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Short-Term Response of Soil Microbial Biomass to Different Chabazite Zeolite Amendments

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ABSTRACT

Zeolitites (ZT) are rocks containing more than 50% of zeolite minerals and are known to be a suitable material for agricultural purposes by improving soil physicochemical properties and nitrogen (N) use efficiency. However, little is known about their effects on soil microbial biomass. This study aimed to evaluate short-term effects of different chabazite ZT amendments (CHAZT) on soil microbial biomass and activity. A silty-clay agricultural soil was amended in three different ways, including the addition of natural (5% and 15%) and NH_4^+-enriched (10%) CHAZT. Dissolved organic carbon (C), total dissolved N, NH_4^+, NO_3^-, NO_2^-, microbial biomass C and N, and ergosterol were measured periodically over 16 d in a laboratory incubation. To verify the microbial immobilization of the N derived from NH_4^+-enriched CHAZT, a high ^{15}N source was used for enriching the mineral to measure the microbial biomass $\delta^{15}N$ signature. An increase in the ergosterol content was observed in the soil amended with 5 % natural CHAZT. However, no similar result was observed in the soil amended with 15% natural CHAZT suggesting that the fungal biomass was favored at a lower application rate. In the soil amended with NH_4^+-enriched CHAZT, microbial biomass N was related to NO_3^- production over time and inversely related to NH_4^+, suggesting high nitrification process. Isotopic measurements on microbial biomass confirmed immediate assimilation of N derived from NH_4^+-enriched CHAZT. These results suggest that the NH_4^+-enriched CHAZT used in this study supplied an immediately available N pool to the microbial biomass.

Key Words: ergosterol, microbial biomass $\delta^{15}N$, natural zeolite, nitrification, NH_4^+-enriched zeolite, slow release fertilizer

INTRODUCTION

The application of organic and inorganic amendments has been recognized as a possible method for improving soil physicochemical properties and fertility (Waltz *et al*., 2003; Lima *et al*., 2009; Colombani *et al*., 2014). Among them, natural zeolite-bearing rocks are known to be a suitable material for agricultural purposes owing to their very high cation exchange capacity (CEC), reversible dehydration, and molecular sieving properties (Reháková *et al*., 2004; Passaglia, 2008; Misaelides, 2011). Zeolites are aluminosilicates with an open three-dimensional framework, which delimits channels and cavities where different kinds of polar and non-polar molecules can be exchanged, involving both inorganic and organic compounds, with a particular affinity to NH_4^+ (Reháková *et al*., 2004). Furthermore, zeolites can be easily modified from their natural state by enrichment processes, which cause the adsorption of specific cations, *e.g*., NH_4^+ and Na^+) (Dittert *et al*., 1998; Leggo, 2000; Faccini *et al*., 2015).

Since natural chabazite zeolite (CHA) is less abundant than clinoptilolite worldwide (Passaglia, 2008), the latter has been investigated in the majority of agricultural and environmental studies. CHA are commonly found in volcanoclastic deposits, especially in the Italian Peninsula, where many quarries are exploiting zeolite-rich tuffs for the production of construction bricks (Passaglia, 2008). These tuffs are generally dominated by potassium (K)-rich CHA, and thus can be classified as zeolitites (ZT) owing to their high zeolite content (>50%) (Galli and Passaglia, 2011). During the cutting process of these construction bricks, the high amount of zeolite-rich material remains unused, constituting a waste for the quarry. However, it is an interesting and precious granular by-product, which can be used for many purposes, including the use as a soil amendment as demonstrated by ZeoLIFE project, European Union (LIFE10 ENV/IT/000321) (Ferretti *et al.* 2017a).

The use of different kinds of natural and enriched ZT as soil amendment has been studied extensively in terms of modification of the soil physicochemical characteristics (Passaglia, 2008; Colombani *et al*., 2015, 2016), reduced N leaching, increased N use efficiency, increased water use efficiency, and improved crop yield (Reháková *et al*., 2004; Sepaskhah and Barzegar, 2010; De Campos Bernardi *et al*., 2013; Gholamhoseini *et al*., 2013; Li *et al*., 2013; Di Giuseppe *et al*., 2016). Some studies have defined NH_4^+-enriched ZT as a slow-release fertilizer, where NH_4^+ is released slowly over time and becomes available for plant uptake, thus reducing potential N losses (Barbarick and Pirela, 1984; Lewis *et al*., 1984; Dwairi, 1998). Except for a few studies such as Mühlbachová and Šimon (2003), the effects of ZT amendments on the soil microbial biomass (MB) are mostly unexplored. Concerning amendments with NH_4^+-enriched ZT, Leggo (2000) carried out an investigation of plant growth in an organo-zeolitic substrate and observed an increase in NO_3^- after the use of natural clinoptilolite enriched by composting with poultry manure. He concluded that the Ca^{2+} present in the soil solution has probably been exchanged with the NH_4^+ adsorbed by the zeolites, making it immediately available to nitrifier microorganisms. However, this outcome is contradictory to the view of NH_4^+-enriched ZT as a slow-release fertilizer. To the best of our knowledge, no studies exists on the effects of natural and NH_4^+-enriched CHA rich ZT (CHAZT) amendments on soil MB.

The present study aimed to investigate the effects of different typologies of CHAZT amendments on soil MB and C-N dynamics over a short-term period. To this end, this study was designed to simulate the conditions occurring in the ZeoLIFE experimental field (ZeoLIFE project), an on-going field-scale experimentation, in which natural and NH_4^+-enriched CHAZT are being tested at the field-scale (Ferretti et al., 2017a). We hypothesized that: i) amendments with CHAZT at natural state will reduce N availability to soil MB in a short-term period owing to their high CEC and NH_4^+ affinity, thus favoring the development of fungi rather than bacteria due to the lower nutrient requirement of fungi (McGill *et al*., 1981; Strickland and Rousk, 2010); and ii) NH_4^+-enriched CHAZT, acting as a slow-release fertilizer once added to soil, will not affect soil MB in the short-term period.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected during spring 2015 from ZeoLIFE project experimental field, consisting of a 6-ha agricultural field where different CHAZT amendments are being tested since 2012. The field is located in the Po River Delta Plain near Codigoro town in Ferrara Province, Italy (44°50′33″ N, 12°05′40″ E), and lays

on clayey-silty soil of alluvial origin classified as Calcaric Gleyic Cambisol (Di Giuseppe *et al*., 2014; IUSS Working Group WRB, 2014). The experimental field has been subdivided into different plots (0.5--1.5 ha) in which both natural and NH_4^+-enriched CHAZT have been applied in various amounts (5--15 kg m^{-2}). Soil samples for this study were collected from an unamended parcel from the top 0.3 m depth layer and amended with different types of CHAZT in the laboratory immediately before the beginning of the experiment, in order to reproduce the short-term effects of zeolite application. Approximately 5 kg soil was brought to the laboratory immediately after sampling, sieved to < 5 mm and air-dried. Main soil characteristics are given in Table I, and soil mineralogical composition has been reported in Malferrari *et al*. (2013). The soil is mainly characterized by quartz, illite, chlorite, K-feldspar, plagioclase, calcite and amorphous residues, thus lacking of clay minerals with very high CEC (*e.g*., smectite).

TABLE I. Basic properties of the soil used in this study

Property[a)]	Value
pH	7.6 ± 0.2[b)]
EC (mS cm^{-1})	1.0 ± 0.1
$CaCO_3$ (g kg^{-1})	64.5 ± 3.5
CEC (mmol kg^{-1})	325 ± 1
TN (g kg^{-1})	2.33 ± 0.31
TOC (g kg^{-1})	22.76 ± 3.2
TOC/TN ratio	9.76 ± 0.34
Bulk density (kg m^{-3})	1 247 ± 81

[a)]EC = electrical conductivity; CEC = cation exchange capacity; TN = total N; TOC = total organic C.

[b)]Means ± standard deviations (*n* = 3)

Natural and NH_4^+-enriched CHAZT used

The ZT used in the present study is a byproduct from a quarry located near Sorano Village (central Italy) that is mainly exploited to obtain blocks and bricks for construction and gardening purposes.

The quarried material is a zeolitized tuff (a weathered rock of volcanic origin) composed by more than 68% of K-rich CHA, 1.8 % of phillipsite and 0.6 % of analcime resulting in a total zeolitic content of 70.9 % (Malferrari *et al.,* 2013). The CEC of the whole rock was determined by Malferrari et al. (2013) and resulted 1420 mmol kg^{-1}. CHAZT with a grain size of 3--5 mm was selected and used both at natural state (NZ) and pre-enriched with NH_4^+ as soil amendment. After sieving, a part of the NZ was subjected to an enrichment process, which allowed the enrichment of the CHA contained in the ZT with NH_4^+, thus creating an NH_4^+-enriched CHAZT (CZ). The enrichment process involved mixing of pig-slurry and CHAZT in a specifically conceived prototype (Faccini *et al*., 2015) produced the CZ with an average NH_4^+-N load of 3.014 g kg^{-1}.

Zoo-technical effluents, such as pig-slurry that are commonly used as organic fertilizers, are generally strongly enriched in the heavier ^{15}N isotope due to NH_3 volatilization that causes depletion of the lighter ^{14}N. The N isotope ratio is expressed in the standard (δ) notation in per mil (‰) relative to the atmospheric air (AIR) isotope standard (Gonfiantini *et al*., 1995). The above mentioned process result in $\delta^{15}N$ values generally >10 ‰ or even >20 ‰ in pig slurries (Table II) (Dittert *et al*., 1998; Schmidt and Ostle, 1999; Lim *et al*.,

2007) implying that in some cases they can be employed as an isotopic tracer for studies on natural N abundance (Dittert *et al*., 1998). The main properties of the NZ and CZ used in this study are presented in Table II.

TABLE II Main properties of the natural (NZ) and NH_4^+-enriched (CZ) zeolitites used in this study

Property[a)]	NZ	CZ
Grain Size (mm)	3--5	3--5
Air-dry GWC (%)	14.2	21.8
TN (g kg^{-1})	0.01	4.27
TOC (g kg^{-1})	0.08	1.24
$\delta^{15}N$ (‰)	--	43.6
TDN (mg kg^{-1})	14.6	3 611
DOC (mg kg^{-1})	--	118
TOC/TN ratio	8.42	0.29
pH	7.58	6.95
MBC (mg kg^{-1})	22.2	23.8
MBN (mg kg^{-1})	9.69	388
NO_3^--N (mg kg^{-1})	--	146
NH_4^+-N (mg kg^{-1})	--	3 014
Ergosterol (mg kg^{-1})	--	--

[a)]GWC = gravimetric water content; TN = total N; TOC = total organic C; TDN = total dissolved N; DOC = dissolved organic C; MBC and MBN = microbial biomass C and N, respectively.

[b)]Below the detection limit.

Experimental set-up

The experiment was conducted in the laboratory in order to mimic the treatments and conditions of the ZeoLIFE experimental field immediately after the application of NZ and CZ, resulting in four different treatments in triplicates. Two treatments were composed by a mixture of soil and NZ in the NZ weight proportion of 5 % (5NZ) and 15 % (15NZ), respectively. One treatment composed of a mixture of soil and CZ in the CZ weight proportion of 10% (10CZ), and the treatment without any amendment served as a control (CNTR). For each treatment, 1 kg of 5-mm sieved material was incubated in open ceramic jars (200-mm diameter) for 16 d at room temperature (*ca*. 20 °C) adjusting the moisture level to 45% water-filled pore space (WFPS) with Milli-Q (Millipore USA) water. These conditions reflected the ZeoLIFE field average temperature and moisture level, based on a 4-year (2011--2015) monitoring record. As the present study aimed to verify the immediate effects after the amendments with NZ and CZ, no further N inputs were applied into CNTR, 5NZ, and 15NZ. On days 2, 7, 9, 11, and 16 of the incubation period, a representative subsample was collected to analyze a set of parameters mentioned below.

Analytical techniques

Inorganic N forms

Soil NH_4^+-N was extracted with 1 mol L^{-1} KCl in a 1:10 (weight/volume) ratio, the solution was shaken for 1 h, and then filtered with Whatman no. 40 filter paper. The solution was diluted and analyzed with an Orion 95-12 ion selective electrode (ISE) connected to a Orion 4star pH/ISE benchtop meter (Thermo Scientific, Beverly, USA) (Banwart *et al.*, 1972 modified; Ferretti *et al.*, 2017b). The NO_3^--N and NO_2^--N were extracted with Milli-Q water (Millipore USA) in a 1:10 (weight/volume) ratio, the solution was shaken for 1 h, and then filtered with Whatman no. 40 filter paper (Myers and Paul, 1968). Contents of NO_3^--N and NO_2^--N were determined by ion chromatography (Ferretti *et al*., 2017b) as indicated by the Italian law according to D.M. 13/09/1999 with an isocratic dual pump ICS-1000 Dionex (Thermo Fisher Scientific, Sunnyvale, USA) equipped with an AS9-HC 4 mm × 250 mm high-capacity column and an ASRS-Ultra 4-mm self-suppressor. An AS-40 Dionex auto-sampler was used to run the analysis. A quality control (QC) sample was run every 10 samples. The standard deviation of all the QC samples run was less than 4%. Dissolved inorganic N (DIN) was calculated as the sum of NH_4^+-N, NO_3^--N, and NO_2^--N.

Ergosterol determination

Ergosterol content was determined following the method proposed by Gong *et al*. (2001) with some modifications. Zeolite and soil samples were freeze-dried at –50 °C and 6 mL methanol (Me-OH) was added to 4 g sample. The suspension was homogenized with a hand vortex, placed in an ultrasound bath for 15 min, and centrifuged at 10 518 × *g* for 15 min. The supernatant was filtered using a syringe membrane filter (4 mm, 0.45 µm polytetrafluoroethylene (PTFE) and then kept in the dark until analysis with an Agilent Technologies Infinity 1290 high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, California, USA). The injection volume of the sample was 5 µL, while the flux rate was 0.5 mL min^{-1} with 95% Me-OH in H_2O as an eluent phase and a Zorbax Eclipse Plus C18 rapid resolution 2.1 mm × 50 mm column with 1.8-µm porosity as a solid phase. Ergosterol was determined using a UV detector at 282 nm.

Soil pH, dissolved C and N, and microbial biomass C (MBC) and N (MBN)

Soil pH was determined using 0.01 mol L^{-1} $CaCl_2$ extract in a 1:10 (weight/volume) ratio with a lab pH meter inoLab® pH 196 Level 2 (WTW, Weilheim, Germany). Chloroform fumigation-extraction method was employed according to Brandstätter *et al*. (2013) and Öhlinger (1996) to determine MBC, MBN, dissolved organic C (DOC), and total dissolved N (TDN). Fumigated and non-fumigated samples were prepared with 1 mol L^{-1} KCl in a 1:10 (weight/volume) ratio. The suspension was shaken for 1 h and then filtered through an N-free filter paper. Filtrates were stored at --20 °C prior to analysis with a TOC-L TNM-L Analyzer (Shimadzu, Kyoto, Japan) equipped with an ASI-L auto sampler. Before the analysis, inorganic C was eliminated by acidification. The C and N extracted from non-fumigated sample represented DOC and TDN, respectively. The C and N extracted from chloroform-fumigated samples minus those extracted from non-fumigated samples represented the C and N immobilized by soil microorganisms, respectively. Correction factors of 0.45 and 0.54 were used according to Brookes *et al*. (1985) and Beck *et al*. (1997) to determine

MBC and MBN, respectively. Dissolved organic N (DON) was calculated as the difference between TDN and DIN.

Calculation of microbial biomass $\delta^{15}N$ and net ^{15}N microbial immobilization rates

To measure the N isotopic signature of the soil MB (Dijkstra *et al*., 2006), we exploited the very high $\delta^{15}N$ of the pig-slurry employed in the NH_4^+-enrichment process of CZ in order to trace and verify its interactions with MB. Microbial biomass isotopic signature (MB$\delta^{15}N$) was determined only for CNTR (at the beginning of the incubation) and in 10CZ treatment (on days 2, 9, and 16) as no differences in isotopic signature were expected between CNTR, 5NZ, and 15NZ. Extraction-fumigation-extraction (EFE) method was employed to determine MBC and MBN isotopic signature (Widmer *et al*., 1989). Briefly, 30 mL of 0.1 mol L^{-1} K_2SO_4 was added to 2 g soil. The suspension was shaken for 1 h and then filtered through ash-free paper. The residual soil in the vial was then transferred to the filter paper by adding new extractant, shaking, and pouring the suspension on to the same filter. The soil was then re-extracted by adding 15 mL of 25 mmol L^{-1} K_2SO_4 and 1 mL $CHCl_3$, shaking for 1 h, and filtering with ash-free filter paper. The extract was then freeze-dried and analyzed with a Vario Micro Cube elemental analyzer (Elementar, Langenselbold, Germany) coupled with an ISOPRIME 100 isotopic ratio mass spectrometer (Isoprime, Cheadle, England) operating in a continuous-flow mode. The amount of ^{15}N incorporated into MB (^{15}N MB, mg ^{15}N kg^{-1} MB) over time was back calculated from MB $\delta^{15}N$ and the amount of MBN according Eq. (1 and 2).

$$^{15}N\ atom\%_{\ sample} = ((\delta^{15}N_{\ sample} / 1000) * {}^{15}N\ atom\%_{\ std}) + {}^{15}N\ atom\%_{\ std} \quad (1)$$

$$MB^{15}N = (MBN * {}^{15}N\ atom\%_{\ sample}) / 100 \quad (2)$$

Where $^{15}N\ atom\%_{\ std}$ is the amount of ^{15}N in the standard (0.3663%), $^{15}N\ atom\%_{\ sample}$ is the amount of ^{15}N in the measured sample, $\delta^{15}N_{\ sample}$ is the MB$\delta^{15}N$ of the measured sampled, MBN is the amount of N of soil MB and $MB^{15}N$ is the amount of ^{15}N incorporated by soil MB.

Microbial ^{15}N net immobilization rate ($^{15}N_{imm}$, $\mu g\ ^{15}N\ d^{-1}$) was calculated according to Eq. (3):

$$^{15}N_{imm} = (MB^{15}N_t - MB^{15}N_{t0})/t \quad (3)$$

where $MB^{15}N_t$ and $MB^{15}N_{t0}$ are the amounts of ^{15}N atoms assimilated by soil MB at time t (d) and initial time point, respectively.

Statistical analysis

To evaluate significant differences between the treatments, data were checked to meet parametric statistic assumption. Successively, repeated measures analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were conducted at a P level of 0.05 for each sampling time. SigmaPlot 12.0 was employed to run statistical analyses.

RESULTS

Inorganic N forms

Soil NH_4^+-N content ranged from 4.3 (in 5NZ) to 68.8 (in 10CZ) mg kg^{-1} (Table III). The NH_4^+-N content was similar throughout the incubation period for CNTR, 5NZ, and 15NZ with no significant differences ($P > 0.05$). Concerning 10CZ, exchangeable NH_4^+-N was always significantly higher than the other treatments ($P < 0.05$), attributed to the N adsorbed by CZ. A decrease in NH_4^+-N ($P < 0.05$) in 10CZ was observed from days 9 to 16, reaching half of the initial amount.

TABLE III

Contents of N[a)] in different forms in the soil without addition (CNTR) or with addition of 5% (5NZ) and 15% (15NZ) natural zeolite and 10% NH_4^+-enriched zeolite (10CZ) on days 2, 7, 9, 11, and 16 of the incubation period

Treatment	TDN	NH_4^+-N	NO_3^--N	NO_2^--N	DIN	DON	MBN
	mg kg^{-1}						
				2 d			
CNTR	49.8 ± 2.4[b)]a[c)]	13.9 ± 3.4a	30.2 ± 1.7a	0.7 ± 0.1a	44.8 ± 1.8a	4.99 ± 3.95a	19.1 ± 7.0a
5NZ	48.7 ± 0.6a	12.2 ± 1.0a	35.9 ± 1.2b	--[d)]	48.1 ± 0.9a	0.52 ± 0.30a	16.3 ± 1.8a
15NZ	48.9 ± 1.2a	10.9 ± 0.5a	34.7 ± 0.9ab	--	45.7 ± 1.5a	3.25 ± 0.22a	18.6 ± 1.5a
10CZ	320.8 ± 29.8b	68.7 ± 4.1b	151.4 ± 4.2c	20.0 ± 0.5b	240.0 ± 8.3b	80.71 ± 37.20b	74.0 ± 3.0b
				7 d			
CNTR	48.5 ± 2.6a	7.6 ± 0.7a	39.5 ± 2.6b	0.2 ± 0.1a	47.3 ± 2.2a	1.15 ± 1.10a	24.0 ± 6.1a
5NZ	47.0 ± 1.1a	11.0 ± 1.0b	33.9 ± 0.9a	0.6 ± 0.2a	45.5 ± 1.1a	1.55 ± 0.21a	19.5 ± 2.9a
15NZ	57.8 ± 3.0a	10.8 ± 0.2b	43.8 ± 2.7b	0.4 ± 0.2a	55.0 ± 2.8b	2.74 ± 2.23a	19.0 ± 7.4a
10CZ	382.2 ± 8.0b	68.8 ± 4.0c	145.4 ± 1.6c	4.3 ± 0.3b	218.5 ± 2.5c	163.72 ± 10.21b	40.1 ± 28.1a
				9 d			
CNTR	50.5 ± 3.4a	9.7 ± 0.7b	25.0 ± 4.6a	7.6 ± 1.0c	42.2 ± 5.3b	8.29 ± 5.91a	11.0 ± 3.9a
5NZ	47.5 ± 0.9a	6.2 ± 0.7a	29.4 ± 0.9a	0.3 ± 0.1a	35.8 ± 0.3ab	11.65 ± 0.78a	9.71 ± 5.9a
15NZ	52.1 ± 0.2a	7.7 ± 1.0ab	24.2 ± 0.3a	--	32.0 ± 0.8a	20.05 ± 0.65b	6.9 ± 4.2a
10CZ	405.6 ± 79.5b	59.1 ± 2.7c	189.7 ± 3.5b	1.9 ± 0.1b	250.6 ± 3.8c	155.04 ± 82.36c	131.9 ± 5.8b
				11 d			
CNTR	39.2 ± 2.7a	4.8 ± 0.5a	29.5 ± 0.4a	0.3 ± 0.2a	34.6 ± 0.5a	4.63 ± 2.73a	22.2 ± 13.6a
5NZ	50.1 ± 14.4a	4.3 ± 0.8a	31.4 ± 0.3a	0.3 ± 0.1a	36.0 ± 0.8a	14.06 ± 14.79a	11.1 ± 4.3a
15NZ	46.4 ± 1.3a	5.1 ± 0.9a	30.2 ± 0.7a	0.2 ± 0.1a	35.6 ± 1.6a	10.84 ± 2.39a	14.1 ± 3.3a
10CZ	534.5 ± 154.4b	46.7 ± 2.5b	300.5 ± 5.7b	2.4 ± 0.2b	349.6 ± 3.3b	184.89 ± 151.14b	220.4 ± 70.0b
				16 d			
CNTR	49.4 ± 2.8a	5.0 ± 0.7a	36.5 ± 0.1a	--	41.7 ± 0.5a	7.72 ± 3.24a	20.6 ± 14.8a
5NZ	68.0 ± 0.1a	5.4 ± 0.5a	49.8 ± 0.2b	3.3 ± 0.3b	58.6 ± 0.8c	9.44 ± 0.83a	21.7 ± 5.9a
15NZ	54.7 ± 2.8a	6.5 ± 0.5a	40.1 ± 0.1a	--	46.6 ± 0.5b	8.11 ± 2.63a	42.0 ± 5.3a
10CZ	375.5 ± 6.7b	29.0 ± 0.8b	274.2 ± 10.1c	2.2 ± 0.1a	305.4 ± 9.2d	70.10 ± 2.71b	237.5 ± 15.6b

[a)]TDN = total dissolved N; DIN = dissolved inorganic N; DON = dissolved organic N; MBN = microbial biomass N.

[b)]Means ± standard deviations (n = 3).

[c)]Means in the same column followed by different letters are significantly different on each sampling day ($P < 0.05$) based on analysis of variance and Fisher's least significant difference test.

[d)]Below the detection limit.

Soil NO_3^--N content ranged from 24.2 (in 15NZ) to 301 (in 10CZ) mg kg^{-1} (Table III). It is noteworthy that NO_3^--N content in 10CZ was entirely out of scale if compared with the other treatments throughout the incubation period. Furthermore, just after 2 d of incubation, 10CZ exhibited a very high NO_3^--N content (151 mg kg^{-1}), approximately five-times higher than that of the other treatments ($P < 0.05$). The content of NO_3^--N in 10CZ increased significantly from days 9 to 16, doubling its initial value.

After an incubation period of 2 d, all the treatments exhibited a significantly lower NO_2^--N content compared with that of 10CZ with a peak of 20 mg kg^{-1} ($P < 0.05$) (Table III). On day 7, soil NO_2^--N content in 10CZ remained high ($P < 0.05$); however, it was considerably lower than that on day 2. From day 9, small differences were observed among the treatments ($P < 0.05$); however, there was no particular trend.

Soil pH and dissolved C and N

Soil pH ranged from 7.73 to 7.90 with no significant differences between the treatments and no variation during the incubation period ($P > 0.05$, Table IV).

Soil DOC content ranged from 26.7 (in 15NZ) to 67.9 (in 10CZ) mg kg^{-1} (Table IV). Dissolved organic C was always higher in 10CZ than other treatments with an increase with time from day 9 until the end of the experiment ($P < 0.05$). Other treatments did not show significant differences in DOC ($P > 0.05$).

TABLE IV

pH, dissolved organic carbon (DOC), ergosterol, and microbial biomass C (MB-C) in the soil without addition (CNTR) or with addition of 5% (5NZ) and 15% (15NZ) natural zeolite and 10% NH_4^+-enriched zeolite (10CZ) on days 2, 7, 9, 11, and 16 of the incubation period

Treatment	pH	Ergosterol	DOC	MBC
		---------- mg kg^{-1} ----------		
		2 d		
CNTR	7.74 ± 0.05[a)]a[b)]	2.78 ± 0.29a	35.0 ± 5.1b	233 ± 16a
5NZ	7.88 ± 0.01a	2.69 ± 0.23a	38.9 ± 0.3b	257 ± 47a
15NZ	7.90 ± 0.02a	4.46 ± 1.39b	27.6 ± 2.0a	231 ± 21a
10CZ	7.84 ± 0.03a	2.91 ± 0.99a	47.7 ± 5.4c	226 ± 15a
		7 d		
CNTR	7.84 ± 0.03a	3.39 ± 0.50b	36.3 ± 3.9b	347 ± 93b
5NZ	7.78 ± 0.01a	2.35 ± 0.16a	34.8 ± 2.3b	212 ± 11a
15NZ	7.85 ± 0.03a	2.26 ± 0.32a	28.9 ± 1.3a	252 ± 58a
10CZ	7.73 ± 0.06a	2.36 ± 0.28a	45.2 ± 2.5c	237 ± 43a

		9 d		
CNTR	7.84 ± 0.04a	2.10 ± 0.05a	48.7 ± 27.4ab	194 ± 61a
5NZ	7.76 ± 0.07a	3.77 ± 1.80a	40.0 ± 5.4a	202 ± 7a
15NZ	7.86 ± 0.03a	2.96 ± 0.38a	38.8 ± 16.2a	197 ± 48a
10CZ	7.86 ± 0.01a	1.98 ± 0.55a	66.7 ± 32.5b	165 ± 70a
		11 d		
CNTR	7.78 ± 0.06a	2.30 ± 0.41a	30.1 ± 2.2a	260 ± 82a
5NZ	7.85 ± 0.01a	5.47 ± 1.73b	32.3 ± 1.2a	191 ± 9a
15NZ	7.88 ± 0.01a	2.25 ± 0.13a	26.7 ± 2.6a	207 ± 12a
10CZ	7.84 ± 0.03a	2.03 ± 0.20a	60.4 ± 0.6b	184 ± 12a
		16 d		
CNTR	7.87 ± 0.04a	3.27 ± 0.58a	47.6 ± 16.8a	240 ± 102a
5NZ	7.77 ± 0.05a	6.71 ± 3.20b	39.7 ± 6.1a	227 ± 9a
15NZ	7.84 ± 0.03a	2.76 ± 1.15a	34.3 ± 2.8a	330 ± 58b
10CZ	7.80 ± 0.02a	2.38 ± 0.32a	67.9 ± 10.5b	190 ± 8a

[a)]Means ± standard deviations (n = 3).

[b)]Means in the same column followed by different letters are significantly different on each sampling day ($P < 0.05$) based on analysis of variance and Fisher's least significant difference test.

Soil TDN content ranged from 39.2 (in CNTR) to 534 (in 10CZ) mg kg^{-1} (Table III). No significant differences in TDN were observed among CNTR, 5NZ, and 15NZ ($P > 0.05$); however, 10CZ exhibited higher TDN content throughout the whole incubation period ($P < 0.05$). Dissolved inorganic N was calculated as the sum of NH_4^+-N, NO_3^--N, and NO_2^--N, while DON was calculated as the difference between TDN and DIN (Table III).

Ergosterol

Ergosterol content was quite similar in CNTR, 15NZ, and 10CZ with no significant differences ($P > 0.05$), except for on day 2, when the ergosterol content was significantly higher in 15NZ than CNTR and 10CZ ($P < 0.05$, Table IV). Ergosterol content increased significantly in 5NZ from day 9 until the end of the incubation period ($P < 0.05$) reaching the highest value on day 16.

MBC and MBN

Soil MBC ranged from 164 (in 10CZ) to 348 (in CNTR) mg kg^{-1} (Table IV). On day 2 of incubation, no significant differences were observed among the treatments ($P > 0.05$); however, on day 7, MBC was significantly higher in CNTR than other treatments. Furthermore, similar to day 2, no significant differences among the treatments were observed on days 9 and 11. However, by the end of the incubation period, 15NZ MB-C content increased significantly ($P < 0.05$).

Soil MBN ranged from 6.9 (in 15NZ) to 238 (in 10CZ) mg kg^{-1} (Table III). Throughout the incubation period, 10CZ was the only treatment that showed significant differences in MBN content with respect to other

treatments ($P < 0.05$). On day 2 of incubation, MBN was significantly higher in 10CZ treatment compared with CNTR and both the two NZ treatments ($P < 0.05$). On day 7, albeit the average values remained very high, a high variability among the three analyzed replicates exhibited no significant differences among the treatments ($P > 0.05$). From day 9, MBN in 10CZ increased significantly to values higher than 130 mg kg^{-1}, differing significantly from the other treatments ($P < 0.05$), and further increased on days 11 and 16, reaching the maximum values recorded during the incubation period. The MBN in 10CZ was strongly correlated with NO_3^--N and NH_4^+-N dynamics during the incubation period (Fig. 1).

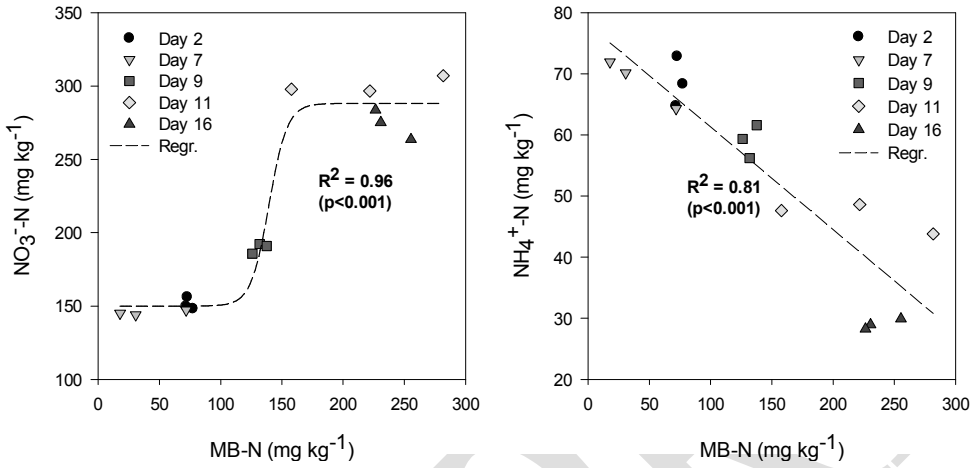

Fig. 1 Relationship of microbial biomass N (MBN) with NO_3^--N and NH_4^+-N in the soil with addition of 10% NH_4^+-enriched zeolite during the 16-d incubation period.

By relating MBC and ergosterol results (Fig. 2) it is clear that samples from 10CZ treatment are show a tendency in low MBC and ergosterol content while especially samples from 5NZ treatment are characterized by high ergosterol.

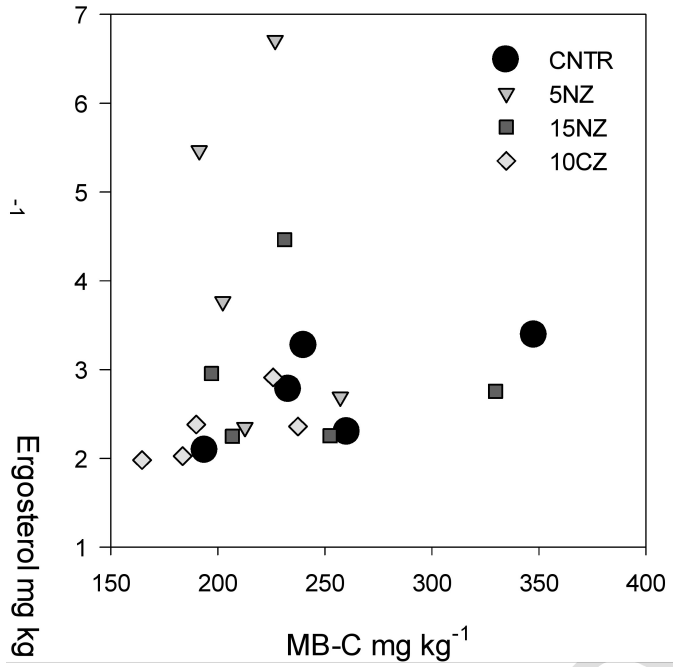

Fig. 2 Scatter plot of ergosterol content against microbial biomass C (MBC) in the soil without addition (CNTR) or with addition of 5% (5NZ) and 15% (15NZ) natural zeolite and 10% NH_4^+-enriched zeolite (10CZ) during the 16-d incubation period. Black dashed line defines samples from 5NZ, in which a substantial increase in ergosterol content occurred during the incubation; Light gray dashed line defines samples from 10CZ, in which MBC tended to be lower than CNTR.

Microbial biomass $\delta^{15}N$ and net ^{15}N immobilization net rates

The analysis of MB isotopic signature by the EFE method revealed significant differences ($P < 0.05$) between CNTR and 10CZ. The CNTR exhibited a marginal negative MBδ^{15}N value of -4.2‰ (Table V), and it was assumed constant throughout the incubation period as there was no further addition of N, while the isotopic signature of pure (bulk) CZ was 43.6‰ (Table II). On day 2, MBδ^{15}N in 10CZ was highly variable; however, it was significantly higher ($P < 0.05$) than CNTR with an average value of 12.9‰ (Table V). On day 9, MBδ^{15}N in 10CZ increased to 25.6‰, while on day 16, the isotopic signature decreased to 15.3‰, still significantly higher with respect to that in CNTR ($P < 0.05$) (Table V). The amount of MB^{15}N calculated from Eq. 1 and 2, resulted 265 ± 11 µg ^{15}N kg^{-1} MB at day 2, 495 ± 22 µg ^{15}N kg^{-1} MB at day 9 and 883 ± 58 µg ^{15}N kg^{-1} MB at day 16, respectively (Fig 3A). The net rate of ^{15}N imm in 10CZ, calculated from Eq. 3 was 31.5 µg ^{15}N d^{-1} between days 2 and 9, while the rate almost doubled to 55.4 µg ^{15}N d^{-1} between days 9 and 16 (Fig 3B).

TABLE V MBδ^{15}N values (‰) for CNTR (begin of the incubation), CZ, and 10CZ at day 2, 9 and 16.

Treatments	Value (‰)
CNTR	- 4.2
10CZ day 2	+ 12.9
10CZ day 9	+ 25.6

10CZ day 16	+ 15.3

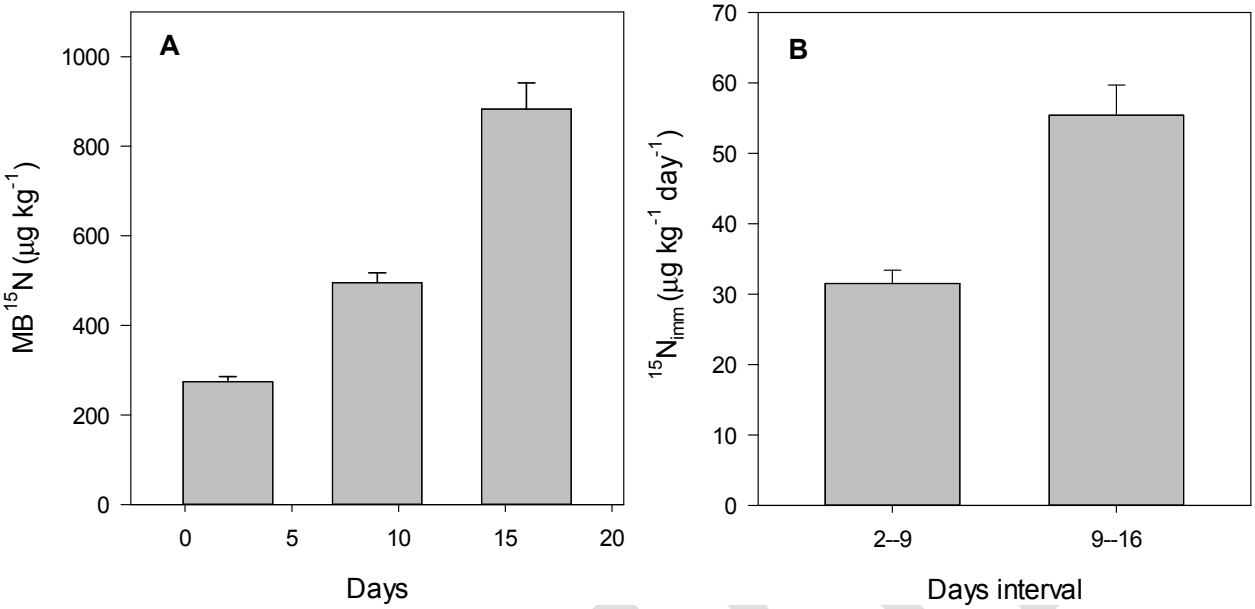

Fig. 3 A) Amounts of ^{15}N immobilized in the soil microbial biomass N ($MB^{15}N$) after addition of 10 % NH_4^+-enriched zeolite during the 16-d incubation period calculated from Eq. 1 and 2. B) Net ^{15}N immobilization rates ($^{15}N_{imm}$) in the soil microbial biomass N in soil treated with 10 % of NH_4^+-enriched zeolite occurred between days 2--9 and days 9--16 of incubation, calculated from Eq. 3. Error lines and number within brackets represent standard deviation.

DISCUSSION

NZ effects

In the present study, pure NZ was characterized by very low MBC and MBN, indicating a weak colonization CHAZT at natural state by microorganisms (Table II). Ergosterol measurement is commonly employed as a marker for fungal biomass, as it is a membrane sterol in fungi, and used to study fungi in various ecosystems, including temperate soils (Johnson and McGill, 1990; Gessner and Chauvet, 1993; Ruzicka *et al*., 2000). Ergosterol was not detected in NZ (Table II), thus fungal abundance in pure NZ was below the detection limit, suggesting that fungi were not introduced into the soil by the amendment.

Addition of NZ to soil did not affect MBC and MBN dynamics during the incubation period, hence microbial immobilization was likely not significantly influenced by NZ amendment in the short term (Tables III and IV). However, ergosterol measurements suggested that the relative amount of fungal biomass compared to the total MB was influenced by NZ amendments, especially at a lower application rate (5NZ) as indicated by the relationship between ergosterol and MBC (Fig. 2). It was, in fact, evident that 5NZ was characterized by a high ergosterol content from day 9, suggesting an increase in fungal population. Fungi are known to have a lower nutrient (N) requirement compared to bacteria (Güsewell and Gessner, 2009), because of their higher cellular C/N ratio. A broad set of factors, such as agricultural management, soil pH, moisture,

and temperature, and atmospheric CO_2, that are known to influence fungal abundance in agricultural soils (Strickland and Rousk, 2010) were maintained constant during the incubation period. Therefore, a possible explanation for the observed result might be the realtively lower immediate nutrient availability, because of a competition for the dissolved mineral N species between NZ (adsorption) and MB (assimilation) in the short term. It is plausible that the addition of NZ with a CEC equal to 1 420 mmol kg^{-1} (Malferrari *et al.*, 2013) increased the soil CEC, as reported by Gholamhoseini *et al*. (2013) after using zeolite-amended cattle manure in sunflower field. Similar observation was reported by Ferretti (2017c) directly in the ZeoLIFE experimental field. However, CEC was not measured in the soil-zeolite mixture used. The addition of an initially N-deficient mineral with a very high CEC and affinity for NH_4^+ might have established a sort of competition among soil microorganisms in the short term for the dissolved mineral N. However, the increase in ergosterol content was not observed in 15NZ despite a very high NZ application rate. Following the previous hypothesis, the higher the NZ in the soil, the higher the fungal development because of the lower available mineral N for microbes. A possible explanation for this behavior may reside in the relative DOC availability in these two treatments (5NZ and 15NZ). The higher DOC content in 5NZ might have favored fungal biomass development, while the relatively lower DOC at the beginning of the incubation in 15NZ might have prevented the development of fungal biomass. This indicates that the amount of NZ added to the soil influenced nutrient availability in the short term, with varying effects on fungal biomass. The findings of the present study support the first hypothesis for 5NZ but not for 15NZ.

CZ effects

Pure CZ was actively colonized by microorganisms; however, fungi were not introduced into the soil by pure CZ (Table II). The addition of CZ to soil exhibited no effects on fungal biomass during the incubation period but significantly increased both DIN and DON, and thus TDN (Table III), suggesting a strong mineralization process. In particular, a high NO_2^--N content on day 2 suggests the occurrence of ammonia oxidation, the first step in nitrification (Ruiz *et al*., 2003). The high NO_2^--N accumulation indicated that the total nitrification process, and therefore the production of NO_3^-, might have been inhibited at the early stages of incubation. It is plausible that this inhibitory effect was due to high NH_3 levels, also favored by the sub-alkaline pH, which decreased the activity of nitrite oxidizing bacteria (NOB), thereby promoting NO_2^- accumulation (Stojanovic and Alexander, 1958; Morrill and Dawson, 1967; McGilloway *et al*., 2003). Considering the amount of CZ added to 1 kg soil (100 g) and its residual NO_3^--N load (146 mg kg^{-1}), the addition of CZ to the soil incorporated a total of 14.6 mg NO_3^-N kg^{-1}. This amount represent only 9.6% of the total soil NO_3^--N for 10CZ on day 2 of incubation. This minimal addition of residual NO_3^--N might have partially increased microbial biomass and stimulated decomposition process.

It was apparent that after 9 d, MBN started to increase further in CZ, along with contemporaneous decline of NH_4^+-N and increase of NO_3^--N (Fig. 1) suggesting an increase in nitrification. However, this high availability of NH_4^+ might have stimulated not only NO_3^- production, but also microbial immobilization into biomass. This was supported by the isotopic analysis conducted on three different samples collected on days 2, 9, and 16 of 10CZ (Table V). The δ^{15}N of the pure CZ was 43.6‰, and well representative of the pig-slurry isotopic signature employed in the enrichment process, while the MBδ^{15}N in CNTR was -4.2‰ at the beginning of the incubation. In this respect, the MBδ^{15}N in 10CZ was strongly influenced by CZ isotopic signature since day 2, especially on days 9 and 16. This indicates that a high amount of ^{15}N was assimilated by

MB (Dittert *et al*., 1998). This is better reflected by the amount of ^{15}N in the soil MB (Fig. 3A). The rates at which ^{15}N atoms were incorporated almost doubled from day 9, concomitantly with a high net NH_4^+ decrease. The decrease in NH_4^+ levels by microbial immobilization might have also reduced the substrate for NH_3 production, resulting in lower inhibitory effects on NOB, and thus a more favorable condition for NO_3^- production.

Notwithstanding the high nitrification occurred, soil pH did not decrease suggesting the excellent buffering capacity of CZ (Colella, 1999; Rădulescu, 2013) together with soil carbonates. These results partially agree with the findings of McGilloway *et al*. (2003), where they found that in a zeoponic substrate consisting of NH_4^+-enriched clinoptilolite zeolites, nitrification was higher than that in soil systems. They also found that ammonium oxidizing bacteria (AOB) were higher than NOB causing NO_2^- accumulation. However, they did not observe a good buffering effect of the substrate. Leggo (2000), who used NH_4^+-enriched clinoptilolite after mixing with poultry manure to produce an organo-zeolitic substrate, stated that a possible explanation for the high NO_3^--N concentration visible from day 1 after the application might be due to the interactions of CZ with the soil solution. In this light, the high natural salinity of the soil employed in the present study might have induced cation exchange reactions with the NH_4^+ adsorbed into the zeolites, thus increasing the availability of the substrate required for nitrification (Di Giuseppe *et al*., 2014).

High microbial activity during incubation, and thus high consumption of O_2 might have caused anaerobic microniches towards the end of the incubation period (Mastrocicco *et al*., 2011) causing a decrease in NO_3^--N content observed from days 11 to 16 *via* reduced nitrification or increased denitrification.

The increase in soil DOC visible on day 2 was probably due to the residual DOC caused by CZ amendment, as the addition of 10% CZ might have raised soil DOC to around 12 mg kg^{-1}. However, the NO_3^--N content started to increase on day 9, along with increased DOC, supporting enhanced mineralization process. These results further suggest that CZ addition supplied an immediately available N source to microorganisms that were able to trigger degradation of soil organic matter, thus significantly increasing soil DOC and DON (Jokubauskaite *et al*., 2015). Considering the above mentioned points, the combination of the following events: i) the supply of a minimal amount of residual NO_3^- and DOC, ii) the probable exchange processes with soil cations, and iii) the colonization of CZ, might have caused a positive priming effect on MB in 10CZ (Kuzyakov *et al*., 2000). A part of the N introduced was thus immediately available to microorganisms for immobilization into their biomass, resulting in high cellular N levels that sharply increased during the incubation period.

The results of the present study did not support the second hypothesis, because the CZ employed did not act as a slow release N source but caused a priming effect on soil MB.

CONCLUSIONS

Soil amended with 5% NZ increased ergosterol content over time, suggesting an increase in fungal biomass, and thus indicating a possible positive practice for increasing soil C sequestration. However, a similar result was not observed when the soil was amended with 15% NZ, suggesting that the application rate of NZ can influence the nutrient availability to soil MB in the short period with different effects on fungal biomass development. The N incorporated with CZ were immediate available to soil microorganisms which should be taken into account for the potential application of CZ in the agricultural context, as this specific CZ will act not as a slow-release fertilizer, but a pool of immediately available N to soil MB that may trigger both

immobilization and mineralization processes. It is thus recommendable to apply CZ immediately before the growing season to minimize N losses or, alternatively, use it as a component of greenhouse cultivation systems. The present study could serve as a basis to foster long-term experiments, both in the laboratory and field.

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