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Potential for agricultural recycling of struvite and zeolites to improve soil microbial physiology and mitigate CO_2 emissions

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ABSTRACT

Recycling nutrients in agroecosystems is becoming increasingly important to promote agricultural sustainability. Struvite and nitrogen (N)-enriched zeolites produced via wastewater treatment offer the potential for nutrient recycling. However, their effects on soil properties, particularly on microbial physiology, remain largely unknown; especially regarding microbial feedback, from which losses or sequestration of essential elements may result. This study investigates the short-term (three days) physiological responses of soil microorganisms, changes in available nutrients, and the immediate effects on soil organic matter (SOM) and carbon dioxide (CO_2) emissions following the application of struvite and N-enriched zeolites derived from liquid digestate, alongside natural zeolites amendments in an acidic sandy soil. All treatments increased soil pH, which emerged as a driving factor in the dissolution of labile organic carbon (C) and the microbial production of N-, C-, and phosphorus (P)-acquiring extracellular enzymes. As soil pH increased, the stoichiometric ratio of microbial biomass C (C_{mic}) to microbial biomass N (N_{mic}), along with the enzymatic C:N ratio decreased, suggesting a superior effect on microbial N-cycling compared to C-cycling. Carbon dioxide emissions increased, particularly with the application of organic fertilizer (digestate), where the highest microbial metabolic quotient reflected increased catabolic activity due to the immediate availability of organic C. Overall, zeolitized tuffs demonstrated the potential to mitigate CO_2 emissions, likely due to CO_2 adsorption capacity.

1. Introduction

Most European soils are considered unhealthy or degraded (European Commission, 2021), having experienced fertility loss, structural weakening (Brevik, 2023; Delang, 2018; Ferreira et al., 2022), and biological decline (Nunes et al., 2012). In degraded soils, a decline of microbial activity, diversity and abundance is often observed. However, soil microorganisms enhance agricultural productivity by playing key role in supplying plants with essential nutrients (Jacoby et al., 2017; Mohanty and Swain, 2018), maintaining soil stability (Chatterjee et al., 2022), and mediating C and nutrient release and turnover (Dobrovol'skaya et al., 2015; Xu et al., 2018). Mineral fertilizers contribute to soil degradation, environmental pollution, and climate change, particularly in sandy soils, which are notably low in soil organic carbon (SOC) and prone to nutrient leaching (Ozores-Hampton et al., 2011; Yost and Hartemink, 2019), as well as in acidic soils, which face nutrient limitations and toxicity issues (Butterly et al., 2022). Moreover, the continued use of N-fertilizers leads to further soil acidification in extensively cultivated areas of the world (Tian and Niu, 2015).

In response to current soil degradation and fertility loss, the

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Abbreviations: AP, Acid phosphatase; BG, β-glucosidase; Dig, soil fertilized with anaerobic liquid digestate; LAP, Leucine aminopeptidase; NAG, N-acetyl-β-D-glucosaminidase; N–Cha, soil amended with N-enriched chabazite-rich tuff; N–Cli, soil amended with N-enriched clinoptilolite-rich tuff; non_fert, unfertilized soil. Soil that received no amendment and no nutrient input; qCO_2, Microbial metabolic quotient (mg CO_2–C/g C_{mic}/h); Stv, soil fertilized with struvite; Stv+Cha, soil amended with virgin chabazite-rich tuff and fertilized with struvite; Stv+Cli, soil amended with virgin clinoptilolite-rich tuff and fertilized with struvite.

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European Green Deal aims to mitigate the impact of fertilizers by enhancing use efficiency and promoting nutrient recycling (European Commission, 2023). Indeed, the objectives set for 2030 include: i) increasing land under organic management by 25 % (conversion from traditional to organic management); ii) reducing fertilizer inputs by at least 20 %; and iii) reducing nutrient losses by 50 % (United Nations, 2015). Recovering nutrients from agricultural waste through struvite and natural zeolites is emerging as a preferred approach (Le Corre et al., 2009; Tasić et al., 2019).

Struvite, which includes the isomorphic phases NH_4- and K-struvite ($MgNH_4PO_4 \cdot 6H_2O$ and $MgKPO_4 \cdot 6H_2O$, respectively), is promising for nutrient recovery (Kumar and Pal, 2015). Once applied to soils, struvite releases N, P, Mg, K, and, if non-pure struvite is used (e.g., struvite produced from wastewater treatment plants), also Ca, residual organic C, and small amounts of micronutrients such as Fe, Cu, Mn, Co, and Zn (Galamini et al., 2024a; Taddeo et al., 2018).

Other interesting materials for nutrient recovery are zeolites: a tectosilicate mineral family composed of $[SiO_4]^{4-}$ and $[AlO_4]^{5-}$ tetrahedra arranged in three-dimensional frameworks that form microporous structures with large specific surface areas. Along the internal surfaces of zeolites, negatively charged sites are balanced by weakly bound exchangeable cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) whose concentrations depend on the depositional setting (Gottardi, 1989). Natural zeolites are found in sedimentary-volcanic rock deposits called tuffs, referred to as zeolitized tuffs if the zeolite mineral content exceeds 50 % (w:w) of the rock (Passaglia and Galli, 1991). Among zeolitized tuffs, clinoptilolite- and chabazite-rich tuffs are the most used.

Current research is focusing on the effects of struvite and zeolites on agricultural soils. For example, the dynamics of struvite solubilization and P redistribution have been recently evaluated, suggesting distinct roles for bacteria and fungi (Ruiz-Navarro et al., 2023). A recent *meta*-analysis linked struvite with higher yields and P uptake compared to conventional P-fertilizers (Hertzberger et al., 2020). Soil fertilized with struvite showed a reduction of about 50 % in N_2O emissions compared to conventional N-fertilizers while maintaining the same crop yield (Wang et al., 2023). Decreased N_2O emissions were also observed by Yang et al. (2023), who noted that powdered struvite dissolves faster compared to granules. Positive effects on Gram-positive bacteria in a semiarid soil were recorded by Bastida et al. (2019) with the use of struvite fertilizer. Both struvite and N-enriched zeolitized tuff showed reduced N leaching compared to urea and digestate fertilizers in sandy soil (Ferretti et al., 2023).

Moreover, due to the intrinsic adsorption capacities of zeolites, these materials can be applied in water and wastewater treatment plants for recovering NH_4^+ (Chen et al., 2018), anions (Lu et al., 2022), and for reducing the contents of heavy metals (Velarde et al., 2023) and organic molecules (Jiang et al., 2018) in polluted waters. When used as a foliar film, pre-enriched zeolites have demonstrated a slow release of micronutrients to facilitate plant uptake and resistance to leaching due to rain (Galamini et al., 2024b). Soils amended with zeolitized tuffs have shown increased cation exchange capacity, as well as enhanced nutrient retention (Ferretti et al., 2024, 2021), while zeolite amendments have improved fertilization efficiency in olive (*Olea europaea*) trees (Medoro et al., 2022) and have been associated with increased soil aggregation (Choo et al., 2020), potentially providing habitats for soil life (Bodner et al., 2023).

Despite recent findings, many aspects of struvite and zeolite use still require further investigation to identify the best management practices for agricultural soils. Several studies have examined N emissions from struvite applications, but there is limited research on the impact of struvite and N-enriched zeolitized tuffs, as well as virgin zeolitized tuff, on CO_2 emissions. Moreover, the effect of struvite and zeolitized tuffs on different SOM fractions and microbial responses remains largely unknown.

To address these knowledge gaps, this study investigated the short-term (three days) physiological response of soil microorganisms in an acidic sandy soil following fertilization with struvite and two N-enriched zeolitized tuffs produced from liquid digestate via wastewater treatment. As a control, the effects of liquid digestate on the soil were assessed. Zeolitized tuffs were also investigated in virgin state (devoid of N) in combination with struvite fertilizer.

This work builds on the findings of Galamini et al. (2023), following the same experimental design. In the previous publication, the focus was on N dynamics, N-genes expression and N-gaseous emissions. In this study, the immediate (three-day) effects of struvite and zeolitized tuffs are investigated with respect to microbial biomass C and N, metabolic C investment, potential extracellular activities of C-, N- and P-acquiring enzymes, derived stoichiometric relationships, and potentials for CO_2 mitigation.

Due to the input of external organic C, it was hypothesized that: 1) increased CO_2 emissions would occur under organic fertilization (digestate) compared to the sole addition of struvite (Lai et al., 2017; Y Wang et al., 2023); due to the input of N in bioavailable forms and greater P bioavailability, it was hypothesized that: 2) a general decrease in the potential activity of N-acquiring enzymes would occur, as well as decreased P-related activity with struvite. Particularly concerning the soil amended with clinoptilolite tuff, in combination with struvite fertilization, it was hypothesized that 3) negligible exoenzymatic responses would occur, as the pronounced decline in microbial biomass previously reported in Galamini et al. (2023) would result in lower activities.

This study provides a first indication of short-term effects of struvite, zeolitized tuffs, and N-enriched zeolitized tuffs on an acidic sandy soil, focusing on key factors for soil fertility and health, such as CO_2 emissions and microbial physiological responses. Clearly, longer timeframes are necessary to elucidate the long-term effects of these materials on agricultural soil ecosystems.

2. Materials and methods

2.1. Soil, zeolitized tuffs and fertilizers

A full description of the materials (soil, struvite, zeolitized tuffs, and liquid digestate) is provided in Galamini et al. (2023). Briefly, the soil was an acidic sandy loam (pH in $CaCl_2 = 5.3$), sampled with an excavator to a depth of approximately 50 cm from arable land in Lackendorf (47°58′98′′ N, 16°50′31′′ E, Austria). After sampling, the soil was homogeneously mixed and sieved to 2 mm. The soil's cation exchange capacity was approximately 38 mmol(+)/kg, with the following exchangeable cations (mg/kg): Ca^{2+} (0.29), Mg^{2+} (0.20), and K^+ (0.12). Total N was approximately 1.17 g/kg, and SOC was 8.77 g/kg. The dissolved inorganic P was about 250 mg/kg.

The clinoptilolite-rich zeolitized tuff was quarried in Salaj County (Romania), while the chabazite-rich zeolitized tuff was sourced from Italy (Grosseto Province). Both materials were industrially milled to a micronized size. The dominant fractions were in the range of 63–3.9 μm (73.4 % and 57.1 % for the clinoptilolite- and chabazite-rich tuffs, respectively), and less than 3.9 μm (21.9 % and 42.7 %, respectively). The zeolitized tuffs were used both in their virgin state, therefore devoid of N, and enriched with N, following treatment with liquid digestate, as described by Ferretti et al. (2023) and Galamini et al. (2024a).

Data concerning the chemical composition of the zeolitized tuffs, determined by Wavelength Dispersive X-ray Fluorescence (Advant'XP, Thermo Scientific, MA, USA) are presented in Table 1. The cation exchange capacity of zeolites arises from the presence of charge-unbalanced $[AlO_4]^{5-}$ tetrahedra in addition to the charge-balanced $[SiO_4]^{4-}$ structures. The resulting negative charge is balanced by exchangeable cations located on the internal surfaces within the microporous structure. Thus, the SiO_2/Al_2O_3 ratio is indicative of the cation exchange capacity (Gili and Conato, 2019). The SiO_2/Al_2O_3 ratios of the clinoptilolite- and the chabazite-rich zeolitized tuffs (Table 1) were consistent with the corresponding cation exchange capacity values

Table 1
Chemical composition of the employed zeolitized tuffs at their natural state (not enriched in N). The clinoptilolite- and chabazite-rich tuffs are referred to as Cli and Cha respectively.

	Cli	Cha		Cli	Cha		Cli	Cha
analyte	%	%	**analyte**	mg/kg	mg/kg	**analyte**	mg/kg	mg/kg
SiO_2	68.6	55.1	**Ba**	813	685	**Pb**	19.6	75.0
TiO_2	0.14	0.52	**Ce**	34	176	**Rb**	102	430
Al_2O_3	10.0	16.3	**Co**	b.d.l.	0.40	**S**	9393	192
Fe_2O_3	1.32	3.43	**Cr**	3.1	30.9	**Sc**	3.30	4.90
MnO	0.04	0.10	**Cu**	2.70	7.10	**Sr**	374	1833
MgO	0.86	2.00	**Ga**	10.6	17.7	**Th**	12.5	72.2
CaO	4.77	5.40	**Hf**	2.70	11.0	**V**	11.8	84.2
Na_2O	0.91	0.80	**La**	39	172	**Y**	15.2	36.1
K_2O	2.54	5.65	**Nb**	6.80	22.8	**Zn**	26.0	50.5
P_2O_5	0.03	0.18	**Nd**	14.9	95.0	**Zr**	88	424
LOI	9.89	8.15	**Ni**	5.70	20.3			
Tot.	99.2	97.6						
						Total mineral C		
SiO_2/Al_2O_3	6.85	3.39				mg/g	0.40	4.37

LOI refers to the Loss of Ignition, namely the weight loss between 105 and 1000 °C. Major elements are expressed as oxides. The total mineral C was measured by Elemental Analyzer (Vario Micro Cube, Elementar, Langenselbold, Germany). b.d.l. stands for below detection limit.

(2.17 and 0.78 mEq/g, respectively) and with the zeolite mineral content (total zeolitic content = 75.3 % and 53.9 % w:w, respectively; Galamini et al., 2023).

Liquid digestates are byproducts of biogas plants, whose management is often associated with environmental pollution (Lamolinara et al., 2022). Digestates are commonly used as organic fertilizer; however, due to the high nutrient loads (Häfner et al., 2022), it is often necessary to treat digestates before agricultural use. The effects of the digestate in soil were therefore investigated and compared with the same N-enriched zeolitized tuffs and struvite, which were previously produced from the digestate itself via a wastewater treatment process, as described in Galamini et al. (2024a). The digestate was sampled from a biogas plant in Ferrara Province (44°58′47′’ N, 11°85′86′’ E, Italy), resulting from the anaerobic digestion of triticale (*x Triticosecale* Wittm.) and dairy manure. The Total Kjeldahl N was approximately 3.30 g/L, consisting of NH_4^+–N (2.52 g/L) and organic N (0.79 g/L). Total P was 0.11 g/L, with approximately 80 % in dissolved form (Galamini et al., 2023).

The characteristics of the N-enriched zeolitized tuffs and struvite are reported in Galamini et al. (2023). Briefly, N-enriched zeolitized tuffs had N concentrations of 6.39 and 4.76 mg/g, for clinoptilolite- and chabazite-rich tuffs, respectively. The struvite precipitate was composed of 90 % struvite, consisting of 63 % NH_4-struvite and 27 % K-struvite, along with calcite and hydrocalcite (9.6 %). Total C and N were approximately (mean ± SD) 39.4 ± 4.1 and 34.3 ± 0.1 mg/g, respectively, with N almost entirely represented by NH_4^+–N. Total P was 110 mg/g (as PO_4–P), K was 16.6 mg/g, while Mg and Ca were 66.9 and 29.3 mg/g, respectively. Organic C was 7.12 ± 0.76, 3.16 ± 0.69 and 28.3 ± 0.4 mg/g in the N-enriched clinoptilolite and chabazite tuffs, and struvite, respectively. The organic C/ total N ratio (mol:mol) was approximately 1 for all the fertilizers.

2.2. Treatments

Fertilized treatments were balanced for N input, meaning that the same amount of N was used. The amount of fertilizers applied was therefore determined using the following equation:

$$N\ dose_{mass}\ (mgN/kg) = N\ dose_{surf}\ (kgN/ha)*\frac{1}{bulk\ density\ (g/cm^3)}*\frac{1}{volume\ of\ 1\ ha\ (m^3)}*1000$$

where N $dose_{mass}$ is the dosage of N added with fertilizers per unit mass of soil (mg N/kg), and N $dose_{surf}$ is the N added per unit surface of the soil (kg N/ha). Thirty cm corresponds to the average ploughing depth and is commonly used in calculations of fertilizer application rates. The bulk density was set to 1.5 g/cm^3, and the N $dose_{surf}$ was set to 300 kg N/ha. Such a high N input is often unrealistic in real conditions, except in rice cultivation, where 270 kg N/ha has been reported (Qiao et al., 2022). However, 300 kg N/ha was chosen to amplify the observed processes. Therefore, the input of N in the fertilized treatments per unit mass of soil was 66.7 mg N/kg.

The treatments were as follows: i) soil that received no amendment and no fertilizer (non_fert); ii) soil fertilized with anaerobic liquid digestate (Dig); iii) soil fertilized with struvite (Stv); iv) soil amended with natural-state (untreated with digestate) clinoptilolite-rich tuff + struvite fertilization (Stv + Cli); v) soil amended with natural-state chabazite-rich tuff + struvite fertilization (Stv + Cha); vi) soil fertilized with N-enriched clinoptilolite-rich tuff (N–Cli); and vii) soil fertilized with N-enriched chabazite-rich tuff (N–Cha). For the preparation of Stv + Cli and Stv + Cha, the soil was initially amended with the respective N-free zeolitized tuffs at 10 % (m:m), and struvite was subsequently added.

2.3. Incubations and measurements

A series of incubations were conducted in open tubes or steel cylinders (refer to Galamini et al., 2023 for further details). To prepare soil samples, precise masses of moist soil, corresponding to known masses of dry soil, were weighed and fertilized at 66.7 mg N/g soil. Milli-Q water was then added to 20 % (w:w), corresponding to approximately 65 % water-filled pore space of the soil. The bulk density was adjusted to 1.5 g/cm3 by slowly compacting each soil sample with a piston until a known volume was reached. Samples were subsequently incubated at a constant temperature of 20 °C in the dark. The water content was kept constant by daily refills. Three replications were used.

Three days after the start of the incubation, potential extracellular enzyme activities (EEAs) were evaluated for four hydrolytic enzymes involved in the release of C-, N- and P-rich organic substrates, namely leucine aminopeptidase (LAP), N-acetyl-β-D-glucosaminidase (NAG), β-glucosidase (BG) and acid phosphatase (AP), following the protocol outlined in Rosinger et al. (2019), with minor modifications (Mayer et al., 2022). Briefly, aliquots of 0.5 g fresh soil were mixed with 50 mL of 0.05 M Na–acetate solution at pH 5.3. After homogenization in a sonication bath for 90 s, 200 μL of soil slurry was transferred to black 96-well microtiter plates in four replicates, under constant stirring. Then, 50 μL of fluorogenic substrate (optimal concentrations, determined beforehand, ranged between 0.5–2 mM) were added, and the plates were briefly mixed horizontally. After an incubation period of 2 h at 20 °C in the dark, fluorescence was measured using an EnSpire multi-plate reader (Perkin Elmer), with an excitation wavelength of 365 nm and emission at 450 nm. Quenched standard curves (50 μM) were prepared for the amino-methylcoumarin-based substrate (LAP) and methylumbelliferone-based substrates (NAG, BG, AP) for each sample to account for soil quenching. Potential EEAs were calculated based on the quenched standard curves (in the ranges of 10–500 μM) and were expressed both as absolute (nmol/g soil/h) and specific (nmol/g C_{mic}/h) potential activity. Enzymatic C:N (BG:LAP + NAG), C:P (BG:AP) and N:P (LAP + NAG:AP) ratios were also calculated from the EEAs.

Carbon dioxide fluxes were measured throughout the entire incubation period using soil cores placed in glass chambers, connected to an automated incubation system (Deltedesco et al., 2019). In each chamber, the air in the headspace was exchanged at a flow rate of approximately 0.9 L/min. Fluxes were determined by calculating the difference between inlet and outlet concentrations (Butterbach-Bahl et al., 2016). For each measurement, the inlet concentration was assessed by passing gas through an empty chamber. A steady state in the headspaces was achieved by equilibrating each chamber for 10 min after each valve switch (controlled by a custom-made relay system), before the outlet concentrations were measured. Carbon dioxide was measured using an infrared analyser (WMA-2, PP System, Amesbury, MA, USA). Fluxes were converted to surface emissions (E; mg CO_2–C/m^2) using the trapezoidal rule: $E_{\Delta t/2} = \Delta t/2\ (f_{t1} + f_{t2})$, where $E_{\Delta t/2}$ is the estimated surface emission between two consecutive measurements at times t_1 and t_2, and f_{t1} and f_{t2} are the measured fluxes; Δt is the sampling intervals (t_2–t_1). Cumulative CO_2C emissions were then obtained by summing the calculated surface emissions.

In this study, parameters previously published by Galamini et al. (2023) were referenced, as new insights and relationships were gathered and evaluated with unpublished data. The previously published parameters included: i) soil pH, ii) C_{mic} and N_{mic} and the total dissolved N (TDN). Since dissolved organic C (DOC) was mentioned in earlier publication, the measurements were not reported.

2.4. Statistical analyses

Statistical analyses were performed using R software (v4.3.1; R Core Team, 2021), employing the following packages: MASS (Venables and Ripley, 2002), ggplot2 (Wickham, 2016), agricolae (Mendiburu and Yaseen, 2020), ggpubr (Kassambara, 2020), ggfortify (Horikoshi and Tang, 2016), corrplot (Wei and Simko, 2021) and AICcmodavg (Mazerolle, 2023).

Distribution and homoscedasticity were assessed using Shapiro–Wilk and Bartlett's tests. Significant differences ($p < 0.05$) were evaluated with ANOVA and Tukey's HSD tests, while the Kruskal–Wallis test was applied for non-normally distributed and non-homoscedastic data. We refer to significant differences at the $p < 0.05$ level. Principal component analysis (PCA) was performed with scaled and centred data. Pearson correlation coefficients were calculated to assess general relationships among the measured responses. Regression models were applied and evaluated using mean absolute error (MAE), root mean squared error (RMSE), and R–square (R^2). The Akaike information criterion (AIC) was used to compare the quality of model fits.

Carbon dioxide data underwent locally estimated scatterplot

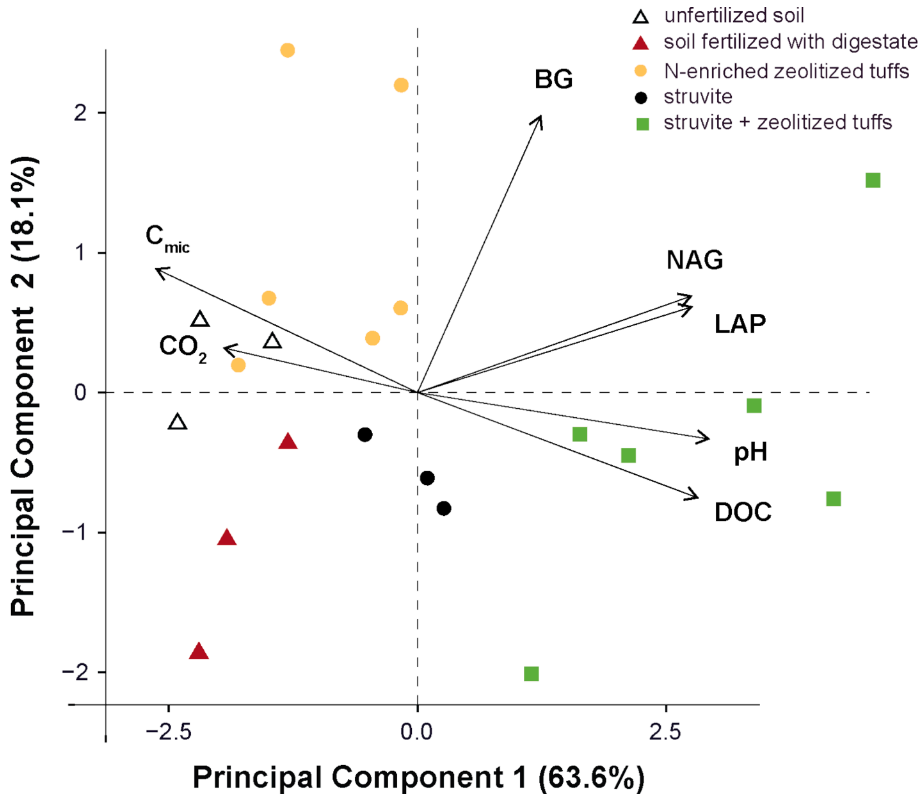

Fig. 1. The first two dimensions of a principal component analysis to evaluate the effect of digestate, zeolitized tuffs and struvite addition on soil biochemical parameters. N-enriched zeolitized tuffs comprise both the N-enriched clinoptilolite and N-enriched chabazite. Struvite + zeolitized tuffs comprise both struvite + natural-state clinoptilolite tuff and struvite + natural-state chabazite tuff.

smoothing and 3rd grade polynomial regressions were used to assess inflection points and slopes. Inflection points were calculated from the second derivative $f''(x)$, imposing the following conditions: i) $f''(x) = 0$, ii) $f''(x)$ changes sign on either side of p where p is the x-coordinate of a given inflection point and x is time.

3. Results

3.1. Trends and correlations

Results of the PCA indicated that the first two principal components (PC1 and PC2) together accounted for 81.7 % of the total variance, with PC1 contributing 63.6 % and PC2 contributing 18.1 % (Fig. 1). Positive contributions to PC1 included pH (17.2 %), DOC (16.5 %), LAP (16.2 %), NAG (16.1 %), while C_{mic} and CO_2 had negative contributions of 15.4 % and 11.4 %, respectively. Principal Component 2 was mainly positively influenced by BG (35.5 %), C_{mic} (15.9 %), NAG (12.4 %) and LAP (11.0 %), and negatively by DOC (13.5 %). Pearson correlation coefficients (r), corroborated by the AIC test, revealed positive relationships between pH–NAG ($R^2 = 0.64$) and pH–LAP ($R^2 = 0.49$).

3.2. DOC, CO_2 and microbial quotients

The DOC contents were significantly higher in the soil treated with clinoptilolite tuff + struvite compared to chabazite tuff + struvite (Table 2); the lowest values were observed in the non-fertilized soil. No differences were observed in samples treated with N-enriched clinoptilolite and N-enriched chabazite tuffs; however, they were not significantly different compared to the soil fertilized with digestate and the non-fertilized soil.

Carbon dioxide emissions increased after fertilization, displaying distinct maximum rates, as illustrated by inflection points and slopes in Fig. 2A. The soil fertilized with digestate and the soil fertilized with N-enriched chabazite exhibited maximum emission rates earlier than the other treatments, approximately after 7 and 3 h, respectively, with rates of about 0.12 g CO_2–C/m^2/h (Fig. 2A). The soil fertilized with N-enriched clinoptilolite showed maximum emissions at 14 h, while the other treatments peaked at around 30 h.

Significantly higher cumulative emissions (mean ± SD) were recorded in the soil fertilized with digestate (5.77 ± 1.09 g CO_2–C/m^2) compared to the non-fertilized soil (2.63 ± 0.46 g CO_2–C/m^2). The other treatments did not show significant differences from the unfertilized soil, which had an average of 3.11 ± 1. 46 g CO_2–C/m^2 (Fig. 2B). Significantly lower cumulative emissions were measured in soils treated with natural-state zeolitized tuffs + struvite fertilizer (2.00 ± 0.31 and 1.43 ± 0.08 g CO_2–C/m^2 for clinoptilolite and chabazite tuffs, respectively) compared to the soil fertilized with digestate (5.77 ± 1.09 g CO_2–C/m^2).

Considering the metabolic quotient (qCO_2: mg CO_2–C/mg C_{mic}/h), the only significant variation occurred in the soil fertilized with digestate, which exhibited a pronounced increase (1.18 ± 0.19, compared to an average of 0.45 ± 0.20 for the other treatments; Fig. 2C). The C_{mic}/SOC ratio (‰) significantly decreased in the soil fertilized with digestate (4.93 ± 0.54 ‰), and the treatments amended with natural-state zeolitized tuffs (both clinoptilolite and chabazite tuffs) + struvite (3.59 ± 0.14 and 4.81 ± 0.16 ‰, respectively), compared to the non-fertilized soil (6.28 ± 0.19; Fig. 2D). Since the SOC remained constant across the treatments (SOC = 8.77 ± 0.25 g/kg), the differences in the C_{mic}/SOC ratio likely resulted from shifts in C_{mic}.

Table 2
Dissolved organic carbon (DOC; mg/kg soil) of the treatments. The acronyms are as follows: non_fert (non-fertilized soil), Dig (soil fertilized with digestate), Stv (soil fertilized with struvite), Stv + Cli (soil pre-amended with 10 % clinoptilolite-rich tuff in its natural-state and fertilized with struvite), Stv + Cha (soil pre-amended with 10 % chabazite-rich tuff in its natural-state and fertilized with struvite), N–Cli (soil fertilized with N-enriched clinoptilolite tuff), and N–Cha (soil fertilized with N-enriched chabazite tuff).

Treatment	non_fert	Dig	Stv	Stv + Cli	Stv + Cha	N–Cli	N–Cha
DOC (mg/kg soil)	51.7^{d}	70.0^{c}	100^{b}	134^{a}	112^{b}	61.0^{cd}	58.3^{cd}

3.3. Absolute and specific EEAs

The soil fertilized with liquid digestate exhibited significantly lower (mean ± SD) BG activities (0.16 ± 0.01 μmol/g soil/h) compared to both N-enriched zeolitized tuffs (0.23 ± 0.04 and 0.22 ± 0.04 μmol/g soil/h for the clinoptilolite and chabazite tuffs, respectively) (Fig. 3A). Greater BG activity compared to the digestate fertilizer was also observed in the soil amended with natural-state clinoptilolite + struvite fertilizer (0.23 ± 0.04 μmol/g soil/h), which also displayed the highest absolute NAG and LAP activities (0.033 ± 0.003 and 0.079 ± 0.007 μmol/g soil/h, respectively). The potential activity of AP was unaffected by the treatments, averaging 0.64 ± 0.05 μmol/g soil/h across all experimental conditions. Specific NAG activity was higher in the soil amended with natural-state clinoptilolite tuff + struvite (2.46 ± 0.43 μmol/mg C_{mic}/h), than natural-state chabazite tuff + struvite (1.31 ± 0.14 μmol/mg C_{mic}/h); both were higher than the non-fertilized soil (0.63 ± 0.15 μmol/mg C_{mic}/h). Specific LAP activity showed a similar trend (1.03 ± 0.15, 0.55 ± 0.05 and 0.32 ± 0.06 μmol/mg C_{mic}/h for the soil treated with natural-state clinoptilolite + struvite, natural-state chabazite + struvite, and the non-fertilized soil, respectively).

3.4. Soil pH and microbial parameters

Without considering the treatment amended with natural-state clinoptilolite tuff + struvite due to its peculiar effect on C_{mic} (see section 4.2), the C_{mic}:N_{mic} ratio (expressed as mol:mol) decreased linearly with soil alkalinization (Fig. 4A). A reduced C_{mic}:N_{mic} ratio compared to the non-fertilized soil (6.93 ± 0.27; mean ± SD) was observed in the soil amended with natural-state chabazite tuff + struvite fertilizer (2.60 ± 0.57), the soil fertilized with digestate (5.18 ± 0.33), N-enriched chabazite tuff (4.81 ± 1.02), and N-enriched clinoptilolite tuff (3.28 ± 0.39). Regarding the enzymatic C:N ratio, a negative linear relationship was observed with soil pH (Fig. 4B). Significantly lower enzymatic C:N ratios were recorded with digestate (2.57 ± 0.35), struvite (2.44 ± 0.10), struvite + clinoptilolite tuff (2.09 ± 0.36), and struvite + chabazite tuff (2.26 ± 0.04) compared to the non-fertilized soil (3.09 ± 0.08).

Excluding the unfertilized soil, the DOC:TDN ratio also demonstrated a positive relationship with soil pH (Fig. 4C). Positive relationships were also observed between soil pH and enzymatic N:P (Fig. 4D). In this regard, significant differences for N:P were as follows: non-fertilized soil (0.092 ± 0.004) < struvite (0.12 ± 0.01) < struvite + clinoptilolite tuff (0.16 ± 0.01) = struvite + chabazite tuff (0.16 ± 0.01).

4. Discussion

4.1. CO_2 emissions

Organic fertilizers may enhance microbial heterotrophic respiration (catabolism) compared to mineral fertilizers by increasing the amount of bioavailable C. Indeed, the DOC commonly represents a preferential C source for soil microorganisms, often resulting in immediate increases in CO_2 emissions after fertilization (Marschner and Kalbitz, 2003). It was therefore hypothesized that the input of DOC with digestate would lead to increased CO_2 emissions compared to the other treatments. In line with this consideration, the soil fertilized with digestate exhibited greater emissions than the soil fertilized with struvite (inorganic) fertilizer, along with a higher qCO_2.

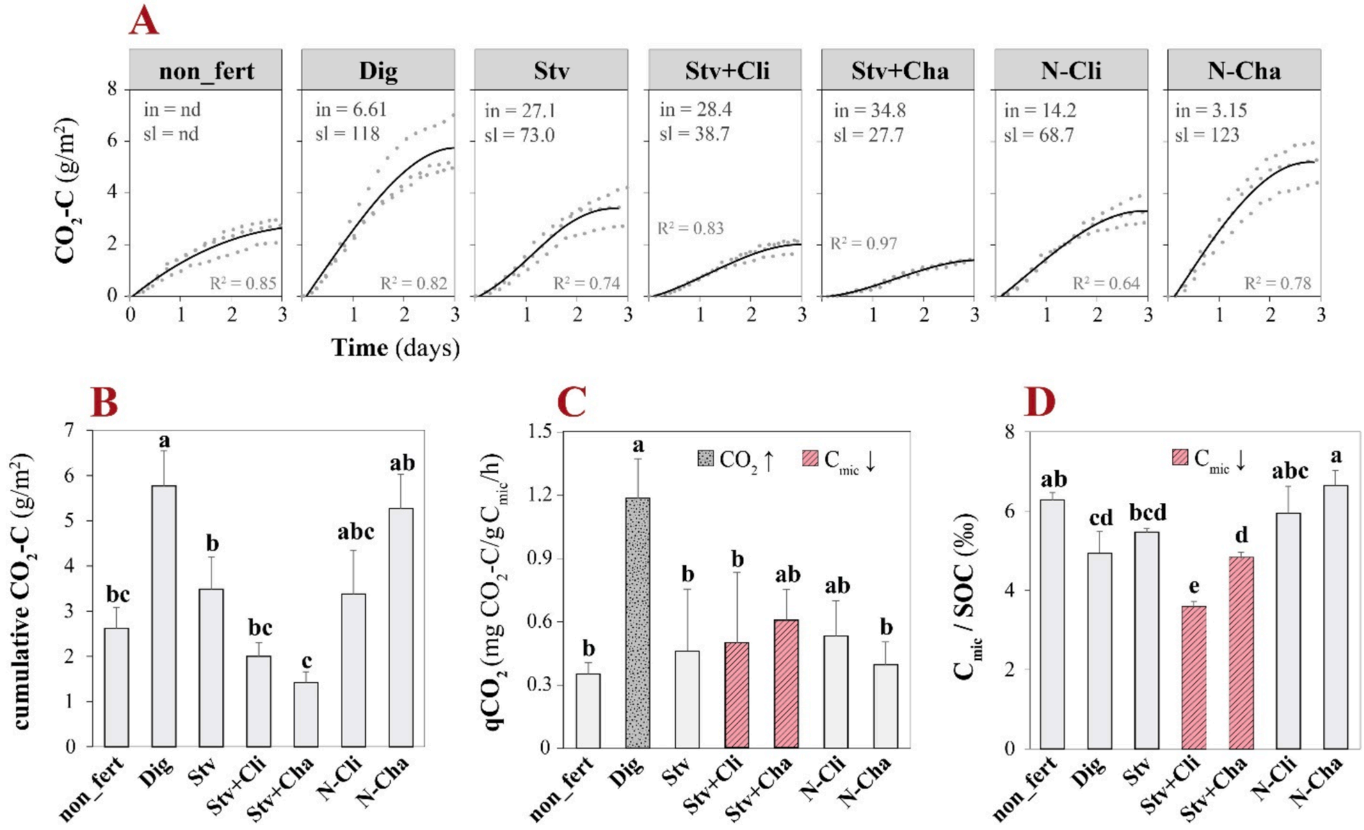

Fig. 2. A) CO_2–C emission rates over three days of incubation and 3rd grade polynomial regressions with relative R^2. Dots represent single measurements carried out on three biological replicates. 'in' is the x coordinate (time) at the inflection point, while 'sl' is the slope (mg CO_2–C/m/h). B) cumulative CO_2–C emissions. C) Microbial metabolic quotients (qCO_2). D) C_{mic}/SOC ratio (‰). In Fig. B, C and D, the two treatments significantly differ ($p < 0.05$) if no letter is in common. To highlight which parameters significantly affected qCO_2 (Fig. C) and the C_{mic}/SOC (Fig. D), significant differences are also reported for the C_{mic} and CO_2 compared to the non-fertilized soil by pink columns (lower C_{mic}) and a gray dotted column (greater CO_2). Acronyms represent distinct treatments as follows: non_fert. (non-fertilized soil), Dig (soil fertilized with digestate), Stv (soil fertilized with struvite), Stv + Cli (soil pre-amended with 10 % clinoptilolite-rich tuff at natural-state and fertilized with struvite), Stv + Cha (soil pre-amended with 10 % chabazite-rich tuff at natural-state and fertilized with struvite), N–Cli (soil fertilized with N-enriched clinoptilolite tuff), and N–Cha (soil fertilized with N-enriched chabazite tuff). The error bars represent standard deviations ($n = 3$).

Our first hypothesis is thus validated; however, the soil fertilized with digestate had lower DOC compared to the treatments fertilized with struvite (section 3.2). The relatively lower DOC, in addition to the largest CO_2 emissions, prompted questions regarding the actual bioavailability of the newly dissolved C observed in the struvite treatments.

As discussed in Galamini et al. (2023), the treatment-induced change in pH resulted in increased DOC. These organic compounds were plausibly mobilized from fractions of the SOC that were weakly bound to the mineral and organic soil particles. This effect was particularly evident in samples amended with clinoptilolite and chabazite tuffs, in the presence of struvite fertilizer. Indeed, the combined effect of zeolitized tuffs and struvite led to pronounced increases in soil pH, resulting in a stronger release of DOC.

The different nature of the DOC provided by the digestate (extrinsic DOC), and the intrinsic fraction of DOC that was directly derived from the SOC may not have been equal in terms of energy requirements for microbial use (Sae-Tun et al., 2023). Indeed, the organic molecules contained in liquid digestates are composed of residues from anaerobic decomposition, including large polymers such as cellulose and hemicellulose, which are degraded in biogas plants into smaller compounds (Siró and Plackett, 2010; Tambone et al., 2019). Considering the liquid digestate used in this study, plant polysaccharides like cellulose and hemicellulose were introduced into the biogas plant, as triticale served as a substrate for anaerobic digestion. However, the resulting digestate was likely depleted in cellulose and hemicellulose. The DOC contained in the digestate was likely in a more advanced state of degradation and therefore more available for soil microorganisms. This argument is supported by the relatively low activity of BG enzymes recorded in the soil fertilized with liquid digestate compared to the other treatments, which may indicate that soil microorganisms reduced C-acquisition due to a better C supply.

In summary, despite the lower DOC in the soil fertilized with digestate compared to most of the other treatments, microbial respiration was greatly affected. The chemical nature of the organic C present in the digestate may explain such immediate C availability, as well as the large qCO_2 recorded in the soil fertilized with digestate.

An effect related to the dosage of zeolitized tuff was also observed: the 10 % (w:w) dose of either clinoptilolite or chabazite tuff showed significant reductions in CO_2 emissions from the soil, while no effect was observed when approximately 1.3 % of zeolitized tuff was added, namely in the soil treated with N-enriched zeolitized tuffs. The immediate reduction in soil CO_2 emissions upon the amendment of zeolitized tuffs may result from several factors, including i) the intrinsic adsorption capacity for CO_2 by zeolites (Cheung and Hedin, 2014; Ferretti et al., 2017; Regufe et al., 2019), ii) the protection of the SOM through particle adhesion onto zeolites and the promotion of aggregates (Lehmann and Kleber, 2015; Pukalchik et al., 2017), iii) the provision of a new substrate for microbial growth, and iv) the establishment of a different microbial habitat that enhances C-use efficiency (e.g., more aerobic conditions, effects on soil pH, etc.), which could also possibly affect the composition of the microbial community (Rousk et al., 2011).

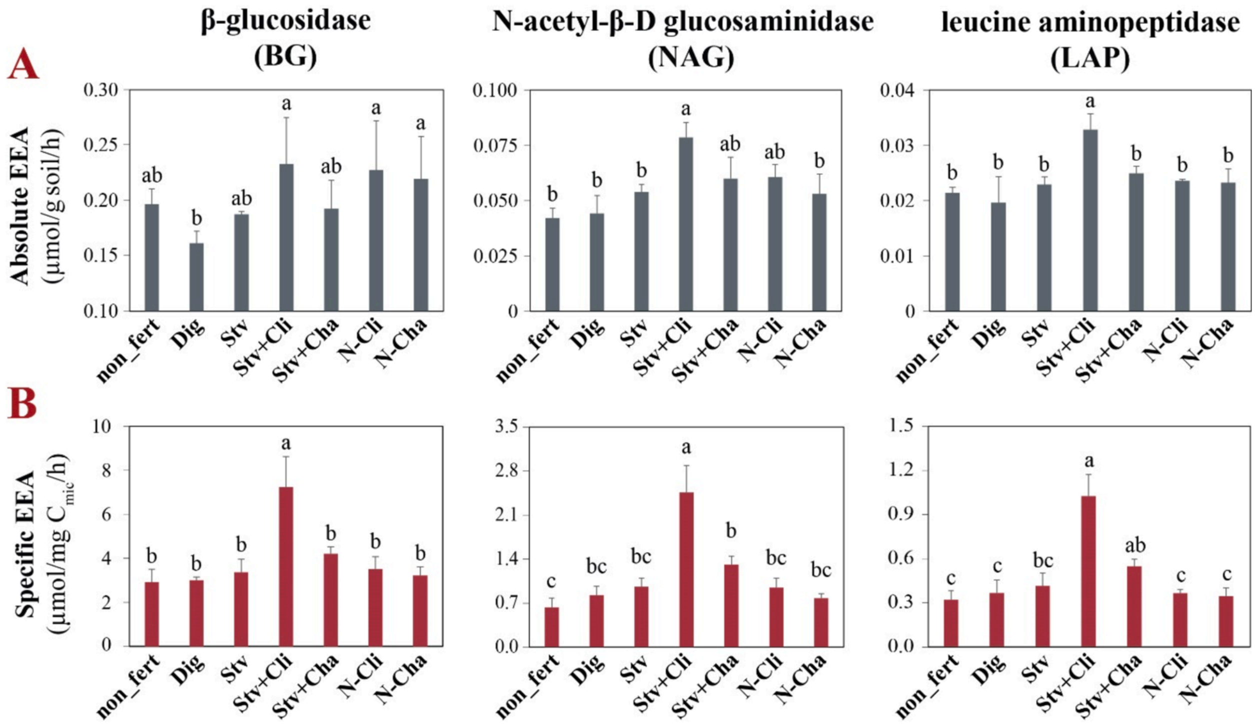

Fig. 3. Extracellular Enzyme Activities (EEA): A) Absolute EEA (μmol/g soil/h); B) Specific EEA (μmol/mg C_{mic}/h). Enzymes are β-glucosidase (BG), N-acetyl-β-D-glucosaminidase (NAG) and leucine aminopeptidase (LAP). The acronyms of treatments are: non_fert (non-fertilized soil), Dig (soil fertilized with digestate), Stv (soil fertilized with struvite), Stv + Cli (soil pre amended with 10 % clinoptilolite-rich tuff at natural-state and fertilized with struvite), Stv + Cha (soil pre amended with 10 % chabazite-rich tuff at natural-state and fertilized with struvite), N–Cli (soil fertilized with N-enriched clinoptilolite tuff), and N–Cha (soil fertilized with N-enriched chabazite tuff). Two treatments are significantly different ($p < 0.05$) if no letter is in common. The error bars represent standard deviations ($n = 3$).

In addition to the results on CO_2 fluxes, we also previously observed decreased NO_x emissions in the same treatments amended with zeolitized tuffs (Galamini et al., 2023). In this regard, and considering the possible abiotic controls of zeolite minerals, namely, adsorption capacity for polar and polarizable gases such as CO_2 and NO_x (Boer et al., 2023), questions arise about the traditional interpretation of qCO_2 as an indicator of soil microbial C-use efficiency when amendments with gas adsorption capacity are used. In these cases, the measured emissions may represent a fraction of the respired C, where total CO_2 efflux could be hindered by the increased gas adsorption capacity of the amended soil. This aspect is discussed in Ameloot et al. (2013) concerning biochar, which – like zeolites – exhibits significant adsorption capacity for CO_2 and other gases of environmental concern.

4.2. Potential extracellular enzymes activities

Microorganisms secrete extracellular enzymes to acquire nutrients from the SOM (Gebhardt et al., 2017). Their activity reflects a complex interplay between the availability of nutrients in the soil ecosystem, biological demands, and stoichiometric constraints (Mori et al., 2023).

A significant increase in the activity was observed only in the soil amended with clinoptilolite tuff + struvite fertilizer. The second hypothesis was therefore rejected, namely the decrease in the potential EEA of N- and P-acquiring enzymes due to the input of nutrients from fertilizers for enzyme production.

To evaluate the effects of plant residues decomposition on C-cycling, BG is among the most widely used enzymes (Adetunji et al., 2017), as it plays a key role in the final stages of cellulose degradation, resulting in the release of readily available simple sugars (glucose). The input of organic substrates rich in plant residues, such as compost, vermicompost, et cetera, has been associated with increased BG activity (Crecchio et al., 2004; Meyer et al., 2015). Here, the presence of readily available C in the digestate, as indicated by increased heterotrophic respiration, likely reduced the need for BG production compared to other treatments, potentially indicating a certain release of microbial C limitation (Rosinger et al., 2019).

In the soil amended with natural-state clinoptilolite tuff + struvite the C_{mic} decreased by about 50 %, while this did not occur in the soil treated with natural-state chabazite tuff + struvite, revealing an intrinsic effect of the clinoptilolite tuff, plausibly related to sulfur toxicity when the tuff was applied at the highest dose (Galamini et al., 2023). The two treatments involving natural-state zeolitized tuffs + struvite also showed increased C- and N-acquiring enzyme activity, as well as the strongest declines in the enzymatic C:N ratio. Two plausible scenarios arise: i) a greater EEAs due to the activity of opportunistic decomposers that were resistant to the sulfur released by the clinoptilolite tuff; or ii) the increased EEAs did not reflect the requirements of the microbial community but were instead indicative of the presence of intracellular enzymes released from lysed cells (Mori et al., 2023). Unfortunately, the data provided do not allow for the separation of these two possibilities.

The second scenario, discussed in works such as Baltar (2017), Skujiņš and Burns (1976), and Wallenstein and Burns (2011), highlights the role of free enzymes in total activity. Compared to intracellular or membrane-bound enzymes, extracellular enzymes can perform functions without the presence of the original organism. Their products are available to the overall microbial community as universal resources (Smith and Schuster, 2019). Organisms that benefit from the products of others are referred to as “cheaters” (Kaiser et al., 2015). For example, Schneider et al. (2012) observed that the main decomposing enzymes for leaf litter were produced by fungi. However, bacteria benefited from the fungal production of extracellular proteins.

Since lower EEAs were expected in the soil amended with clinoptilolite tuff + struvite fertilizer due to microbial decay caused by the clinoptilolite tuff, the third hypothesis was rejected. In the soil amended with clinoptilolite tuff + struvite, the increased EEAs likely indicated the presence of beneficial substrates for the remaining living

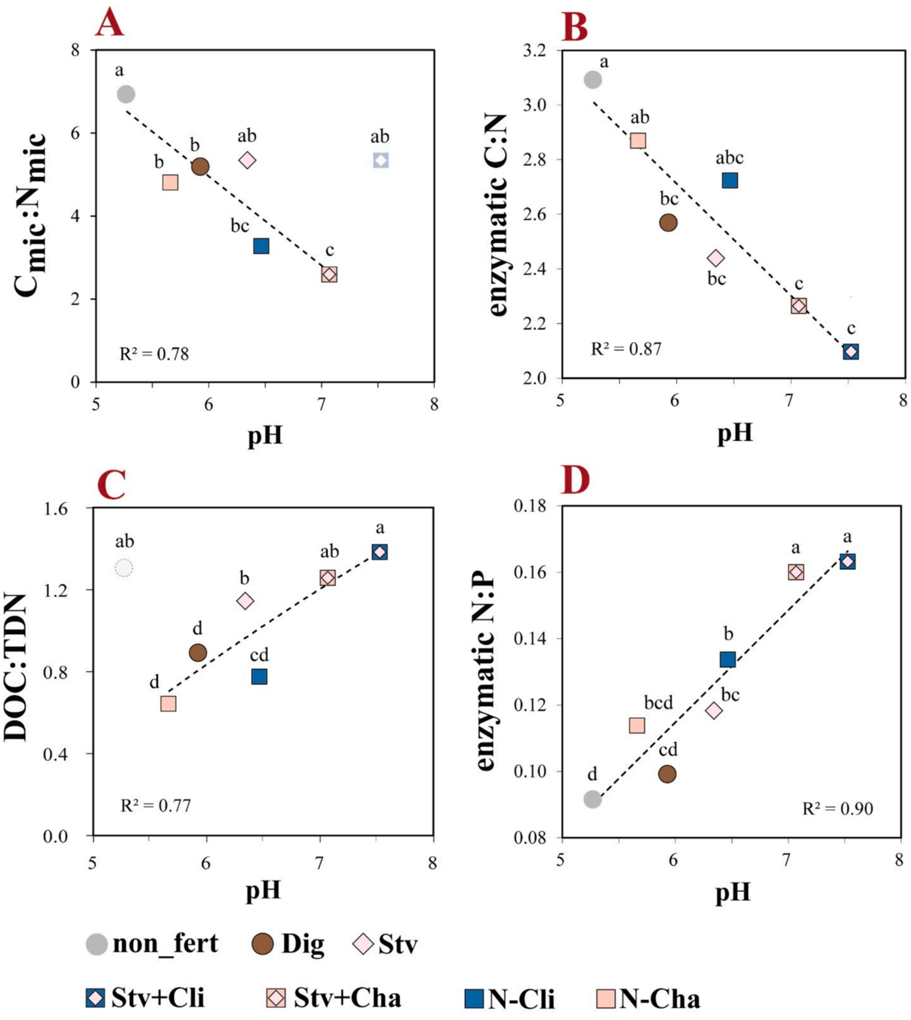

Fig. 4. Linear relationships between soil pH and microbial-related parameters. Acronyms for treatments are: non_fert (non-fertilized soil), Dig (soil fertilized with digestate), Stv (soil fertilized with struvite), Stv + Cli (soil pre amended with 10 % clinoptilolite-rich tuff at natural-state and fertilized with struvite), Stv + Cha (soil pre amended with 10 % chabazite-rich tuff at natural-state and fertilized with struvite), N–Cli (soil fertilized with N-enriched clinoptilolite tuff), and N–Cha (soil fertilized with N-enriched chabazite tuff). A) C_{mic}:N_{mic} (mol:mol; Stv + Cli is not considered in linear regression due to its inconsistencies, as outlined in section 4.2); B) exoenzymatic C:N = BG/(NAG + LAP); C) DOC:TDN is the ratio between the dissolved organic carbon and the total dissolved nitrogen (mol:mol; Cnt is not considered in linear regression due to no nutrient input); D) exoenzymatic N:P = (NAG + LAP)/AP. Two treatments significantly differ in the *y* axis value ($p < 0.05$) if no letter is in common.

microorganisms.

While microbial decay occurred in the soil amended with clinoptilolite tuff + struvite fertilizer, it did not occur in the soil treated with chabazite tuff + struvite. In contrast, the increased specific activity of N-acquiring enzymes compared to the non-fertilized soil and the pronounced decline in the C_{mic}:N_{mic} ratio were indicative of immediate effects on microbial functioning, suggesting N immobilization in the microbial biomass (Rosinger et al., 2022).

The final step of chitin degradation involves the role of NAG, which allow the release of amino sugars, while LAP catalyse the hydrolysis of leucine residues at the N-terminus of proteins and peptides (Daunoras et al., 2024). The input of inorganic N into soil has been observed to decrease NAG and LAP activities, as microorganisms can utilize the inorganic N supply. However, NAG and LAP activities generally increase under alkaline conditions (Carroll et al., 2013; Uwituze et al., 2022). The pH optima of hydrolytic enzymes generally do not align with soil pH but are often site-specific (Daughtridge and Margenot, 2024). For LAP, pH optima were found to be higher than soil pH, likely due to pH-dependent catalytic mechanism such as partial enzymes denaturation and the protonation of functional groups. In four arable soils, LAG exhibited pH optima in the range of 7–9, whereas NAGs showed pH optima more aligned with soil pH values, ranging from 5.4 to 8 (Daughtridge and Margenot, 2024).

Here, the input of inorganic N (NH_4^+–N from struvite), which is generally associated with reductions in chitinase and aminopeptidase activity (Uwituze et al., 2022), resulted in an increase of specific NAG and LAP activities in samples amended with zeolitized tuffs (both clinoptilolite and chabazite) + struvite fertilizer. Evidently, the rise in soil pH and subsequent shifts in soil buffering capacities (Frankenberger and Johanson, 1982) played a pivotal role in the immediate enhancement of key enzymes in N- and C-cycling. This was confirmed by the linear relationships (validated with the AIC test) found between pH and specific EEAs ($R^2 = 0.95, 0.84, 0.78$ for NAG, BG, and LAP, respectively). Our results suggest that upon zeolite and struvite addition, soil microorganisms start producing extracellular enzymes due to a higher bioavailability of C and N, a process referred to as substrate-induced enzyme production (Mori et al., 2023). The observed increases in NAG activities with increasing soil pH are somewhat unexpected, given the fact that fungi are considered the major degraders of chitinase (Kellner and Vandenbol, 2010), and that fungal growth is superior to bacterial growth under more acidic conditions (Rousk et al., 2010). However, recent studies suggest that soil pores of different sizes harbour specific microbial communities (Li et al., 2024), with soil fungi proliferating in larger soil pores (Rosinger et al., 2025). Therefore, the addition of zeolitized tuffs may have improved the soil pore distribution (towards a higher share of meso- and macropores), subsequently favouring soil fungi. Clearly, more studies are required to decipher the effect of tuff and zeolite amendments on microbial community composition shifts.

4.3. *Microbial response to soil alkalinization*

It has been reported that C_{mic}:N_{mic} increased under soil acidification (e.g., Li et al., 2021). In this study we found a similar relationship, as C_{mic}:N_{mic} decreased with soil alkalinization, except for the soil amended with clinoptilolite tuff + struvite, likely due to the negative effect on C_{mic} (Galamini et al., 2024). It has been recently reported that soil pH increase caused by liming decreased the C_{mic}:N_{mic} ratio, also strongly increasing bacterial diversity and relative abundance in acidic forest soils (Cha et al., 2021). In contrast, changes in the C_{mic}:N_{mic} ratio may not necessarily reflect changes in the bacterial-fungi ratio, as outlined e.g., in a study conducted on the Hoosfield acid strip (a 200 m long strip with a pH gradient ranging from 4.0 to 8.3 that resulted from a one-time uneven application of chalk in the mid 1800) where C_{mic}:N_{mic} remained very stable across the gradient, while the fungal-to-bacterial ratio decreased along with pH increasing, together with greater bacterial diversity (Rousk et al., 2010) along the strip. The independency between C_{mic}:N_{mic} and the fungal-to-bacterial ratio was observed also by (Zhou et al., 2019). A shift in the C_{mic}:N_{mic} ratio may also be associated with the incorporation of N into microbial biomass (Rosinger et al. 2022).

Concerning the relationships between EEAs and nutrient limitations, recent studies have indicated that such connections are not always straightforward (Mori et al., 2023; Zheng et al., 2022), especially in fertilized soils (Rosinger et al., 2019). However, it is still possible to make some further considerations, as outlined below.

The release of organic C from less-protected SOM, induced by shifts in soil pH, affected the ratio of dissolved, and likely easily available, C to N, expressed here as DOC:TDN. Under soil alkalinization, the augmented DOC:TDN ratio, coupled with lower requirements for assimilating C compared to N by the microbial community, suggested by the decreasing C_{mic}:N_{mic}, likely translated into the lower enzymatic C:N ratio. In other words, the liming effect of struvite and zeolites (Degryse et al., 2017; Tsadilas et al., 1997) likely resulted in different stoichiometric requirements for C and N, which was further reflected in the ratio of C- and N-acquiring exoenzymes.

The increased demand for organic N under soil alkalinization is also indicated by the rising enzymatic N:P ratio. Nevertheless, none of the treatments exhibited significant mineralization of the organic N substrate, as evidenced by the negligible rates of gross NH_4^+–N production (Galamini et al., 2023). Lastly, it should be noted that the effects on soil pH, and consequently the significant impacts observed on microbial biomass, were due to the high dosage of natural-state zeolitized tuff (10 %), and/or the high dosage of nutrients, especially struvite, with approximately 660 mg P/kg soil (equivalent to about 1500 kg P_2O_5/ha) added. Lower application rates are unlikely to impact soil pH, resulting in a more stable environment (Shen et al., 2019). High N application rates can lead to soil acidification, negatively impacting soil activity and diversity. Excess P can cause nutrient imbalances, affecting microbial processes and potentially leading to the accumulation of insoluble mineral P phases. Furthermore, high levels of N and P can shift the microbial community composition towards certain bacterial taxa, while others decline (Wang et al., 2016). Therefore, in the context of actual soil application, if the goal is to supply N to crops while avoiding excess P, struvite should be used in combination with other N fertilizers that have a lower P content.

5. Conclusions

This study demonstrates that the addition of struvite and zeolitized tuffs alters soil pH, resulting in immediate and substantial effects on shifts in microbial stoichiometry and physiological responses. Under conditions of alkalinization, both the C_{mic}:N_{mic} and enzymatic C:N ratios decreased, while the DOC:TDN ratios increased, suggesting a greater microbial demand for N relative to C. Higher bioavailability of C and N subsequently induced the production of extracellular enzymes. About the working hypotheses, the following considerations can be made: 1) Microbial C use was incentivized with organic fertilization (digestate) as indicated by increased heterotrophic respiration and qCO_2, whereas a less pronounced response was observed with sole struvite, and struvite in combination with zeolite amendments; 2) Lower potential EEAs were expected in fertilized soil; however, no differences were recorded between fertilized and unfertilized soils; 3) Lower enzymatic activity was expected in the soil amended with 10 % clinoptilolite-rich zeolitized tuff due to microbial decay; however, significant increases were recorded, plausibly due either to opportunistic decomposers or the release of intracellular enzymes from lysed cells.

This study highlights the potential of zeolitized tuffs to mitigate fertilizer-derived CO_2 emissions from arable soils, their implications for short-term soil C and N dynamics, and provides recommendations for the use of struvite and zeolitized tuffs in the context of soil health and sustainable farming. However, it is important to acknowledge the limitation of this study to short-term observations during a brief incubation period. Further research is required to elucidate the long-term impacts on microbial activity, soil biochemical processes, and broader ecosystem dynamics.

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CRediT authorship contribution statement

G. Galamini: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **G. Ferretti:** Visualization, Methodology, Conceptualization. **C. Rosinger:** Writing – review & editing, Formal analysis, Data curation. **S. Huber:** Visualization, Formal analysis. **A. Mentler:** Visualization. **E. Diaz–Pines:** Writing – review & editing, Resources, Methodology, Data curation. **B. Faccini:** Visualization. **K.M. Keiblinger:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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