	1.18 ± 0.02	1.14 ± 0.02[a]
	TOC	1.7 ± 0.1	1.8 ± 0.1	2.1 ± 0.1[a]
	$SUVA_{254}$	1.0 ± 0.9	1.4 ± 0.2	1.0 ± 0.1

[a] Significant differences between the same treatments from F0 to F2 (Kruskal Wallis one-way ANOVA; Dunn's test, $p < 0.05$).

Table 3
Productivity results in the tomato field. (GA1) Control sample, (GA2) Soil treated only with biochar: about 0.1%, (GA3) Soil treated with biochar and Micosat F®, (GA4) Soil treated with biochar and AMF, and (GA5) Soil treated with biochar, AMF and Consortium B, respectively.

Sample name	Marketable production (t ha^{-1})	Brix refractometry production (kg °brix ha^{-1})	Refractive Index (°brix)
GA1	120	6.5	5.4
GA2	101[a]	5.2	5.1
GA3	122	6.4	5.3
GA4	129	6.7	5.2
GA5	145[a]	7.4	5.1

[a] Significantly different from control soil GA1 by Scott-Knott's test ($p < 0.05$).

GA2 and GA4 soils had an increase of 56% and 92%, respectively. The increase is not easy to interpret, but, among the different causes, it could be also explained by an increased metabolic activity of the rhizosphere (Li et al., 2022; Feng et al., 2023). Variations of DOC at different times were not related to those of TOC, which remained almost constant, as evidenced upon normalization (Fig. 2b). A contrasting trend was noticed for soils GA4 and GA5, which showed higher levels of TOC but lower concentrations of DOC at the end of the experiment. The differences could be due to the organic carbon being more stable and water insoluble.

In addition to total nitrogen, nitrate, nitrite, and ammonium ions were determined. In all the samples, the concentrations of ammonium ions were below the detection limit (0.1 mg L^{-1}). Similarly, nitrite was not detectable in soils at the beginning and the end of the experiments, suggesting that the addition of biochar and biostimulants did not influence the redox system of nitrogen forms. Low concentrations of nitrite (1.3–1.8 mg L^{-1}) were found in the soils collected in the first growing phase (F1), and they were characterized by high values of DOC and DN (Figs. 2 and 3). Concentrations of nitrates were variable (13–71 mg L^{-1})

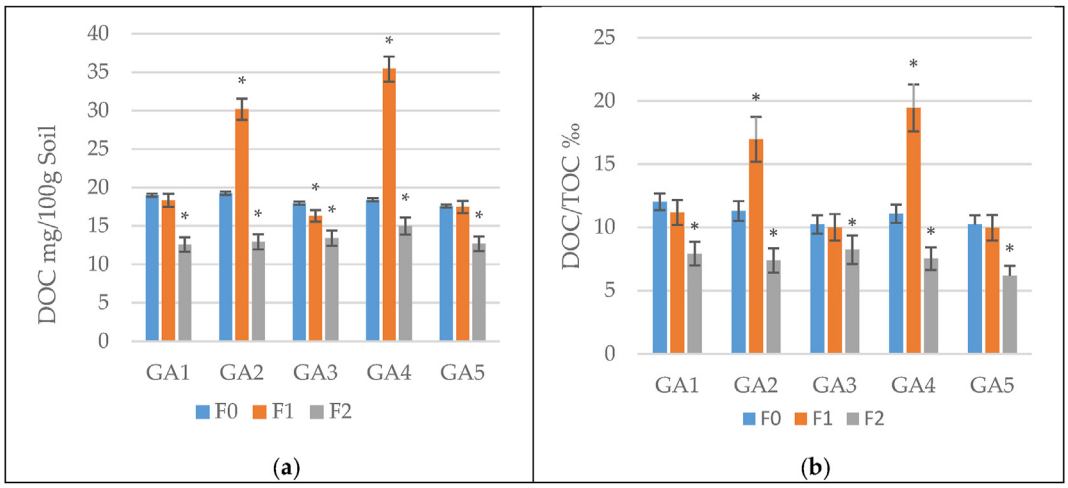

Fig. 2. (a) Dissolved organic carbon concentration (DOC) and (b) ratio of DOC respect the total soil organic carbon before (F0), during (F1) and after (F2) tomato growth determined in (GA1) Control sample, (GA2) Soil treated only with biochar: about 0.1%, (GA3) Soil treated with biochar and Micosat F®, (GA4) Soil treated with biochar and AMF, and (GA5) Soil treated with biochar, AMF and Consortium B, respectively. Mean value ± sd (n = 3). *, *Significant differences between the same treatments from F0 to F2 (Kruskal Wallis one-way ANOVA; Dunn's test, $p < 0.05$).*

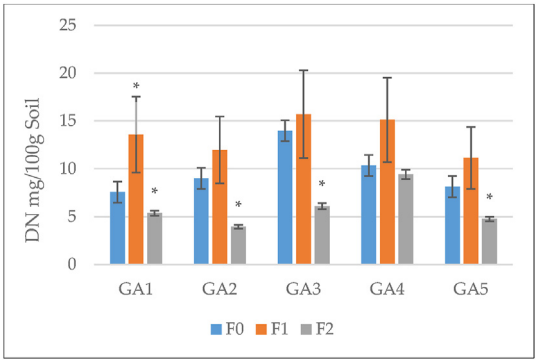

Fig. 3. Dissolved nitrogen concentration (DN) before (F0), during (F1) and after (F2) tomato growth determined in (GA1) Control sample, (GA2) Soil treated only with biochar: about 0.1%, (GA3) Soil treated with biochar and Micosat F®, (GA4) Soil treated with biochar and AMF, and (GA5) Soil treated with biochar, AMF and Consortium B, respectively. Mean value ± sd (n = 3). *, *Significant differences between the same treatments from F0 to F2 (Kruskal Wallis one-way ANOVA; Dunn's test, $p < 0.05$).*

but paralleled those of the dissolved nitrogen. The DN/N-nitrate ratios ranged from 0.84 to 1.0, suggesting that all the nitrogen released into the water can be associated with nitrate, with only a small fraction of organic nitrogen. As recorded for DOC, there was a decrease in the concentrations of DN between F0 and F2 (Fig. 3). This agrees with what is reported in soil, concerning the dynamics of carbon and nitrogen, including soil acidification (Hagemann et al., 2017; Wang et al., 2023). However, there was no linear correlation between DN and DOC, and variability in the values were found for the different treatments at time 0. Nevertheless, in all soils, the temporal dynamics were the same. That is, dissolved nitrogen increased during tomato growth and decreased at the end of the growth (Fig. 3).

3.2. Molecular spectroscopy of dissolved organic matter (DOM)

The optical properties of DOM, specifically the UV–visible absorbance and fluorescence, can be used to obtain information about the composition of DOM. SUVA is defined as the absorbance at a specific wavelength (254 nm) normalized for dissolved organic matter. The parameter provides a general characterization of the nature of natural organic matter and is closely correlated with the presence of aromatic compounds in DOM (Weishaar et al., 2003; Abd Manan et al., 2020). Results obtained at different sampling times showed no significant differences regarding the $SUVA_{254}$ (Table 2), and this may suggest that there was no great variation in the aromatics, as a result of the use of biochar and biochar plus biostimulants. The low $SUVA_{254}$ value, on average about 1.0, indicates a low amount of the aromatic fraction (Weishaar et al., 2003).

Fluorescence excitation/emission maps were used to compare different sampling times, which were before treatment (F0), during growth (F1), and at the end of growth (F2). EEM maps for the GA3 treatment are shown in Fig. 4. The EEMs showed a band with a maximum at 360 nm in excitation and at 450 nm in emission. Comparing the sampling times, changes in the shape of the excitation/emission band were not noticed, but a change in the emission intensity. The intensity decreased from the value before treatment to the value after growth for all the samples, as observed for the release of DOC. Biochar used in this experiment was also analyzed under the same conditions. For biochar, components at 320 nm (EX) and 420 nm (EM) and another one, with less intensity, at 370 nm/470 nm, were observed. In Fig. 4, for example, the spectra obtained from EEMs with the PARAFAC model for GA3 at different sampling times are shown.

Spectral analysis pointed to three components under the same region, which could be noted for each sample: 350 nm (EX)/405 nm (EM), 370 nm (EX)/455 nm (EM), 430 nm (EX)/505 nm(EM). These components were all in the region of humic acid, fulvic acid, and an overlap with those of DOM. Biochar EEMs were also analyzed with PARAFAC. In this case, four components were found, three of them in the regions of humic and fulvic acids and one at 285 nm (EX)/345 nm(EM) corresponding to amino acids (Weishaar et al., 2003). This component was not observed in the soils, as expected due to the small biochar load. However, the protein fraction was not detected even in soils treated with high biochar loads (Guo et al., 2013).

3.3. Column leaching test

The experiment was designed to simulate the leaching due to the percolation of rain into the topsoil during treatments. This preliminary experiment was performed with only two sample columns, because the aim was to demonstrate how the results could show the mobility of C and N and could provide an example as to how a leaching test could be done with conditions nearest to real ones.

To simulate natural conditions, the soil was subjected to “very intense rain” for a short time (40 min) and “intense rain” for a longer time (3h)

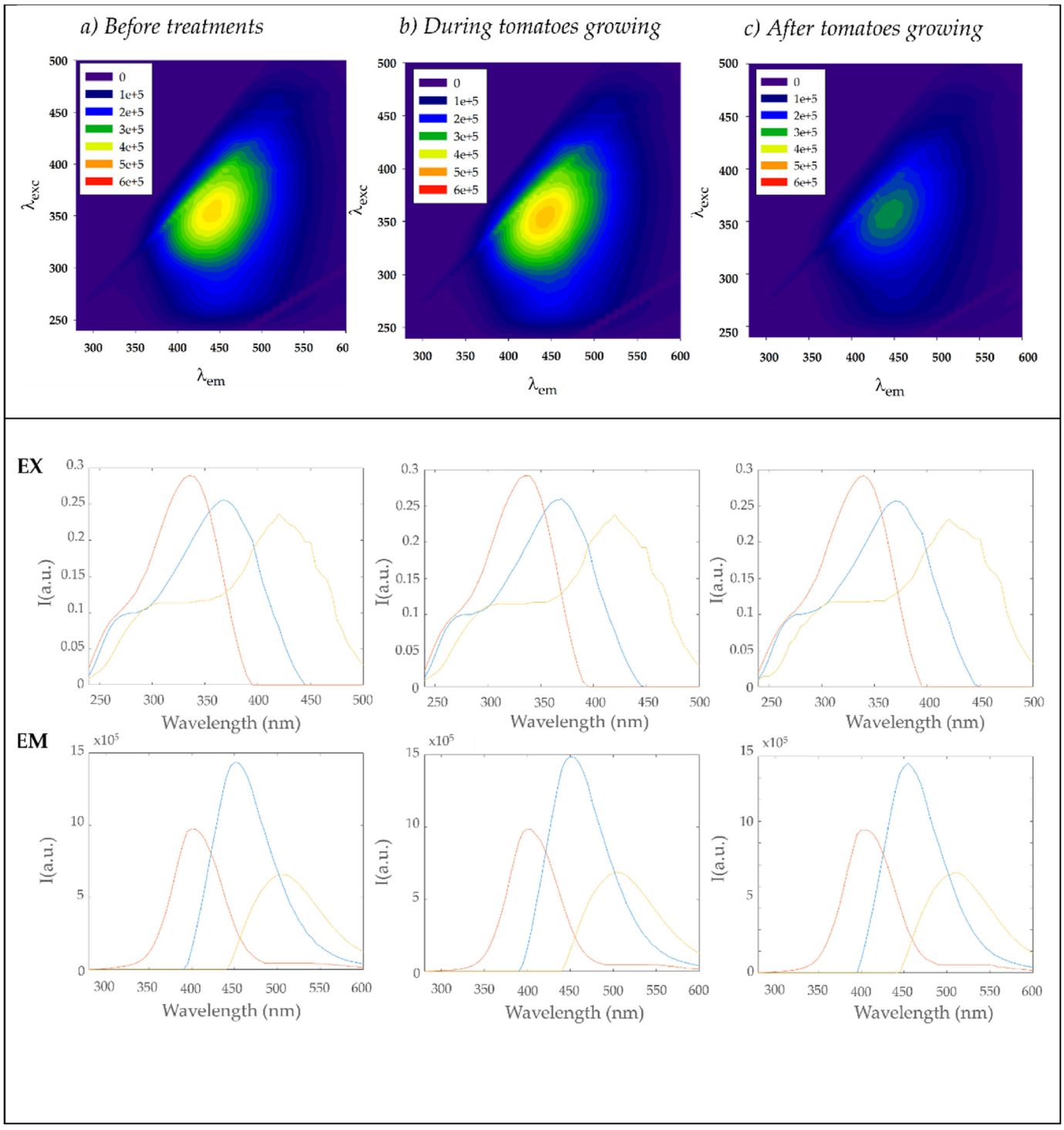

Fig. 4. Excitation/emission (EEM) spectra of GA3 (soil treated with biochar and Micosat F®), before F0(a), during F1(b), and after F2(c) tomato growth. Excitation (EX) and emission (EM) signals obtained through PARAFAC statistical analysis are reported.

(Table 1). The rainfall corresponded to 100 mm per all rainy days and to 600 mm in eight days. This experiment was performed on the control soil (GA1, F1) and soil amended with biochar and mycorrhiza (GA4, F1).

For both soils, similar trends of leached DOC (Fig. 5a) were found. There was a low release of DOC on the first day, associated with the retention of about ¾ of the volume of rain by the dry soil. Then there was a trend of slightly decreasing release going into rainy days. From day 2 to day 8 in both soils, there was a halving of the flow of DOC. The 2-day break included in the analysis slightly affected the release, with an increase in DOC mobility between day 4 and day 7.

Among samples, differences in DOC were moderate. The addition of biochar and mycorrhizae increased the release of DOC. These results conflict with the existing literature, as biochar-derived DOM can be adsorbed onto soil particles after application (Rombolà et al., 2022; Qiu et al., 2023; Yuqing Sun et al., 2021). In general, the effects of DOC reduction due to biochar supplements were visible in the medium or long-term, while in the short-term the soluble fraction of biochar contributed to a DOC increase. The cumulative DOC values released during the column leaching test were about one order of magnitude lower than that measured as DOC (for GA1 2.8 instead of 18.3 mg/100g

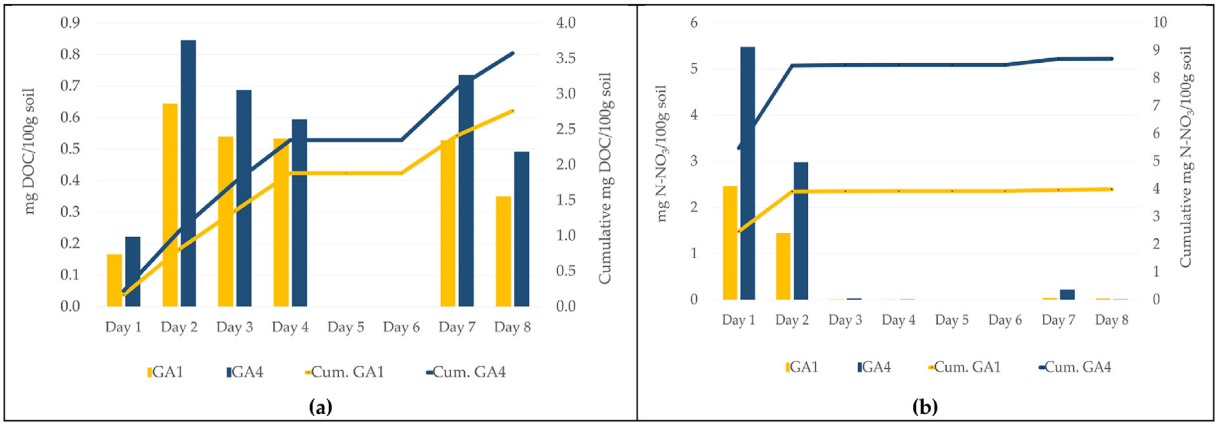

Fig. 5. Dissolved organic carbon (DOC) (a) and nitrate (b) released day-by-day and cumulatively over the whole period of the leaching column test, simulating variable rain conditions (reported in "Material and methods"). GA1 and GA4 represent the control sample and soil treated with biochar and AMF, respectively.

soil; for GA4 3.6 instead of 35.5 mg/100g soil), indicating that the latter parameter gives an estimation of the fraction of organic carbon soluble in water, but it was not representative of its mobility. The soil/water ratio and the wetting time are important factors that can influence the absorption balance (Heasman and van der Sloot, 1997).

Although the release of DOC was constant over the time, the behavior of nitrate was different showing different leaching kinetics. In fact, after the first two days of rain, a consistent drop in its release was observed (Fig. 5b).

Soil GA4 showed a greater release of nitrates in comparison with GA1. Even after the 2 days without rain (days 5 and 6), nitrates were released (day 7), but their concentrations were negligible on the last day. The dry-wet regimes seemed to influence the nitrogen mobility during the test. The cumulative release of nitrates in the treated soil are less retained, similar to what was observed for the DOC.

3.4. Agronomic results

Commercial yield (Table 3) showed significant differences among treatments. High commercial yield was obtained from the soil treated with biochar, mycorrhizae, and Consortium B (GA5) (145.5 t ha^{-1}), whereas the treatment with only biochar (GA2) was the least productive, below the GA1 (untreated) control. Not only do economic outcomes of this new type of fertilization need to be considered, but also ecological implications, including a life cycle analysis, which will be the subject of a more specific study.

4. Conclusions

Agricultural applications of biochar in combination with biostimulants can positively affect agricultural production. In this study, different forms of nitrogen and carbon in soil were evaluated after the application of biochar and biostimulants to a tomato crop using different analytical techniques. Due to the low loads of biochar, in-depth information on quantitative variation of different forms of C and N was not possible. The analysis of the water-soluble fractions of organic carbon and dissolved nitrogen compounds gave useful information to comparatively evaluate the effects of different soil treatments. Specifically, in all soils, an overall reduction in mobility of C and N was recorded at the end of the crop cycle. DOC (dissolved organic carbon) and DN (dissolved nitrogen) did not provide a realistic value for the mobility of C and N. Column leaching tests highlighted different results in terms of mobilized quantities. In the column studies, while cumulative leaching of N was comparable to DN, C was about one order of magnitude lower than the measured DOC values. Additionally, rain cycles affected their leaching. Thus, it is important to design leaching tests based on local and seasonal weather conditions. Spectroscopic analysis, in addition to ion chromatography, provided a detailed characterization of the nature of the solubilized forms. Specific absorption spectra showed that humic and fulvic acids constituted the most important source of soluble carbon, whereas nitrate was the most important form of soluble nitrogen. Regarding agronomic results, positive effects were observed in marketable production of tomato when soil was amended with biochar, AMF, and Consortium B.

Although the results come from a preliminary experiment and further research will be necessary to investigate the mechanisms behind the C and N behaviors and confirm the agronomic data, this research demonstrates the importance of the proposed analytical approach.

Author contributions

Conceptualization, Daniele Fabbri and Ivano Vassura; methodology, Alessandro Rombolà, Sandro Cornali, Roberto Reggiani, Ivano Vassura; formal analysis, Beatrice Rizzi, Alessandro Rombolà, Arianna Menichetti, Sandro Cornali, Roberto Reggiani, Maria Roberta Vecchi; writing, review and editing, Ivano Vassura, Daniele Fabbri, Beatrice Rizzi, Luca Pagano; funding responsibilities, Nelson Marmiroli, Daniele Fabbri. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by PSR 2014–2020 – Misura 16.1.01 – Focus Area 4B Regione Emilia-Romagna for the project 5150147 FERTILIAS «Strategie green di biorisanamento dei suoli per una migliore gestione idrica, dei fertilizzanti e dei pesticidi».

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.

Acknowledgments

We owe our special thanks to the "Ganazzoli Filippo" farm where the experimental trial was conducted. The authors would like to thank Prof. Marco Montalti University of Bologna for his contribution in the interpretation of spectroscopic data and Mia Marchini, Azienda Agraria Sperimentale Stuard SCRL, for her contribution to field activities and interpretation of agronomic data.

The authors would like to thank the Horizon 2020 SIMBA project (Sustainable Innovation of Microbiome Applications in the Food System) for the availability of the use of the Consortium B, as well as Connectfarms, MicroEco, and DeliSoil projects.

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