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Gross Ammonification and Nitrification Rates in Soil Amended with Natural and NH_4-Enriched Chabazite Zeolite and Nitrification Inhibitor DMPP

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Abstract: Using zeolite-rich tuffs for improving soil properties and crop N-use efficiency is becoming popular. However, the mechanistic understanding of their influence on soil N-processes is still poor. This paper aims to shed new light on how natural and NH_4^+-enriched chabazite zeolites alter short-term N-ammonification and nitrification rates with and without the use of nitrification inhibitor (DMPP). We employed the ^{15}N pool dilution technique to determine short-term gross rates of ammonification and nitrification in a silty-clay soil amended with two typologies of chabazite-rich tuff: (1) at natural state and (2) enriched with NH_4^+-N from an animal slurry. Archaeal and bacterial *amoA*, *nirS* and *nosZ* genes, N_2O-N and CO_2-C emissions were also evaluated. The results showed modest short-term effects of chabazite at natural state only on nitrate production rates, which was slightly delayed compared to the unamended soil. On the other hand, the addition of NH_4^+-enriched chabazite stimulated NH_4^+-N production, N_2O-N emissions, but reduced NO_3^--N production and abundance of *nirS-nosZ* genes. DMPP efficiency in reducing nitrification rates was dependent on N addition but not affected by the two typologies of zeolites tested. The outcomes of this study indicated the good compatibility of both natural and NH_4^+-enriched chabazite zeolite with DMPP. In particular, the application of NH_4^+-enriched zeolites with DMPP is recommended to mitigate short-term N losses.

Keywords: sustainable agriculture; soil amendment; N_2O; N cycle; gross N transformation rates; ^{15}N pool dilution; nitrate production; enriched zeolite; microbial biomass; N processes

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1. Introduction

Unsustainable farming practices are unquestionably impairing soil, water, and air quality as a consequence of nutrient leaching and emission of harmful greenhouse gases (GHGs). This deterioration of natural resources makes the achievement of sustaining the future food demand for the global population improbable. Fertile soils are becoming increasingly scarce due to soil degradation and further expansion of agricultural areas is not possible anymore. Therefore, it is imperative to improve the sustainability of agricultural practices on lands that are already being cultivated [1].

The development of strategies for reducing nutrient losses from agroecosystems and increasing the fertilizer and nutrient use efficiency (FUE and NUE) by crops is crucial [2–6].

The use of soil amendments is recognized as a valuable possibility to improve soil properties, crop yield, and reduce nutrient losses from the soil system [1,7,8]. A wide spectrum of soil amendments is employable in the agricultural context, ranging from organic (e.g., manure, biochar, etc.) to inorganic (e.g., lime, natural zeolites, etc.) materials [1,9–11]. The nature and characteristics of each soil amendment may vary drastically, but the main purpose is always to improve soil properties, nutrient retention, sustain crop yield and remediate degraded soils. Within soil inorganic amendments, natural zeolites have been studied for decades because of their peculiar properties and relative high availability at low cost around the globe [12–17]. Zeolite minerals are crystalline substances with an open 3D-framework formed by linked tetrahedra of $[SiO_4]^{4-}$ and $[AlO_4]^{5-}$ that delimits open cavities in the form of channel and cages. These cavities are usually occupied by H_2O and other molecules (extra-framework ions) that are exchangeable [18]. Zeolite's main properties can be summarized as follows: (i) high cation exchange capacity (CEC), (ii) reversible dehydration, and (iii) molecular sieve [18,19]. Thus, it is not surprising that zeolites became exceptionally popular after their discovery in a wide range of industrial applications. These attributes are functional also for agronomic purposes as zeolite addition to the soil can improve water and nutrient retention, resulting in increasing water reserve for plants and in reducing N losses (leaching and volatilization) [20–22].

Nowadays, more than sixty types of natural zeolites have been described by researchers (http://www.iza-online.org/natural/default.htm accessed date 9 March 2021), each of which differs in terms of framework structure, chemistry, and ion exchange capacity. However, only a few of them occurs in sufficient quantity and purity to be considered exploitable natural resources [23]. Among them, clinoptilolite is the most frequent and abundant zeolite in nature, followed, in order, by mordenite > chabazite > phillipsite > erionite [24]. Natural zeolites are often constituents of volcanic tuffs [25], then, referring simply to "natural zeolite" when using these products, from a geological perspective, is improper as it should be better substituted by rocks or tuffs rich in zeolite or zeolitite, in case the zeolite content of the rock is greater than 50% [24].

Although less diffused than clinoptilolite, chabazite zeolite (framework type CHA) is particularly attractive for agricultural and industrial applications because of its very higher CEC (3.84 meq g^{-1}) [26,27]. A peculiarity of high agronomic interest exhibited by CHA zeolite is the capacity to bind high amounts of NH_4^+ with weak hydrogen bonds to the framework, making it easily accessible and hence exploitable by various potential users (e.g., microorganisms or plants) [26]. The tetrahedral framework of CHA is composed of parallel layers of double 6-rings in ABC sequence with cavities of different size (the wider one bonded by eight-membered rings), in which H_2O and exchangeable cations are hosted (most frequently Ca, K and Na) [26] (Figure 1).

In central Italy, many pyroclastic deposits have been altered to zeolitites, containing mostly Ca-CHA and K-CHA, and phillipsite. Some of the zeolitic units are tens of meters thick and contain up to 80% of zeolites, and are thereby of commercial interest. Until a few years ago, the resulting material obtained from the cutting of bricks for construction and gardening in zeolitic-tuff quarries was considered a byproduct. Recently, this byproduct was tested as a soil amendment in the framework of the EU project ZeoLIFE (LIFE10 ENV/IT/000321) and revalued. Results from a long term (>3 years) field experiment where this CHA-zeolitite was used as a soil amendment highlighted positive effects on soil N cycling, which promoted the possibility to reduce the N inputs and NO_3^- leaching losses while maintaining or even increasing crop yield (depending on crop type) [8,28].

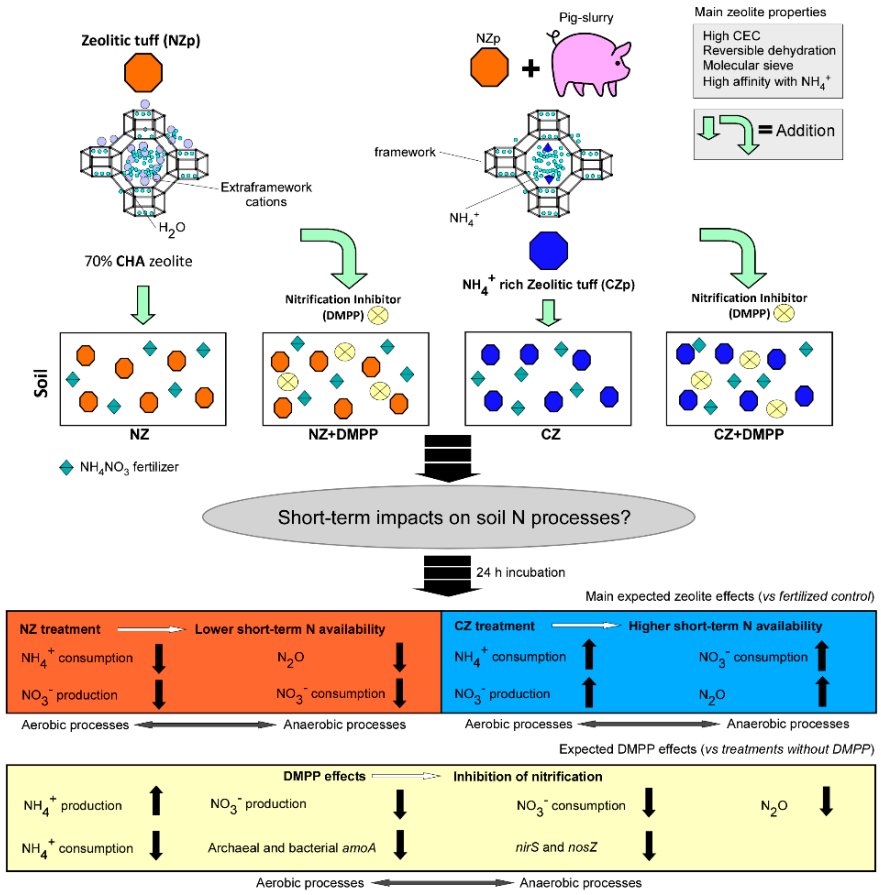

Figure 1. Conceptual scheme of the experiment, including the main expected results for each treatment. Representation of framework structure and location of water molecules and extra-framework cations of the chabazite zeolite were taken and adapted from [26].

The interest of the scientific and industrial communities in natural zeolites increased exponentially in the last decades. Hundreds of papers and several reviews have been published about their use and effects in agriculture [12,19,24,29–31]. However, a mechanistic approach has still not been used to describe how soil N processes are influenced by these highly reactive minerals. Various studies hypothesized that zeolites influence nitrification rates [22,32,33] after measuring net variations of mineral N concentrations over time. Measuring net rates does not allow discrimination between production and consumption processes and hence are not an adequate tool to address the effective impact on soil N cycling. To address mechanistically the effects of zeolite amendments on soil N cycling, it is hence fundamental to evaluate their influence on gross N transformation rates once applied to the soil.

Nitrification consists of a two-step oxidative microbial conversion of first NH_4^+ to NO_2^- by ammonia oxidizer archaea (AOA) and bacteria (AOB) and then to NO_3^- by nitrite-oxidizing bacteria (NOB). Ammonia oxidation is considered to be the rate-limiting step of nitrification and it is catalyzed by bacterial and archaeal ammonium monooxygenase (AMO) encoded by the gene *amoA* [34]. Nitrification is normally favoured under well-aerated conditions and a good NH_4^+ supply [35]. Under oxygen limiting conditions, NO_3^- can be used by several microbes as a terminal electron acceptor and is converted to the gaseous forms N_2O and N_2 [36]. The complete process involves the enzymes nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) [37]. Depending on the environmental conditions, substantial amounts of the potent greenhouse gas N_2O are emitted from soil, unless the whole N_2O is converted to N_2 by the enzyme N_2O reductase encoded by *nosZ*. The abundance of the functional gene

amoA and the genes *nirS* and *nosZ* can give indications on the efficiency of the respective processes [38–41].

CHA zeolite can be also utilized for removing NH_4^+ from wastewaters, including those that originated from livestock [42,43]. These "charged" zeolites, which have adsorbed consistent amounts of NH_4^+, can be eventually exploited as "slow-release" fertilizers to recycle the adsorbed N efficiently [28]. NH_4^+-charged zeolites have been found to increase NO_3^- concentrations over time when used in soil or as cultivation substrate [10,44,45]. In this view, the use of a synthetic nitrification inhibitor (NI) may represent a valid countermeasure. NIs are effective synthetic substances used for promoting NUE and reducing N losses by slowing nitrification [46]. NIs facilitate the retention of soil N in the form of NH_4^+ by inhibiting the AMO through random binding to the membrane-bound enzyme. AMO shows a broad substrate range [47], and NIs compete with NH_4^+ for the active site of this enzyme, causing a delay in the first and rate-limiting step of nitrification [48]. Within commercially available NIs, dicyandiamide (DCD) and 3,4-dimethylepyrazole phosphate (DMPP) are the most widely used [49] but DMPP was found to have higher efficiency at lower application rates (10 times less than DCD) [50,51]. Although DMPP capacity to reduce nitrification rates and N_2O emissions has been well established [52–54], the effects on soil N gross rates (especially ammonification rates) have been poorly investigated as well as the combined effects with natural zeolites in soil. One of the few examples is [55], where a general increase of mineralization of recalcitrant organic N after DMPP addition in two soils was observed, beside a reduction in gross nitrification and N_2O emissions. DMPP as an organic molecule can decompose and hence provide a source of C for microorganisms that may further stimulate mineralization processes. The soil organic C content and amount of clay minerals can also play a role in the performance of DMPP as a consequence of sorption-effects [56]. High sorption of DMPP may result in a lower availability to microorganisms and hence to a lower efficiency; the authors of [55,57,58] evaluated the effects of natural and NH_4^+-charged CHA-zeolite on the sorption of DMPP in soil and concluded that the soil was more efficient in binding DMPP, thanks to the presence of higher levels of organic C that favored the sorption of DMPP. The CHA-zeolite contained in the tuff could not, therefore, retain DMPP by ion exchange processes but instead brought mostly to a reduction in the overall sorption capacity of the soil, due to their lack of organic C. This outcome speculates if this lower sorption capacity in zeolite amended soils also affects the availability of DMPP to soil microbial biomass and consequently the overall efficiency in inhibiting nitrification.

This is the first study to our knowledge that investigates the effects of zeolite-tuff on gross ammonification and nitrification rates. We tested natural and NH_4^+ charged CHA zeolite-tuff with and without the addition of DMPP in short-term incubation (24 h) (Figure 1).

We aimed to address the following research questions (*RQ*) and hypotheses (*Hy*):

RQ 1: Does the addition of CHA in natural or NH_4^+-charged state to soil have any effects on gross N mineralization and nitrification rates?

RQ 2: Is the performance of DMPP improved by the presence of natural or NH_4^+-charged zeolitite?

Therefore, a short-term incubation experiment was performed in which gross N ammonification and nitrification rates were measured by means of the ^{15}N pool dilution technique, coupled with measurements of functional marker genes of nitrification (*amoA AOA* and *amoA AOB*), nitrite and nitrous oxide reduction (*nirS* and *nosZ*), and the quantification of N_2O and CO_2 emissions.

2. Materials and Methods

2.1. Zeolites

The zeolite-rich volcanic tuff used in this study is a by-product obtained from a quarry located near Sorano (Grosseto, Italy). According to [59], the total zeolite content was 70.9 wt% of the whole rock, of which 68.5% is made of K-rich and Na-poor chabazite, 1.8% of phillipsite, and 0.6% of analcime and hence can be defined as a zeolitite (ZT). The ZT

was supplied by a local company and used in a grain size of 3–5 mm. Part of the ZT was used in natural state (NZ_p) and part was used after enrichment with NH_4^+ ions carried out following the protocol indicated by [60]. This method employed a field tank that mixes pig-slurry and ZT for a known amount of time. With this procedure, natural zeolites contained in the tuff adsorb NH_4^+ from the pig-slurry, becoming NH_4^+-enriched zeolites (CZ_p). Chemical characteristics of NZ_p and CZ_p are reported in Table 1.

Table 1. Basic zeolites and soil properties. NZ_p and CZ_p refer to pristine chabazite zeolite tuff (with no soil added) in natural and NH_4^+-enriched state, CNTR is the unamended soil, NZ is the soil + 10% wt of NZ_p while CZ is the soil amended with 10 wt% of CZ_p. EC is electrical conductivity (mS cm^{-1}). Values are expressed as mean ± standard deviation (3 replicates). Different superscript letters express significant differences as a result of ANOVA and Tukey-HSD statistical tests ($p < 0.05$). nd = not detected.

Sample	pH	EC mS cm^{-1}	Total N g kg^{-1}	Total C g kg^{-1}	Total OC g kg^{-1}
CNTR	7.59 ± 0.05^{a}	1.44 ± 0.04^{c}	2.09 ± 0.10^{c}	33.47 ± 0.76^{a}	21.04 ± 0.23^{a}
NZ_p	7.58 ± 0.05^{a}	0.54 ± 0.07^{e}	0.10 ± 0.01^{e}	0.80 ± 0.10^{e}	nd
CZ_p	6.95 ± 0.09^{b}	15.03 ± 0.63^{a}	4.27 ± 0.12^{a}	1.53 ± 0.08^{d}	0.08 ± 0.01^{d}
NZ	7.55 ± 0.03^{a}	1.34 ± 0.02^{d}	1.76 ± 0.01^{d}	27.69 ± 0.75^{c}	16.48 ± 0.03^{c}
CZ	7.63 ± 0.04^{a}	2.56 ± 0.11^{b}	2.27 ± 0.07^{b}	29.78 ± 0.78^{b}	18.10 ± 0.01^{b}

2.2. Soil

Soil samples were collected during spring 2016 from the ZeoLIFE project experimental field, which consists of a 6 ha agricultural arable field where different ZT amendments have been tested since 2012 [7,8,28]. 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) that is considered a Nitrate Vulnerable Zone (NVZ) according to the Nitrate Directive (91/676/EEC). The topsoil layer is of alluvial origin and a clayey-silty texture, classified as Calcaric Gleyic Cambisol [61,62]. The soil is mainly characterized by quartz, illite, chlorite, K-feldspar, plagioclase, calcite, and amorphous residues [59]. Soil samples for this study were collected from an unamended parcel from the top 30 cm depth layer and amended with NZ_p and CZ_p in a laboratory.

2.3. Experimental Setup

Air-dried soil samples were sieved to 5 mm and pre-incubated at 45% water filling pore space (WFPS) at 20 °C for 4 days. NZ_p and CZ_p were applied to the soil at 10 wt% and tested as treatments (NZ and CZ, respectively) versus a control (CNTR). All the treatments received 486 μg g^{-1} of NH_4NO_3, equivalent to 170 kg-N ha^{-1}, which reflects the fertilization rate used in NVZ according to the Nitrate Directive 91/676/EEC. The same fertilization rate was also used in previous field experiments of the ZeoLIFE project [28] and in laboratory studies involving the same soil and zeolites [22]. As an additional factor, in a separate batch, each treatment received 2.916 μ g^{-1} of the nitrification inhibitor DMPP in liquid form, corresponding to 0.6% of the fertilizer added, according to [35,38]. This dosage also reflects the amount of DMPP that is commonly contained in granulated formulations [51,54].The tested treatments thus resulted in the following six combinations in four replicates:

- unamended soil (CNTR);
- unamended soil (CNTR) + DMPP;
- soil with 10 wt% of NZ_p (NZ);
- soil with 10 wt% of NZ_p (NZ) + DMPP;
- soil with 10% of CZ_p (CZ);
- soil with 10% of CZ_p (CZ) + DMPP.

2.4. Basic Soil and Amendment Properties

Unamended and amended soil-ZT mixtures, as well as pure ZT (NZ_p and CZ_p), were analyzed for basic properties such as gravimetric water content (GWC), pH, electrical conductivity (EC), and total C and N concentrations.

GWC was measured by oven-drying at 105 °C to constant weight (all soil parameters have been corrected for GWC content). EC was measured in 1:10 w:v ratio of soil in H_2O extracts with a conductometer (WTW LF191) connected with a probe (WTW LS 1/T-1,5), while pH was measured in H_2O extracts (1:5 w:v ratio) with a WTW inoLab pH Level 2. Total C-N concentrations were measured with an Elementar Vario Micro Cube Elemental Analyzer (EA) by weighing samples in tin cups according to [7].

2.5. ^{15}N Pool Dilution

After pre-incubation, an equivalent of 8 g dry soil was added in two steps (4 + 4 g) into 50 mL centrifuge tubes and fertilizer solution was applied in two steps ensuring uniform distribution of the ^{15}N label. Additional water was applied to achieve the targeted water filling pore space (WFPS) of 65%. Soil bulk density (BD) was adjusted to 1 g cm^{-3}.

Gross ammonification (gross production of NH_4^+-N from the mineralization of organic matter), and nitrification (gross production of NO_3^--N from the oxidative process of NH_4^+), and gross consumption rates were evaluated by ^{15}N pool dilution assays.

NH_4NO_3 fertilizer was applied in solution in the form of $^{15}NH_4{}^{14}NO_3$ for samples for gross ammonification and in the form of $^{14}NH_4{}^{15}NO_3$ for samples for gross nitrification, respectively, at a target ^{15}N enrichment of 10% in both solutions. The samples were prepared in two batches for an incubation time of 24 h. They were extracted with 2 M KCl in a 1:5 w/v ratio by adding 40 mL directly into the centrifuge tubes, homogenizing with a vortex, shaking for 1 h at 150 rpm, followed by centrifugation at 3500 rpm for 10 min. The solution was filtered through Whatman #40 ash-less free cellulose filters. Part of the solution was analyzed for NH_4^+-N and NO_3^--N concentrations using a microplate spectrophotometer Perkin Elmer Enspire, according to [63] and to [64], respectively. The remaining solutions were used for the diffusion of $^{15}NH_4$ and $^{15}NO_3^-$ according to [65]. Briefly, during ammonia diffusion, in samples designed for gross ammonification measurements, 2 g of MgO was added to the extracted solution together with an acid trap consisting of an acidified piece of cellulose-filter acidified with 12 µL of 2.5 M $KHSO_4$ and enclosed in Teflon tape. The containers were closed tightly and shaken for 72 h to raise the pH and transform all the NH_4^+ into gaseous NH_3 and capture it into the acid trap. The same procedure was carried out for samples designed for gross nitrification measurements but with the following modifications: first, NH_4^+ was eliminated by rising the pH and adding MgO and shacking the containers with the lids open; then Devarda alloy was added to the samples to reduce NO_3^- into NH_4^+, acid traps were added, and the samples were shaken for 72 h to ensure NH_3 diffusion into the acid traps. The acid traps were dried in a desiccator containing HCl for trapping atmospheric NH_3 and then closed in tin cups for isotopic analysis. The isotopic signature ($\delta^{15}N$) of the acid traps was determined with an Elementar Vario Micro Cube Elemental Analyzer (EA) in line with an ISOPRIME 100 Isotopic Ratio Mass Spectrometer (IRMS) operating in a continuous-flow mode.

2.6. DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR) of Functional Marker Genes

Immediately after the incubation procedure (24 h), soil samples were homogenized and subsamples of 0.25 g soil were taken to extract total DNA in 3 replicates. DNA was extracted using the PowerSoil® DNA Isolation Kit from MoBio (Mobio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions, with the following modification: the soil was extracted twice by using the same soil and PowerBead Tubes to increase the recovery of DNA [38]. DNA concentration and quality were determined spectrophotometrically (NanoDrop 2000, Thermofisher, MA, USA).

The two DNA aliquots from each sample were pooled before qPCR. For the quantification of functional marker genes of nitrification (*amoA AOA and amoA AOB*) and denitrification (*nirS* and *nosZ*), existing primer sets were used [66,67]. For details on gene-specific qPCR primers and thermal programs, please refer to Electronic Supplementary Information (ESI), Tables S1 and S2. Quantitative real-time PCR (qPCR) assay was carried out in a volume of 10 µL and the assay mixture contained GoTaq® qPCR Master Mix (Promega, USA), 10 µM of each primer (except for the gene *amoA AOA* 20 µM were used), and 1 µL of pooled template DNA. Each sample was quantified in triplicates using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Standard curves were constructed as described in [38].

2.7. N_2O and CO_2 Measurements

For each treatment, a subset of 50 mL centrifuge tubes containing samples was sealed with Suba-Seal septa (Sigma Aldrich, St. Louis, MI, USA) in 4 replicates. After an incubation time of 24 h, 10 mL gas samples were taken with a gas-tight syringe and injected in pre-evacuated glass vials (Agilent Technologies, Santa Clara, CA 95051, USA). The samples were analyzed with a GC-System (Agilent Technologies) equipped with a headspace auto-sampler. N_2O concentrations were detected with an ^{63}Ni electron capture detector while CO_2 concentrations were measured after reduction on a Ni catalyst, with a flame ionization detector [68,69].

2.8. Calculations and Statistical Analysis

Conversion of $\delta^{15}N$ into ^{15}N atom% was performed with Equation (1):

$$^{15}\text{N atom \%} = [(\delta^{15}\text{N}/1000)*0.3665] + 0.3665 \quad (1)$$

where $\delta^{15}N$ is the isotopic signature expressed as "delta" notation and 0.3665 (%) is the ^{15}N natural abundance in the standard (N_2 air).

Net rates, gross production, and gross consumption were calculated with the following equations (Equations (2)–(4)) [70]:

$$\text{Nr} = (C_{t24} - C_{t0})/t \quad (2)$$

$$Gp = Nr^* [\log (APE_{t0}/APE_{t24})/\log(C_{t24}/C_{t0})] \quad (3)$$

$$Gc = Gp - Nr \quad (4)$$

where *Nr*, *Gp*, and *Gr* are net rates, gross production, and gross consumption (mg NO_3^- or NH_4^+-N kg^{-1} day^{-1} dm), respectively. C is the concentration of NH_4^+-N or NO_3^--N in mg kg^{-1}, APE is the atom% excess of ^{15}N, calculated as atom% ^{15}N measured is the sample–^{15}N natural abundance (0.3665%), t_0 and t_{24} are the initial and final extraction points, t is the time intercurred in the incubation (1 day). Nr (Equation (2)) indicates the temporal net variation of the target pool size with incubation time and does not allow the discrimination between production and consumption processes. By relating Nr with the dilution of the introduced ^{15}N pool with newly produced NH_4^+-N or NO_3^--N at natural abundance by ammonification and nitrification processes, gross production rates can be calculated (Equation (3)). Finally, it is possible to calculate the rate of consumption by subtracting Nr from the Gp (Equation (4)).

Gaseous N_2O-N and CO_2-C fluxes were calculated on soil mass basis by considering the linear increase in headspace concentration over the incubation period according to [71]. The ideal gas law was used to convert ppm of N_2O and CO_2 in ppm, to N and C mass. The conversion factor of ppm to µ m^3 was calculated according to Equation (5):

$$\text{Cf} = (P*Mwc*1000)/(R*T) \quad (5)$$

where Cf is the conversion factor (ppm to μg m^3); P is the air pressure (in kPa); Mwc is the molar mass of N or C; R is the gas constant (8.314); T is the incubation air temperature (K) [71].

Then, the flux on the mass basis was calculated according to Equation (6):

$$Fmass = [\Delta(ppm * Cf)/\Delta t)*Hs]/(Vo*Db) \quad (6)$$

where Fmass is the linear gas efflux in the incubation container on soil mass basis (mg N_2O-N or CO_2-C g^{-1} soil h^{-1}); Ppm is the concentration measured with the GC in ppm; Cf, conversion factor calculated from Equation (5); Δt is the incubation time (hours); Δ (ppm * CF) is the change in gas concentration during the incubation period; Hs is the headspace of the container (m^3); Db is the soil bulk density (g cm^{-3}); Vo is the volume of the microcosm (cm^3) [71].

To evaluate significant differences between the treatments, ANOVA and multi-comparison Tukey HSD tests were performed at $p < 0.05$. Normality and homoscedasticity were tested before running ANOVA using Shapiro-Wilk and Bartlett's tests. If data were not normally distributed, data were ln transformed to meet ANOVA assumptions. If normality was not reached, the non-parametric Kruskal Wallis test was applied. All statistical analysis was performed using R studio version 1.3.335. The following R packages have been employed for data analysis and figure building in this paper: "*ggplot2*" [72], "*Agricolae*" [73].

3. Results

Before the addition to soil, NZ_p and CZ_p differed in EC and pH values (Table 1). CZ_p had a neutral pH and high EC, while NZ_p had a weakly alkaline pH and very low EC. No significant differences were observed between CNTR, NZ, and CZ for pH ($p > 0.05$), while significant differences were accounted for EC ($p < 0.05$): the NZ treatment was characterized by lower EC, while CZ showed the higher EC. Significant differences were also observed between the treatments for total N, C, and OC concentrations. The N concentration of NZ_p was very low, while that of CZ_p was 4.27 g kg^{-1}. The C concentration of both pure zeolite tuffs was very low if compared to that of the soil but CZ_p was characterized by a small fraction of residual OC. As for the EC, total N was higher in CZ treatment, followed by the CNTR, while NZ showed the lowest total N ($p < 0.05$). Differences were also observed in terms of total OC between the treatments, with the highest values in the CNTR and the lowest in NZ ($p < 0.05$).

3.1. NH_4^+-N and NO_3^--N Concentrations and Transformation Rates without DMPP

NH_4^+-N and NO_3^--N concentrations and net rate calculations are reported in Table 2. The initial (t_0) NH_4^+-N concentration of CNTR and NZ was very similar ($p > 0.05$), while that of CZ was significantly higher ($p < 0.05$). After 24 h (t_{24}), the NH_4^+-N decreased (similarly) in CNTR and NZ, while it increased in CZ. This resulted in similar negative net rates for CNTR and NZ and a positive net rate for CZ (Table 2). The gross production and consumption rates of NH_4^+-N are reported in Figure 2. In CNTR and NZ treatments, consumption of NH_4^+-N was higher than production, which showed very low rates, while in CZ treatment, the production of NH_4^+-N exceeded consumption.

NO_3^--N concentrations at t_0 were very similar in CNTR and NZ, and significantly higher in CZ (Table 2) ($p < 0.05$). NO_3^--N increased significantly in all the treatments at t_{24} but all net nitrification rates were very similar ($p > 0.05$). The gross production and consumption rates of NO_3^--N are reported in Figure 3. Production of NO_3^--N exceeded consumption in all the treatments. The CNTR was characterized by the highest production of NO_3^--N, followed by NZ and CZ, which exhibited the lowest production rate ($p < 0.05$). Consumption rates were similar between the CNTR and NZ ($p > 0.05$), while no consumption was recorded in the CZ treatment (Figure 3).

Table 2. NH_4^+-N and NO_3^--N concentrations at the beginning (t_0) and end (t_{24}) of the incubation. Net rates are calculated as indicated in Equation (3). Values are expressed as mean ± standard deviation (4 replicates). Different superscript letters express significant differences ($p < 0.05$) between treatments as a result of ANOVA (for NH_4^+-N t_0), Kruskal-Wallis (other variables), and Tukey-HSD statistical tests. Values in bold highlight the significant effect of DMPP.

	Sample	NH_4^+-N t_0 mg kg^{-1}	NH_4^+-N t_{24} mg kg^{-1}	NH_4^+-N Net mg $kg^{-1}day^{-1}$	NO_3^--N t_0 mg kg^{-1}	NO_3^--N t_{24} mg kg^{-1}	NO_3^--N Net mg $kg^{-1}day^{-1}$
No DMPP	CNTR	97.4 ± 2.8^{b}	65.8 ± 1.3^{e}	-31.5 ± 3.8^{e}	123 ± 4^{b}	149 ± 4^{c}	25.7 ± 5.8^{a}
	NZ	96.4 ± 1.0^{b}	68.6 ± 1.9^{d}	-27.8 ± 1.9^{e}	118 ± 2^{bc}	146 ± 2^{c}	27.6 ± 1.4^{a}
	CZ	293 ± 22^{a}	355 ± 15^{a}	61.8 ± 26.9^{b}	303 ± 9^{a}	337 ± 2^{a}	33.6 ± 8.9^{a}
With DMPP	CNTR	96.8 ± 2.3^{b}	$\mathbf{94.3 \pm 1.1^{b}}$	$\mathbf{-2.5 \pm 1.9^{c}}$	$\mathbf{114 \pm 1^{d}}$	$\mathbf{116 \pm 2^{d}}$	$\mathbf{1.7 \pm 1.2^{b}}$
	NZ	95.0 ± 2.8^{b}	$\mathbf{79.0 \pm 4.5^{c}}$	$\mathbf{-16.1 \pm 3.4^{d}}$	116 ± 0.4^{c}	$\mathbf{115 \pm 5^{d}}$	$\mathbf{-1.4 \pm 4.2^{b}}$
	CZ	269 ± 19^{a}	368 ± 6^{a}	$\mathbf{98.3 \pm 17^{a}}$	296 ± 3^{a}	$\mathbf{281 \pm 4^{b}}$	$\mathbf{-14.6 \pm 5.2^{c}}$

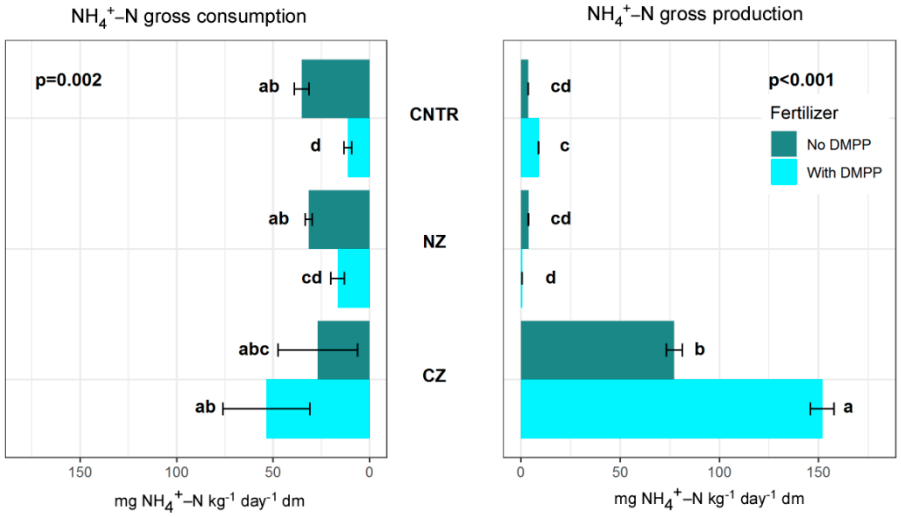

Figure 2. Gross NH_4^+-N consumption (on the **left**) and gross NH_4^+-N production (on the **right**) rates in treatments with and without DMPP addition. CNTR is the soil without any zeolite addition, NZ is the soil + 10 wt% of CHA zeolite in natural state while CZ is the soil + 10 wt% of NH_4^+-charged CHA zeolite. Error bars represent standard deviations (4 replicates) while different letters indicate significant differences between treatments as a result of ANOVA (for gross production), Kruskal-Wallis (for gross consumption) and Tukey (HSD) statistical tests ($p < 0.05$).

3.2. NH_4^+-N and NO_3^-N Concentrations and Transformation Rates with DMPP

The t_0 NH_4^+-N content in treatments that received DMPP was very similar to those without DMPP. However, at t_{24}, the concentrations tend to be lower in CNTR and NZ while they increased in CZ. This resulted in slightly negative net rates in CNTR and NZ treatments and a high positive rate for CZ, with a significant effect of DMPP ($p < 0.05$) (Table 2). Gross production of NH_4^+-N was very low in both CNTR and NZ as in the treatments that did not receive DMPP, while in CZ, the production was further stimulated by DMPP addition ($p < 0.05$), resulting in the highest rates recorded (Figure 2). The consumption of NH_4^+-N was still prevailing over production in CNTR and NZ, but it was lower than in treatments that did not receive DMPP ($p < 0.05$). In CZ treatment, the consumption was similar between with and without DMPP addition.

NO_3^--N at t_0 in treatments that received DMPP was similar to that of treatments without DMPP (Table 2). However, at t_{24}, the concentrations did not change significantly in CNTR and NZ, resulting in very similar and low net rates ($p > 0.05$) and hence in a significant effect of DMPP ($p < 0.05$). The concentration of NO_3^--N in CZ treatment decreased slightly at t_{24}, resulting in a negative net rate and a significant effect of DMPP ($p < 0.05$). Gross production of NO_3^--N was significantly lower in all the treatments which

received DMPP with slightly lower rates in NZ compared to the CNTR ($p < 0.05$), while no production was recorded in the CZ treatment. Consumption rates of NO_3^--N were, on the other hand, higher in treatments that received DMPP with CZ recording the lowest consumption rate ($p < 0.05$), while the CNTR and NZ were similar ($p > 0.05$).

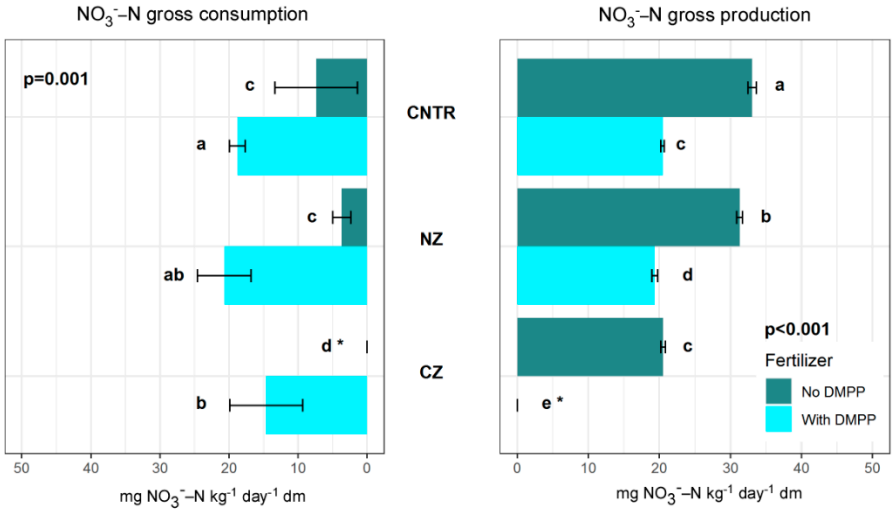

Figure 3. Gross NO_3^--N consumption (on the **left**) and gross NO_3^--N production (on the **right**) rates in treatments with and without DMPP addition. CNTR, NZ, CZ, error bars and letters as in Figure 1. "*" Due to high variability within ^{15}N measurements in CZ treatment, these rates resulted in slightly negative values and are hence assumed as zero. Different letters indicate significant differences between treatments as a result of ANOVA (for gross production), Kruskal-Wallis (for gross consumption) and Tukey (HSD) statistical tests ($p < 0.05$).

3.3. Abundance of Functional Genes (amoA AOA, amoA AOB, nirS, and nosZ)

Results from qPCR (Table 3) showed a higher abundance of *amoA* AOA rather than *amoA* AOB but no significant differences were observed between the treatments ($p > 0.05$). The addition of DMPP did not affect the amount of *amoA* AOA and AOB in the tested treatments ($p > 0.05$). Significant differences were observed in *nirS* and *nosZ* abundance among the treatments: CZ showed a lower abundance of these functional genes compared to the CNTR ($p < 0.05$). The same pattern was observed also in treatments that received DMPP but without any significant effect of the nitrification inhibitor ($p > 0.05$).

Table 3. Copy numbers (CN) of the functional genes for archaeal ammonia oxidation (*amoA AOA*), and bacterial ammonia oxidation (*amoA AOB*), as well as nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*). Values are expressed as mean ± standard deviation (3 replicates). Different superscript letters express significant differences as a result of ANOVA and Tukey-HSD statistical tests ($p < 0.05$).

	Sample	*amoA* (*AOA*) CN $g^{-1} \times 10^{12}$	*amoA* (*AOB*) CN $g^{-1} \times 10^{9}$	*nirS* CN $g^{-1} \times 10^{8}$	*nosZ* CN $g^{-1} \times 10^{8}$
No DMPP	CNTR	10.3 ± 3.19^{a}	2.43 ± 0.47^{a}	5.62 ± 0.41^{a}	8.20 ± 0.87^{a}
	NZ	8.00 ± 1.61^{a}	2.56 ± 0.43^{a}	4.74 ± 1.00^{abc}	6.97 ± 2.85^{a}
	CZ	9.30 ± 2.06^{a}	2.61 ± 0.29^{a}	3.58 ± 0.55^{bc}	3.85 ± 0.90^{b}
With DMPP	CNTR	10.1 ± 1.32^{a}	1.93 ± 0.55^{a}	5.28 ± 0.76^{ab}	6.86 ± 0.58^{a}
	NZ	10.4 ± 1.84^{a}	1.60 ± 0.92^{a}	4.14 ± 0.35^{abc}	5.11 ± 2.03^{ab}
	CZ	7.13 ± 3.18^{a}	2.71 ± 0.19^{a}	2.87 ± 1.04^{c}	3.71 ± 1.19^{b}

3.4. N_2O and CO_2 Emissions

Emissions of N_2O-N and CO_2-C are reported in Table 4. The CNTR and NZ treatments showed a similar magnitude of N_2O-N emissions both with and without the addition of

DMPP ($p > 0.05$), while CZ always showed higher emissions (up to 10-fold higher without DMPP) ($p < 0.05$). The addition of DMPP significantly reduced the N_2O-N emissions in all the treatments ($p < 0.05$).

Table 4. N_2O-N and CO_2-C emission rates throughout incubation. Values are expressed as mean ± standard deviation (4 replicates). Different superscript letters express significant differences as a result of ANOVA (for N_2O-N, ln transformed), Kruskal-Wallis (for CO_2-C), and Tukey-HSD statistical tests ($p < 0.05$). Values in bold highlight a significant effect of DMPP.

	Sample	N_2O-N Emissions μg N_2O-N g^{-1} day^{-1}	*CO_2-C Emissions* μg CO_2-C g^{-1} day^{-1}
No DMPP	CNTR	0.127 ± 0.108^{b}	21.67 ± 19.55^{bc}
	NZ	0.196 ± 0.148^{b}	25.15 ± 6.55^{bc}
	CZ	1.876 ± 0.226^{a}	46.43 ± 4.89^{a}
With DMPP	CNTR	$\mathbf{0.0061 \pm 0.0031^{c}}$	32.62 ± 11.81^{ab}
	NZ	$\mathbf{0.0049 \pm 0.0034^{c}}$	$\mathbf{16.98 \pm 1.55^{c}}$
	CZ	$\mathbf{0.122 \pm 0.068^{b}}$	$\mathbf{28.88 \pm 1.94^{bc}}$

The highest CO_2-C emissions were recorded in CZ treatment without DMPP addition ($p < 0.05$), while the CNTR and NZ showed very similar emission rates ($p > 0.05$). The addition of DMPP had no significant effects on the CNTR, while it led to a significant reduction of CO_2-C emissions in both NZ and CZ treatments ($p < 0.05$).

4. Discussion

From Table 1, it is evident that the EC of the various treatments was closely related to the TN (Pearson coefficient = 0.8672, $p < 0.001$). The high EC shown by CZ_p is a direct consequence of the enrichment process with pig-slurry, which both increased the N content but also left some organic residues on the external surface of the rock, as testified by the higher OC compared to NZ_p (Table 1). NZ_p, being a volcanic tuff in natural state, was characterized by an extremely low EC and by the absence of N. As a consequence, after the addition to soil of CZ_p and NZ_p, the EC and TN of soil-zeolite mixtures were affected, resulting in the following trend: CZ > UA > NZ (Table 1).

4.1. Effects of CHA in Natural State (without DMPP Addition)

Both NH_4^+-N and NO_3^--N concentrations in all treatments were high after the addition of the N fertilizer (Table 2). The NZ treatment showed consistent analogies with the CNTR. Net and gross ammonification rates were very similar (Figures 2 and 3) as well as the amount of *amoA* AOA and *amoA* AOB functional genes (Table 3). In both treatments, the processes associated with the consumption of NH_4^+-N (e.g., volatilization, immobilization, and oxidation) exceeded those responsible for NH_4^+-N production (e.g., mineralization of organic N, DNRA). The observed lack of significant differences in functional genes between CNTR and NZ agrees with the study of [74], which reported no differences in the abundance of functional genes involved in nitrification (*amoA* and *nxrA*) and denitrification (*narG*, *nirK*, *nirS*, and *nosZ*) after natural zeolite addition to sludge composting in aerated reactors.

The consumption of NH_4^+-N was very similar to that of the NO_3^--N production, indicating that the NH_4^+-N consumed over the 24 h of incubation was converted into NO_3^- by nitrification processes (Figures 2 and 3). However, in NZ, the production of NO_3^- was slightly lower than that in the CNTR (Figure 3) (reduction of ~1.8 mg kg^{-1} day^{-1}), suggesting an effect of CHA zeolite on short-term nitrification. This was the only significant effect recorded on NZ treatment, considering that N_2O-N and CO_2-C emissions were also very similar to the CNTR (Table 4). A plausible hypothesis is that given the weak bounds of NH_4^+ with the CHA framework, the microbial biomass was able to access the fraction of N adsorbed by zeolites quite fast, causing a slight delay in short-term NO_3^--N

production rates. Coherently, gross NO_3^--N consumption was also very similar in CNTR and NZ, indicating that processes responsible for NO_3^- removal were unaffected by CHA zeolite application in the short term (Figure 3). This is further supported by comparable N_2O-N emissions as well as *nirS* and *nosZ* functional genes (Figure 4 and Table 3), which are responsible for reducing nitrite and nitrous oxide into NO and N_2, respectively, in the process of denitrification. This finding suggests that the addition of CHA as soil amendment does not significantly affect the abundance of genes from microbes in the short term but instead may have some significant effects on their activity.

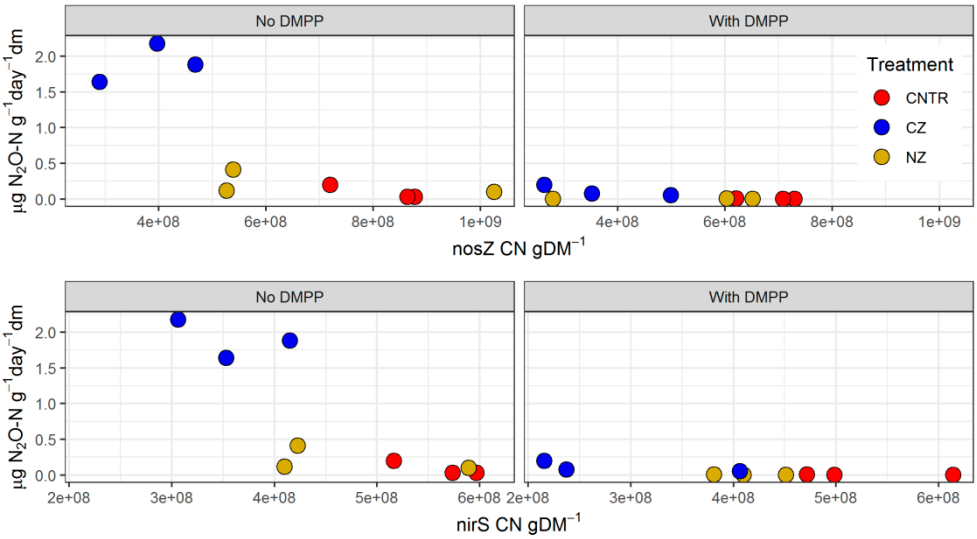

Figure 4. Relationship between *nirS* and *nosZ* functional genes and N_2O emissions in treatments with and without DMPP addition. CNTR (red circles), NZ (dark-yellow circles), CZ (blue circles) like in Figure 1.

It is important to consider that other typologies of zeolites, characterized by stronger binding between NH_4^+ and the framework structure, may show different behaviours and more pronounced effects on nitrification rates.

4.2. Effects of CHA at NH_4^+-Enriched State (without DMPP Addition)

NH_4^+-N and NO_3^--N concentrations were particularly high in CZ because of the additional N input brought by CZ_p (Table 2). Net changes in the pool sizes of both NH_4^+ and NO_3^- were positive throughout the incubation, with a predominance of net production of NH_4^+-N. The predominance of ammonification over nitrification processes in CZ_p is more evident by looking at gross rates (Figure 2). In CZ treatment, NH_4^+-N production was more than three-fold of NO_3^--N production (~75 mg NH_4^+-N kg^{-1} day^{-1} dm compared to the < 25 mg NO_3^--N kg^{-1} day^{-1} dm, respectively). Contrary to the other treatments, in CZ NH_4^+, production exceeded consumption. The possible origin of the NH_4^+ excess is likely related to the increased breakdown of organic N or to Dissimilatory Nitrate Reduction to Ammonium (DNRA) although we did not directly measure these two processes. In particular, DNRA is still a poorly understood process in the N-cycle, which consists of NO_3^- reduction via NO_2^- to NH_4^+ with the last step catalyzed by the cytochrome c nitrite reductase (*NrfA*). DNRA is a process that does not contribute to N removal but instead concurs in retaining N in the soil matrix [75]. DNRA and denitrification are known to occur simultaneously under a wide range of environmental conditions and are generally boosted at WFPS higher than 60%; hence, it is assumed to occur at low soil redox and under high C/NO_3^- ratio. However, this latter assumption has found contradictory evidence in the literature [76]. The addition of a small fraction of labile organic C through CZ_p (Table 1) may have stimulated both processes (hydrolysis of urea and/or DNRA), contributing to the increasing of NH_4^+ levels in this treatment. CZ treatment without DMPP also shows

higher CO_2-C emission rates (Table 4), indicating higher heterotrophic respiration and probably a higher decomposition rate of soil organic matter, which also generates NH_4^+. The higher heterotrophic respiration observed for CZ may reduce oxygen availability in air-filled pore space and hence conditions favourable for DNRA. Further investigation is required to better identify the mechanisms of gross NH_4^+ production in CZ amended soils.

Another relevant aspect observed in CZ involves the amount of N_2O-N emitted over the incubation and the amounts of *nirS* and *nosZ* functional genes (Figure 4, Tables 3 and 4). In this treatment, the emissions of N_2O-N were huge, ten-fold higher than that recorded in the CNTR. Archaeal and bacterial *amoA* were not significantly different from the CNTR, while a significant reduction in the abundance of *nosZ* and *nirS* was recorded (Tables 3 and 4). It is known that the potent GHG N_2O-N is a byproduct of both nitrification and denitrification reactions [77]. High N_2O emissions, thus, indicate normally high rates of denitrification or nitrification. In our experiment, this increase in N_2O is not attributable to enhanced nitrification processes since the gross production of NO_3^- was lower than the rates accounted for in the CNTR. Significantly lower copy numbers of *nirS* and *nosZ* in CZ compared to CNTR provides additional support as more N is lost in the form of N_2O by partial denitrification rather than full denitrification (Figure 4). Within the many environmental constraints, WFPS, substrate NO_3^--N concentrations, and the soil texture are known to play an important role in the fraction of N_2 over N_2O emitted by denitrification (N_2/N_2O ratio). Lower N_2/N_2O ratios generally occur at lower WFPS, high substrate NO_3^--N concentrations, and high clay content [76,78]. This agrees perfectly with the conditions of CZ treatment during the experiment and helps to explain the observed shift toward emissions of N_2O from partial denitrification in CZ.

Unexpectedly, the higher N_2O-N emissions in the CZ treatment are not according to NO_3^--N consumption, and, unfortunately, in this case, the experimental data do not allow a mechanistic understanding of the origin of the accelerated N_2O-N emissions. However, a previous incubation experiment in which the same soil amended with the same CZ_p were tested over 16 days of incubation can provide some clues for the observed high emissions [10]. The authors found an immediate accumulation of NO_2^--N in the soil during the first days of incubation before a drastic increase in NO_3^--N net production. This NO_2^- accumulation was explained as a consequence of the high NH_4^+ level of the substrate (induced by CZ_p), which is known to inhibit NO_2^- oxidation to NO_3^- [45,79–81]. Likely, the same NO_2^- accumulation may have occurred also in this experiment since the experimental conditions were comparable; however, NO_2^- was not measured. The NH_4^+ inhibition of NO_2^- oxidation to NO_3^- could have caused the relatively low gross nitrification rates encountered (both in terms of gross production and consumption). Other evidence in the literature support this hypothesis, for example, in another incubation experiment performed by [22], CZ_p addition to soil induced relatively higher NH_3 and NO_x emissions. NH_3 is the substrate of the 1st step of nitrification reaction where it is converted into first hydroxylamine and then into NO_2^-. Higher NO_x emissions measured by [22] are also supportive of the observed higher N_2O emissions in the present experiment. This suggests that when O_2 levels were relatively low (WFPS of 65%) and NH_4^+ levels were very high, the evolved N_2O may have been generated by nitrifier-denitrification directly from the NO_2^- [82]. Nitrifier denitrification is a pathway of nitrification, where the oxidation of NH_3 to NO_2^- is then followed by its reduction to N_2O (and then likely to N_2) carried out by only one group of microorganisms (autotrophic NH_3-oxidizers) [82].

4.3. DMPP Effects

In the present study, the addition of DMPP had significant effects both in terms of gross ammonification and nitrification. DMPP addition significantly reduced both NH_4^+ consumption and NO_3^- production in the CNTR and NZ treatments, suggesting that nitrification rates were reduced as expected, while NH_4^+ production was unaffected. Lower ammonium consumption and nitrate production rates favour the preservation of the NH_4^+ pool over time and hence the mitigation of N leaching and gaseous losses.

The slightly lower nitrate production rates in NZ (compared to the CNTR) were maintained even after the addition of the nitrification inhibitor (Figure 3). The differences between these two treatments were, however, exiguous (even if statistically significant) and in summary, nitrate production was reduced by ~ 38% after the addition of DMPP in both treatments. As the sorption capacity of DMPP in this soil was already established to be slightly reduced by CHA zeolite application (especially NZ_p) [58], the inhibition efficiency of DMPP was expected to be possibly even increased after CHA application. If the sorption of DMPP is reduced, it is reasonable to hypothesize a higher and faster inhibitory effect on zeolite amended soils. However, the results obtained in this short-term study highlighted no significant differences in DMPP efficiency compared to the CNTR. This indicates good compatibility of DMPP with CHA zeolites, as the inhibition efficiency was not altered.

The authors of [55] tested DMPP in soils with similar properties and at similar WFPS, but they observed a lower efficiency in reducing gross nitrification (from 9.4 to 21.6%). However, other studies reported highly variable DMPP efficiencies, with reductions in net nitrification rates up to more than 90% in arable soils with pH ranging from ~ 5 to ~ 8 during the first days after DMPP addition [52]. Comparison of DMPP performance with other studies in the literature is hardly possible due to the large number of parameters influencing nitrification and potential inhibition, such as soil texture, pH, organic C [83], WFPS, nutrient levels, and incubation time.

No significant effects of DMPP addition on the abundance of the investigated functional genes were observed (Table 3). This evidence supports the fact that in the present study DMPP had no detrimental effects on the abundance of soil microbes but only affected their activity. This finding is in agreement with a recent study by [84], where short-term effects on soil N-cycling genes and transcripts after the addition of nitrification plus urease inhibitor were evaluated. The authors did not observe major effects on the abundance or activity of target and non-target N-cycling microbial groups, despite reducing N_2O emissions; most of the observed inhibition effects on genes and transcripts were only transient.

In our study, DMPP significantly affected N_2O emissions as already observed by other authors in the literature (Table 4) [38,53,55,85]. The mitigation effect on this potent GHG is suggested to function in two ways, one is direct, by the inhibition of nitrification which generates N_2O as a by-product, while reduced substrate (NO_3^-) availability for denitrification can also indirectly lower N_2O emissions. In this study, a reduction of N_2O emissions by more than 90% in the DMPP treated soils was observed. The authors of [86] measured similar reductions (99.2%) in N_2O emissions but as for nitrification rates, the reported efficacy of DMPP in reducing N_2O emissions is very broad and dependent on specific conditions of each case study. Additionally, as the source of N_2O cannot be finally resolved (i.e., nitrification, denitrification, nitrifier denitrification, etc.), the reduction/inhibition efficiency on N_2O is even harder to compare in the literature.

DMPP behaved differently in the CZ treatment compared to CNTR and NZ (Figures 2 and 3, Tables 2–4). In this treatment, when DMPP was added, gross production of NH_4^+ was further stimulated, while NO_3^- gross production was completely inhibited in the first 24 h (reduction of 100% vs the same treatment without DMPP), but a fraction of NO_3^- was still being consumed.

If emissions of N_2O-N of each treatment are based on the total amount of N added (Table 5), it emerges that in CZ, notwithstanding the strong N surplus, DMPP reduced the emissions of N_2O-N by 72% compared to the fertilized control without DMPP. Even though the percentage for reduction of N_2O-N emission with DMPP was higher for CNTR and NZ (with 95, and 96% respectively), the highest total amount of N_2O-N was inhibited in the CZ treatment (1.754 μg N). The absolute efficiency in inhibiting nitrification and N_2O emissions in CZ treatment agrees with other studies in which it was established that DMPP performance is generally higher in treatments that receive organic fertilizers (i.e., the best performance for DMPP has been found to occur when applied in combination with ammonium sulphate or organic fertilizers) and at high application rates [85]. For CZ, both conditions were met, since CZ_p amendment contains additional N originating from

animal slurry (approximately ~427 kg N ha^{-1} organic fertilizer) and thus increased the total N input compared to the other treatments that received only chemical NH_4NO_3.

Table 5. Amount of N_2O-N emitted per unit of N added in each treatment, the entity of reduction operated by DMPP addition compared to the fertilized control, and amount of N_2O-N inhibited in each treatment. The N_2O-N emissions rate used for calculations are shown in Table 4. Different letters express significant differences as a result of ANOVA and Tukey-HSD statistical tests ($p < 0.05$). ¥ Mean values were used for calculations.

	Treatment	N Input NH_4NO_3 kg N ha^{-1}	N Input CZ_p kg N ha^{-1}	N_2O-N/N Added µg g^{-1} day^{-1} (×100)	¥ DMPP Reduction vs. CNTR Fertilized	¥ µg N_2O-N Inhibited by DMPP
No DMPP	CNTR	170	0	0.075 ± 0.064 ^{bc}	-	-
	NZ	170	0	0.115 ± 0.087 ^{b}	-	-
	CZ	170	427	0.314 ± 0.043 ^{a}	-	-
with DMPP	CNTR	170	0	0.004 ± 0.002 ^{d}	95%	0.121
	NZ	170	0	0.003 ± 0.002 ^{d}	96%	0.191
	CZ	170	427	0.020 ± 0.011 ^{c}	72%	1.754

DMPP addition also had a significant effect on CO_2 emissions in both NZ and CZ treatments, reducing the fluxes significantly (Table 4), while no significant effects were found in the CNTR. Significant short-term effects of CHA zeolites on soil CO_2 emissions were already encountered in a previous incubation experiment when zeolites were applied in combination with urea fertilizer (CO_2 emissions reduced by ~21% to approximately ~30%) [22]. To understand the mechanisms behind this CO_2 emission reduction in zeolite amended soil, further focused experiments are required. The effects of DMPP on soil CO_2 emissions and, hence, on C-mineralization and heterotrophic respiration are poorly investigated and contrasting evidence is found in the literature. For example, the authors of [38] found no significant DMPP effect of soil CO_2 emission in a lab-incubation experiment performed on loam and clay soils with different labile C availability, while the authors of [54] measured an average reduction of 28% in a three-year field experiment, suggesting significant effects of DMPP on C-mineralization.

NO_3^- consumption and the enhanced NH_4^+ production coupled with the total inhibition of NO_3^- production may suggest the possibility of an enhanced breakdown of organic N into NH_4^+ or DNRA. In addition, the significantly lower abundance of *nosZ* and *nirS* genes even after DMPP addition in CZ treatment compared to the CNTR or NZ treatments (Table 3) suggest lower denitrification, and hence NO_3^- consumption might be driven by DNRA, as the little fraction of labile OC added with CZ_p could have stimulated respiration and hence the reduced soil redox potential promotes a shift to NO_3^- consumption via DNRA [76]. Although the effects of DMPP on DNRA rates in soils is a nearly unknown subject, in the very recent work of [38], the authors found that DNRA rates increased by a factor > 5 when DMPP was applied to a sandy horticultural soil but had no significant effects on a loamy pasture soil. The authors suggest that the increased labile C availability promoted NO_3^- reduction through DNRA. This evidence further supports our hypothesis that DNRA was probably an important process occurring in CZ treatment after DMPP addition.

5. Conclusions

The outcomes of this study represent a starting point for a mechanistic understanding of the effects of natural and NH_4-enriched CHA-zeolites (used in the form of zeolite tuff) on the short-term soil N cycle, also in combination with DMPP as a nitrification inhibitor. A visual summary of the obtained results is represented in Figure 5. This study highlighted that CHA-zeolite in natural state did not alter considerably short-term gross ammonification but instead slightly reduced nitrate production rates under these specific experimental conditions. However, if CHA zeolite-tuffs are applied at the field-scale, even a short delay

of the nitrification process can be of great agronomic and environmental interest. The addition of NH_4-enriched zeolites improved ammonification but not gross nitrification in the short-term period, indicating that probably other non-targeted N processes were stimulated (i.e., nitrifier-denitrification). The addition of CHA in natural state has not altered the performance of DMPP, while the presence of NH_4^+-charged CHA led to a stronger inhibition effect of DMPP and potentially stimulated other non-targeted N processes such as DNRA. These different effects on the N cycle observed in the soil amended with NH_4^+-charged zeolites are thus not attributable to a zeolite-effect but more likely to the additional N (and C) source introduced with this amendment. We thus suggest taking into consideration the use of DMPP or other nitrification inhibitors when using NH_4^+-charged zeolites as a soil amendment or fertilizer. Further experiments are required for enlarging the dataset to also evaluate the effects of other typologies of zeolites (e.g., clinoptilolite), other soil textures (e.g., sandy soils), longer incubation times, and other important variables such as temperature, moisture, and presence of crops. Given the results obtained in this experiment, particular emphasis should be dedicated in future studies to unveiling the effects of natural and NH_4-enriched zeolite on long-term soil gross N rates.

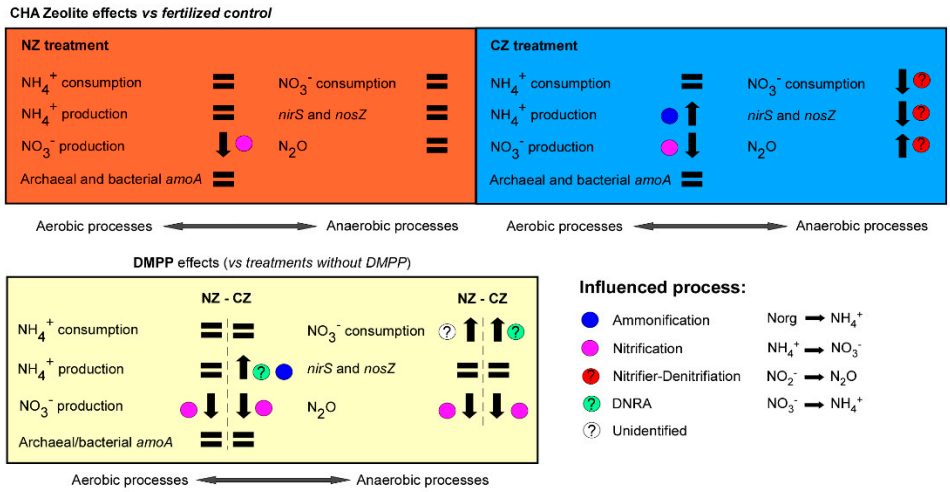

Figure 5. Summary of the main results. "=" symbols indicate no observed variations, arrows indicate an increase or decrease in the parameter. Question mark next to processes depicts a potential pathway that is supported by the data, but not directly measured, and hence the necessity to acquire more data. NZ treatment is the soil amended with 10% of CHA zeolite-tuff in natural state, while CZ is soil amended with 10% CHA zeolite-tuff enriched with NH_4^+.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/6/2605/s1. Table S1: Primers used for quantitative PCR. Table S2: Quantitative PCR thermal profiles for the different target genes.

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