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SOIL MICROBIAL AND PHYSICOCHEMICAL CHANGES AFTER THE ADDITION OF BIOCHAR, BACTERIAL INOCULUMS AND NITROGEN FERTILIZER

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Abstract

Addition of biochar is often proposed as an improving agent of soil properties. The combination of biochar (BCH) with mineral or biological amendments in order to improve its influence on soil-plant properties compared to the unamended BCH was vastly studied. Bacterial inoculums as a promising additive to BCH amendment are highly dependent on BCH quantity, its feedstock and soil state. Luvisol from a protection zone of water sources was used in pot experiment set-up. The changes in physicochemical properties (pH, cation-exchange capacity - CEC) and biological soil activities (soil enzymes: urease, phosphatase and laccase activity and total bacteria content) after the addition of beech wood biochar combined with the addition of bacterial inoculums (Bacofil and Novarefm) and nitrogen fertilizer after two growing cycles of Lactuca sativa var. capitata were studied using spectrophotometry methods. Increased pH and CEC values were detected in biochar amended treatments. The increase of laccase activity claimed on BCH additives promoting effect, especially in a case of Bactofil inoculum amendment. Nevertheless, BCH suppressed acid phosphatase activity in all the BCH additives equally. Whereas urease activity and total soil bacteria extraction remained unchanged in BCH amended treatments compared to control.

Keywords: biochar, enzyme activity, fertilizer, inoculum, lettuce, nitrogen, soil bacteria

INTRODUCTION

Soil degradation stands for a durable descent in soil productivity, quality and its environment moderating ability (Lal, 2001). Widespread degradation factors in Czech Republic include soil erosion, soil compaction, loss of organic matter (OM), soil acidification, and soil contamination (Šarapatka and Bednář, 2015).

Biochar (BCH) has gained widespread attention as a mean to balance soil degradation, while being able to sequester carbon into soils. BCH derives from the carbonization of biomass and is a stable solid material for many years in soil (Lehmann, 2007). It could increase soil fertility (Chan *et al.*, 2007) and enhance agricultural productivity (Major *et al.*, 2010) by increasing cation exchange capacity and

nutrient cycling with the ability of soils to retain plant available water, decrease soil bulk density, increase pH (Laird *et al.*, 2010) and additionally BCH is a habitat for microorganisms which generally increases microbial biomass (Lehmann *et al.*, 2011; Ippolito *et al.*, 2012).

High diversity of microbial community structure is involved in efficient nutrient transfer to crops and nutrient conservation in soil (Gul *et al.*, 2015). BCH alters positively soil microbial abundance and community composition (Domene *et al.*, 2014), affects microbially-mediated nutrients transformation in soil (Kuppusamy *et al.*, 2016) that eventually leads to changes in nutrient availability and crop productivity (Spokas *et al.*, 2012). Generally, BCH effects on soil enzyme activities are variable, depending on soil type and on the particular enzyme (Bailey *et al.*, 2011). For instance, soil extracellular enzymes that are involved in carbon (C) and sulphur cycling, increased while amended with the lower amount of BCH (0.5% by mass), whereas higher BCH amounts decreased their activity (Wang *et al.*, 2015). Lammirato *et al.* (2011) concluded that BCH is inclined to soil enzymes adsorption without complete loss of their potential activity. Particularly some enzymes like laccases, that are playing a central role in OM recycling being involved in lignin degradation and humic substances formation, are immobilized by BCH (Li *et al.*, 2018). BCH also reduced urease activity that release inorganic N into soils (Wu *et al.*, 2013). Other studies claim that BCH improves soil phosphatase activity, which is fundamental to transform organic phosphor (P) into inorganic P, consequently influencing micro-environment (Nèble *et al.*, 2007).

Despite its potential positive effects, BCH could have negative influence on soils activities and on plant growth (Asai *et al.*, 2009). Thus, co-amendment of BCH with fertilizers (Steiner *et al.*, 2008) or bacterial inoculum (Sun *et al.*, 2016) could mitigate its negative impact and ameliorate BCH properties. However, there is still a need to understand BCH influence on soil enzymes, especially after several growing periods, as most of the research focus on the influence of freshly applied BCH.

In this study, we investigated the influence of BCH combined with other soil amendments (bacterial inoculums and nitrogen (N) fertilizers) on soil physicochemical properties (pH, CEC) and biological activities by choosing soil enzymes involved in N, P and C nutrient cycles (urease, phosphatase and laccase activities; total soil bacteria extraction) after two growing periods of lettuce (*Lactuca sativa* L.).

MATERIALS AND METHODS

Soil Sampling

Soil was collected from experimental plots situated in the protection zone of underground drinking water source "Brezova nad Svitavou" (Czech Republic; 49°40.409'N, 16°27.545'E.) Soil was sampled with a spade according to Czech Technical Standard ISO 10 381-6 from the topsoil horizon (till 0–30 cm) in summer 2015. The soil is classified as sandy loam Luvisol (Tab. I). Fresh soil samples have been delivered to the laboratory where they have been air-dried, homogenized and sieved through a 10 mm sieve.

Biochar Material

Beech wood biochar (*Fagus silvatica* L.) has been originated from Czech Republic (company BIOUHEL.CZ s.r.o.) produced by slow pyrolysis with the use of low temperature 470 °C (Tab. II).

Experimental Design

Five different types of treatments including a control have been prepared (Tab. III). Four replications of each treatment resulted into twenty plastic square containers (10 × 10 × 11 cm) filled with 800 g of topsoil.

BCH was freshly applied in the quantity of 6% per pot with the first plant growing cycle. The BCH quantity used for the experiment was chosen as a high concentration in order to obtain distinguished results (Chan *et al.*, 2007).

Pots were split into two groups. Half of them were inoculated with the commercial bacterial inoculums "Bactofil" (BI1) from BioFil Ltd (Budapest, Hungary) while the other half were

I: *Basic properties of soil used in experiment (adapted from Plošek, 2016)*

CEC ($cmol\ kg^{-1}$)	pH (H_2O)	Conductivity ($\mu S\ cm^{-1}$)	N_{tot}	C_{tot}	C_{org}	C/N	Humus content (%)	P	K	Ca	Mg
			($mg\ g^{-1}$)					($mg\ kg^{-1}$)			
10.333	6.3	106.4	1.6	17.7	11.3	19.8	1.95	180.6	167.8	1449	52.5

II: *Physicochemical characteristics of studied biochar*

pH	Conductivity ($mS.cm^{-1}$)	Dry matter	N_{tot}	C_{tot}	Ash	P	K	Ca	Mg
		(%)				($mg\ g^{-1}$)			
10.12	4.22	95.47	0.37	56.05	32.72	2.614	16.36	51.23	6.134

III: *Characteristics of all the applied treatments*

Amendment				Dose per pot	Treatment
Description	Biochar	Bacterial inoculum	Mineral fertilizer		
Without amendment	-	-	-	-	C
"Bactofil" inoculum (I1)	+ (B) 60 g	*Azospirillum brasilense*, *Azotobacter vinelandii*, *Bacillus megaterium*, *Bacillus polymyxa*, *Pseudomonas fluorescens*, *Streptomyces albus*	-	0.1 ml	BI1
			+UAN 390 fertilizer (N)	0.359 ml	BI1N
"Novaferm" inoculum (I2)	+ (B) 60 g	*Azospirillum* spp., *Azotobacter* spp., *B. megaterium*, *Bacillus subtilis*	-	1 ml	BI2
			+UAN 390 fertilizer (N)	0.359 ml	BI2N

inoculated with "NovaFerm" (BI2) from Nova Scienta Kft (Soltvadkert, Hungary) at the beginning of the experiment on lettuce (BBCH-scale: 13). Later (BBCH-scale 15-18), UAN 390 fertilizer was added to half of the inoculated pots (BI1N and BI2N) at the dose recommended by the supplier (140 kg N ha^{-1}). UAN 390 is a liquid fertilizer of ammonium nitrate with urea and with ammonium (NH_4-N) nitrogen, nitrate (NO_3-N) nitrogen and amide (N-NH_2) nitrogen. It contains 30% of nitrogen; the ratio of ammonium, nitrate, and amide nitrogen is 1 : 1 : 2.

Plant Cultivation and Soil Preparation

Lactuca sativa var. capitata L. cv. Kennedy has been chosen as an experimental plant. Each pot contained one plant. The pots were randomly put into a Walk-In Chamber by CLF Plant Climatics GmbH® growth chamber that was set to maintain temperatures of 22 °C by day and 19 °C at night, 65% humidity, with a day length of 12 h and light intensity of 380 µmol $m^{-2}s^{-1}$. All the pots have been watered by adding 50 ml of deionized water every 2 days.

After two month of one growing cycle, leaf biomass was harvested. Root biomass from the first growing cycle of plants was removed and experimental soils were homogenized again and re-filled into containers. Lettuce plants were seeded again. The inoculation by "Bactofil" and "NovaFerm" additives and the application of UAN 390 fertilizer were done at the same BBCH-scale as in the case of the first two month plant growing cycle.

At the end of the experiment, soils were collected for determination of physicochemical and biological activities. Soil samples were cleared from plant residues and sieved to 2 mm. One part of the soil was used for soil enzyme activity determination, while the rest was transferred into plastic trays to dry at 40 °C in the oven for 24 h and used for pH and CEC measurement.

Soil Physicochemical Parameters

The 2 mm-sieved soil samples were prepared according to ISO 11464, 1994. pH (H_2O) was measured after stirring a mixture of soil and deionized water (1 : 5, v/v) in accordance with the ISO 10390 standard. Cationic exchange capacity (CEC) was analysed after percolation of CH_3COONH_4 (1 M, pH = 7) solution into soil samples followed by an extraction of ammonium ions (NH_4^+-N) with sodium chloride (NaCl, 1 M) according to the French NF X31-130 standard.

Soil Biological Parameters

Extraction of indigenous bacterial cells from soil was performed on the two fresh soil samples using a Nycodenz gradient separation method (Lindahl and Bakken, 1995). A white band of bacterial cells were obtained at the interface between the Nycodenz-soil mix particles and the overlying aqueous layer. Ureases activity can be evaluated according to Kandeler and Gerber (1988). The measurement of the urase activity is done through the colorimetric determination of ammonium (NH_4^+-N) release (Saha *et al.*, 2012). Phosphatase activity was measured according to the protocol of Eivazi and Tabatabai (1977) that is based on the colorimetric estimation of the p-nitrophenol released by the acid phosphatases activity upon soil incubation with buffer solution and the p-nitrophenyl phosphate. The protocol to determine laccase activity uses substrate ABTS: 2,2'-azinobis-(-3-ethylbenzthiazoline-6-sulfonate) then quantifying the rate of oxidation of ABTS to $ABTS^+$ in the supernatant (Eichlerová *et al.*, 2012).

Data Analysis

Soil physicochemical properties (pH, CEC) and biological activities (urease, phosphatase and laccase activities; total soil bacteria extraction) are expressed and presented as the means and standard deviations of replicates for each treatment. Analysis of variance (ANOVA) was accomplished to estimate differences within the treatments. The normal distribution of data (Shapiro-Wilk test) and equality of variances (Bartlett test) were checked. When both tests proved conformity, Fisher statistics was considered for significance ($p \leq 0.05$) and the Tukey (HSD) test was used for pair-wise comparisons of statistical groups. The Kruskal-Wallis test was

performed for data that were not distributed normally. All statistic tests were conducted in XLSTAT software (AddinsoftTM software 2016).

RESULTS

Changes in Soil Physicochemical Parameters

Soil pH value was significantly increased from neutral value in control soil to highly alkali in biochar amended treatments (Tab. IV).

Bactofil amended soils had pH values higher by 1.2 times compared to control and by 1.02 times compared to the rest of the treatments. BI1N and BI2N treatments had no significant differences resulting into alkali pH fluctuating from 8.0–8.1 that was 1.2 times higher compared to a control.

Control soil showed low CEC data and Bactofil amended treatment was equal (7.4–7.5 cmol kg^{-1}) resulting into the lowest CEC value compared to the other BCH amended treatments. The rest of the treatments (BI1N, BI2 and BI2N) exposed significantly higher by 1.1 times CEC values compared to control fluctuating from 8.1–8.2 cmol kg^{-1}. Additionally, BI2N treatment was statistically equal to control CEC value.

Changes in Soil Biological Parameters

Soil Bacteria Extraction

The number of bacteria g^{-1} dry soil in the studied BCH amended and inoculated Luvisol soil presented in Fig. 1.

No significant differences were found between the treatments concerning soil bacteria extraction. Bacteria quantity in the control soil revealed 151063 g^{-1} dry soil and this unamended treatment was statistically equal to the rest of BCH amended treatments where the values ranged from 83821–213004 g^{-1} dry soil.

Urease Activity

No significant differences in urease activity were found among all the treatments including control unamended soil (Fig. 2). The control soil exhibited 82.2 mg NH_4^+-N g^{-1} dry soil that was statistically equal to the rest of BCH amended treatments that ranged from 98.3–138.9 mg NH_4^+-N g^{-1} dry soil.

Acid Phosphatase Activity

Acid phosphatase activity where control soil exposed 0.15 µg Np g^{-1} dry soil and the rest of BCH amended treatments (BI1, BI1N, BI2, BI2N) displayed significantly decreased by 2.5 times values compared to control, that fluctuated 0.06–0.07 µg Np g^{-1} dry soil (Fig. 3).

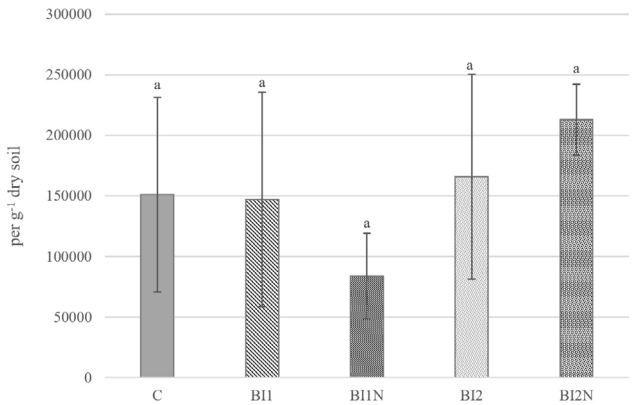
Laccase Activity

Laccase activity changes in amended soils after the second plant harvest (Fig. 4).

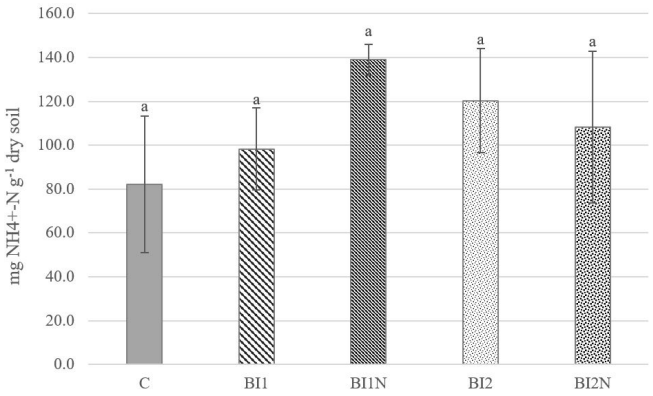
Laccase activity in control soil was 0.16 µmol $ABTS^+$ g^{-1} dry soil and it was equal to BI2 treatment of 0.24 µmol $ABTS^+$ g^{-1} dry soil. Amended B, BI1N and BI2N treatments had significantly higher by

IV: *Soil physicochemical parameters in control soil (C) and in BCH amended soils combined with inoculums (BI1, BI2) and with additional N fertilizer addition (BI1N, BI2N) after the second plant harvest, ± standard deviation. CEC: cationic exchange capacity, pH (For every parameter, different letters in lines refer to significant differences between soils) (Tukey HSD test, n = 4, p ≤ 0.05).*

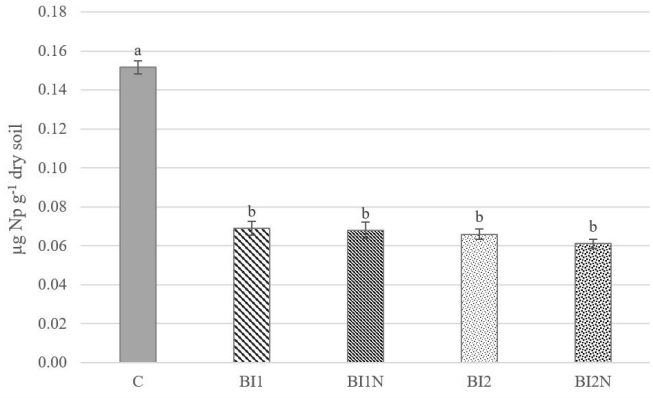
Treatment	C	BI1	BI1N	BI2	BI2N
pH	6.8 ± 0.1c	8.3 ± 0.1a	8.0 ± 0.1b	8.1 ± 0.0b	8.0 ± 0.0b
CEC (cmol kg^{-1})	7.5 ± 0.1bc	7.4 ± 0.2c	8.1 ± 0.2a	8.2 ± 0.3a	8.1 ± 0.3ab



1: *Average counts of bacteria (per g^{-1} dry soil) in control soil (C) and in BCH amended soils combined with inoculums (BI1, BI2) and with additional N fertilizer addition (BI1N, BI2N). Values are presented as means ± SD. Different letters refer to significant differences between treatments (Tukey HSD test, n = 4, p ≤ 0.05).*



2: *Urease activity (mg NH_4^+-N g^{-1} dry soil) in control soil (C) and in BCH amended soils combined with inoculums (BI1, BI2) and with additional N fertilizer addition (BI1N, BI2N). Values are presented as means ± SD. Different letters refer to significant differences between treatments (Tukey HSD test, n = 4, p ≤ 0.05).*



3: *Acid phosphatase activity (µg Np g^{-1} dry soil) in control soil (C) and in BCH amended soils combined with inoculums (BI1, BI2) and with additional N fertilizer addition (BI1N, BI2N). Values are presented as means ± SD. Different letters refer to significant differences between treatments (Tukey HSD test, n = 4, p ≤ 0.05).*

1.6–1.8 values compared to control that ranged from 0.25–0.28 µmol $ABTS^+$ g^{-1} dry soil.

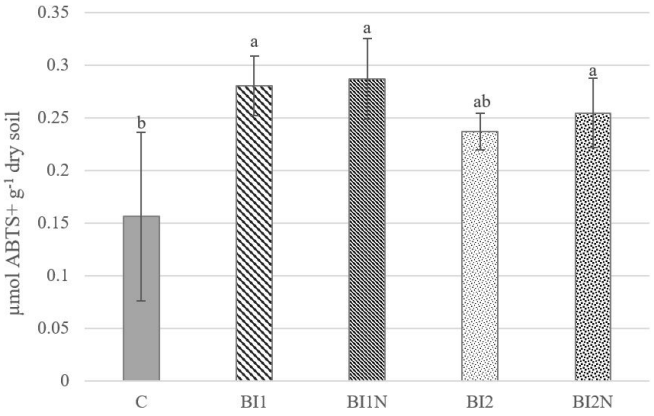
DISCUSSION

The Effect of the BCH, Bacterial Inoculums and N Fertilizer on Soil Physicochemical Parameters

BCH amended soils with bacterial inoculums and N fertilizer had significantly higher pH values compared to control soil (Tab. IV). According to Novak *et al.* (2009) significant pH increase occurred at the higher production temperatures of BCH due to the concentration of nonpyrolyzed inorganic elements in the feedstock. pH increases to alkaline levels in BCH amended soils that leads to micronutrients deficiencies with a consequent yield decrease (Chan and Xu, 2012). From the other side, studies of Hale *et al.* (2011) showed no pH nor CEC changes in plain fine loam soil amended with corn stover BCH, microbial inoculums and nutrient solutions, even in the aged form. CEC values displayed quite similar trend with the higher CEC data in BCH amended soils by 8.5% compared to control soil (Tab. IV), although Bactofil inoculum proved its lower effectiveness being equal to control. Our results are in line with the former report of Liang *et al.* (2006) where CEC per unit soil C were up to 1.9 times higher in Anthrosols amended with BCH compared to the adjacent soils. Studies of He *et al.* (2017) on straw BCH mixed with regular chemical fertilizers showed CEC increase up to 21.8% compared to the other treatments, studying paddy soil used for the production of rice and rapeseed.

The Effect of the BCH, Bacterial Inoculums and N Fertilizer on Bacteria Quantity and Soil Enzyme Activities

The results on total soil bacteria count demonstrated the absence of effect in BCH



4: *Laccase activity (µmol ABTS⁺ g⁻¹ dry soil) in control soil (C) and in BCH amended soils combined with inoculums (BI1, BI2) and with additional N fertilizer addition (BI1N, BI2N). Values are presented as means ± SD. Different letters refer to significant differences between treatments (Tukey HSD test, n = 4, p ≤ 0.05).*

amended soils, as these parameters were equal to control without any additives (Fig. 1). Our results are in line with the studies of Prayogo *et al.* (2014), where addition of BCH in rather low concentrations of 0.5% and 2% had no significant effect on the amount of total, bacterial or fungal phospholipid fatty acid that indicated bacterial development. Sun *et al.* (2016a) reported on pine-wood BCH used as a *Pseudomonas putida* inoculum carrier that does not promote increased shelf life or inoculum efficacy. Whereas former reports of Saxena *et al.* (2013) stated on beneficial BCH influence combined with *Bacillus sp.* as a bioinoculant and commercial fertilizer on phosphate solubilizing bacteria abundance in loamy soil.

Urease enzymes are released during the hydrolysis of urea to carbon dioxide and ammonia, that might be assimilated by microbes and plants (Lloyd and Sheaffe, 1973). No effect was observed on urease activity with any amendments, even with N fertilizers containing urea (Fig. 2). Similar results on urease activity were obtained in Lu *et al.* (2015) with wheat straw BCH and BCH poultry manure compost applied to Aqui-Entisol with maize. Contrary to that, studies of Akça and Namli (2015) demonstrated the opposite effect of the poultry litter BCH on urease activity in clay loam soil which grew lettuce, tomato and pepper plants, promoting its abundance. Our previous studies stated on promoted plant growth in BI1N and BI2N treatments, where N compounds were effectively utilized by plant, and Novaferm treatment demonstrated greater effectiveness in terms of bacterial combination promoting plant growth even without N additives (Mikajlo *et al.*, 2020).

N fertilizer itself, in turn, can influence acid phosphatase activity positively like in the studies on ammonium nitrate additive under winter wheat cultivation (Lemanowicz, 2011), in the same way as the combined inoculation including *Bacillus subtilis* promotes acid phosphatase activity along with rhizosphere microbial population in the studies on lettuce in agricultural soil (Kohler *et al.*, 2007). Although in our studies BCH in higher doses had greater suppressing effect compared to mineral additives.

Phosphatase activity values in BCH amended soils with inoculums and N fertilizer revealed lower by 53.4–60% data compared to non-amended soil (Fig. 3). Generally, phosphatase enzymes hydrolyse organic phosphorus (P) compounds with consequent transformation into different forms of inorganic P, that in turn are assimilable by plants (Margalef *et al.*, 2017). Our former studies on total P content in BCH amended soils showed no significant differences within treated and control soils after the second lettuce harvest, owing to P consumption by control plants in the first harvest (Mikajlo *et al.*, 2020). This can also be related to higher acid phosphatase activity in non-amended soil. Perhaps this decrease in BCH amended soils is related to BCH chemical properties. The results are in line with the former reports on acid phosphatase activity decrease by 18.6% and 34.0% for clay loam and silt loam induced by manure BCH addition that had been detected by Jin *et al.* (2016) explained by possible probability of substrates chemical blocking by BCH. The confirmation of this hypothesis had been also revealed by Sun *et al.* (2016b) studying rice straw BCH and evaluating its effect on the microbial community in biochar niche, while concluding absorption of tested enzymes by BCH including phosphatase and resulting in its quantitative decrease. The other study of Chen *et al.* (2013) detected no differences in acid phosphatase activity within control and wheat straw BCH amended sandy loam soils of rice paddy field in China.

Contrary to phosphatase activity values, laccase activity exhibited the lowest content in control soil (Fig. 4). Generally, laccases play an important

role in the carbon cycle and have been considered mostly like fungal enzymes, that participate in lignin transformation and other polyphenols, being present in dead plant material and humic substances in soils (Eichlerová *et al.*, 2012). Additional soil additives like BCH, N fertilizer and inoculums could alter these processes. Bacterial inoculums along with the UAN fertilizer improved soil laccase activity values. Although Novaferm amended soils revealed laccase activity values similar to the unamended soil, whilst the rest of the BCH amended soils displayed data by 33.4–44.8% higher compared to the control soil. In a previous study, we demonstrated that roots colonization by arbuscular mycorrhizal fungi was equal to control or slightly increased in BCH amended soils without any significant differences between treatments with bacterial and N fertilizer additives (Mikajlo *et al.*, 2016). According to Gibson *et al.* (2016) during BCH abiotic aging process the oxidation of aromatic C and the introduction of aliphatic C-H groups on BCH occurs, that enhances laccase and peroxidase activities with fungal respiration. Similar results on increased laccase activity in BCH amended soil had been also found in the studies of Lauber *et al.* (2009) where no significant shift in the composition of laccase genes between control- and N-fertilized soils had been found nor in the relative abundance of laccase genes. Contrary to these findings, other studies claim on BCH ability to immobilize laccase including its activity decrease owing to BCH porous surface structure (Taheran *et al.*, 2017; Lonappan *et al.*, 2018; Naghdi *et al.*, 2018). Overall, physicochemical and biological soil activities are highly dependent on BCH feedstock, means of production and an initial soil state (Lehmann *et al.*, 2011).

CONCLUSION

Our results showed prosperous effect of inoculums on soil physicochemical properties that was supported by N fertilization. In addition, Novaferm bacterial combination with BCH had higher effectiveness when analysing CEC. Moreover, for laccase activity both Bactofil and Novaferm with mineral N showed increased fungal enzyme values compared to control. Nevertheless, taking into consideration other soil enzyme activities and microbial growth, we may state on BCH and additives combination ineffectiveness regarding urease and acid phosphatase activity along with total soil bacteria amount. Most probably, high BCH concentrations are not prosperous enough for microbial development in soil. Chosen BCH concentration needs reconsideration inclining to its reduction and testing its possible influence in comparison with inoculums and N additives. This investigation needs deeper study aiming to analyse BCH aging in soil for a longer period and its functioning in soil-plant nutrient cycling.

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