Ammonium-charged zeolitite effects on crop growth and nutrient leaching: greenhouse experiments on maize (*Zea mays*)

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ABSTRACT

Nitrate leaching and the resulting groundwater contamination from intensive crop production has become a major concern for long-term farmland efficiency and environmental sustainability in Italy. The aim of this study was to evaluate a water-saving NH_4-charged zeolitite (produced by new design prototype) for minimizing NO_3–leaching from soil and optimising corn growth and yield. Forty-eight zeolitite:soil lysimeters for two trials were installed in a greenhouse to study the growth and yield characteristics of maize (*Zea mays*) as well as the nitrate leaching under different fertilizing conditions (i.e., standard, high or 70%, medium or 50% and low or 30% of conventional fertilization rate) and NH_4-charged zeolitite (control, 0; dose-1, 50 t ha^{-1} and dose-2, 100 t ha^{-1}) treatments. The results implicitly suggest that plants may have a better response if NH_4-charged zeolitite is used with a reduced amount of conventional fertilizer, allowing a reduction of nitrate leaching.

KEYWORDS

zeolitite, nitrate leaching, maize growth, crop, ammonium, fertilizer

INTRODUCTION

Agriculture remains one of the main sources of water pollution, and farmers need to adopt more sustainable practices, as huge efforts are still required in order to restore optimal water quality across the European Union (EU) and abroad (Bijay-Singh et al., 1995; Thorburn et al., 2003; Jalali, 2005; Islam et al., 2011). Generally, farming is responsible for the major N-compound discharges into surface waters and groundwater, and still nowadays farming practices in all Europe use a large amount of chemical fertilizers and animal manure, with large regional differences (Velthof et al., 2014). Of the total nitrogen input in the fields, in fact, a large amount is not absorbed by the crops and resides in the soil (Mastrocicco et al., 2013; Wang et al., 2013a; Sebilo et al., 2013), where it gets transformed into highly soluble nitrates and is flushed away into the water system (Mastrocicco et al., 2009; Arbat et al., 2012; Aschonitis et al., 2012; Wick et al., 2012; Wang et al., 2013b), triggering different degenerative processes and ultimately causing eutrophication phenomena (Del Amo et al., 1997; De Wit et al., 2005; Statham, 2012). Moreover, when denitrification occur in soils (Rivett et al., 2008), greenhouse gases are released into the atmosphere (Smith et al., 2007; Benbi, 2013; Ding et al. 2013; Skinner et al., 2014). Livestock effluents, whose NH_4 concentration could exceed 1000 mg l^{-1}, are also often used as fertilizers as they can also improve soil fertility for crop production (Marinari et al., 2000; Khan et al., 2007); it is known that intensive livestock breeding is another biggest sources of nitrogen pollution in water (Goldberg, 1989; Williams, 1995; Widory et al., 2004) and it heavily contributes to CO_2 and methane emissions worldwide (FAO, 2006). With the Nitrates Directive (Council Directive 91/676/EEC) and the Water Framework Directive (WDF 2000/60/EC) the EU aims at preventing nitrate pollution by promoting the use of good farming practices and established a protocol for protection and management of water, reporting measures that must be taken by each Member State, to favor the restoration of hydrologic resources and reach a good chemical and ecological state of waters, by reducing dumping and toxic substance emissions.

Several previous investigations (Lehmann et al., 2003; Novak et al., 2009; Ding et al., 2010; Islam et al., 2011; Nelson et al., 2011; Sarkhot et al., 2012; Hale et al., 2013) focusing on mixtures of soil and artificial high-CEC fertilizes (i.e. biochars or coating materials) have shown that when they are added to soil they can reduce the leaching of NO_3–N and NH_4–N, which therefore implies that these nutrients are bound to them, and no further transformation reactions take place. For example, applying 20 g kg^{-1} biochar to an agricultural soil amended with swine manure decreased the leaching of NO_3–N and PO_4–P by 11% and 69% respectively (Laird et al., 2010). However, it is currently unclear how long-lasting these effects are (Hale et al., 2013) and if some of them could be toxic to soil (Azeem et al., 2014).

Zeolitites are rocks containing more than 50% of zeolites (Galli & Passaglia, 2011), a kind of minerals with peculiar physical and chemical properties, like high and selective cation exchange capacity (CEC), molecular absorption and reversible dehydration (Bish & Ming, 2001). Natural zeolites have a remarkable selectivity for cations characterized by low ionic potential (i.e., NH_4^+, K^+, Pb^{2+}, and Ba^{2+}) and, in particular, are capable to uptake NH_4^+ from solutions in various environment and to release it under proper conditions (Ahmed et al., 2006; Passaglia & Laurora, 2013), such as slow release fertilizer (SRF). Moreover, a single application to the soil can meet nutrient requirements for model crop growth and increases soil properties for several growth seasons, producing long-term changes in physical properties.

In this context, ZeoLIFE project (LIFE+10 ENV/IT/000321; Coltorti et al., 2012) has been conceived to test an innovative integrated zeolitite cycle aiming at reducing the amount of traditional (both chemical and organic) fertilizers and correcting agricultural soils, with improvement of the yield and economization of fertilizers and water for irrigation, ultimately leading to a reduction of fresh and groundwater pollution and excessive exploitation of the water resource. This study describes the selection of zeolitite/soil ratio to be applied in Maize (*Zea mays*) cultivation through a series of greenhouse experiments, as an ex-situ trial to be subsequently reproduced at large scale in an agricultural field. The NH_4-charged zeolitite (NH4CZ, hereafter), produced by prototype (IT application MO2013A000354) OK, HO CORRETTO SOLO UN ERRORE NELLA REF was mixed to ZeoLIFE experimental field agricultural soil (Codigoro, Ferrara, Italy; Di Giuseppe et al., 2013 and 2014) and to an artificial standard soil in two trials respectively, and in different ratios, in order to reduce NO_3^- leaching in groundwater and to optimize Maize production in comparison to traditional practice (chemical fertilizer addition).

MATERIALS AND METHODS

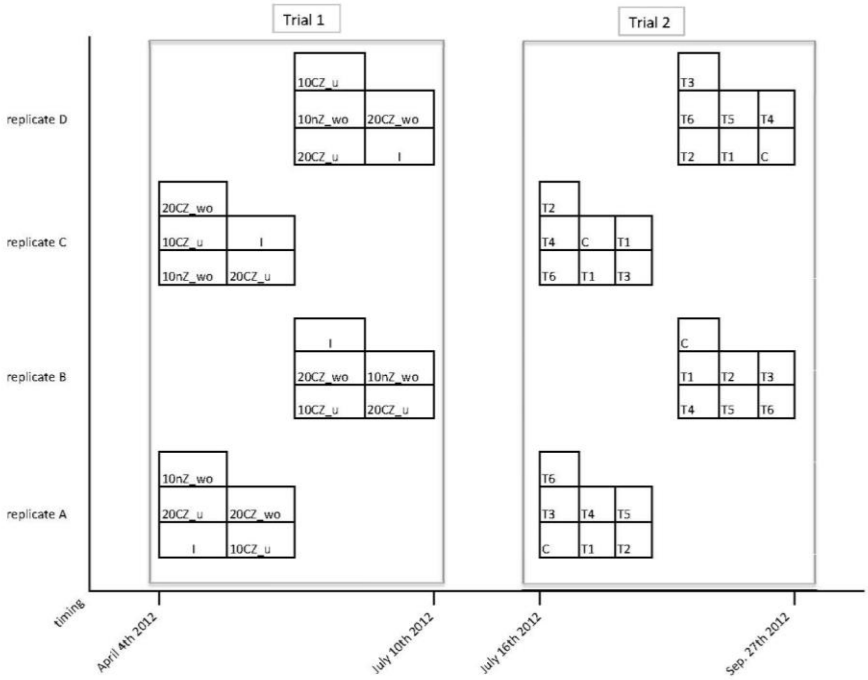
NH_4-charged zeolitite

The natural zeolitite used in this study comes from Sorano (Grosseto, Central Italy); chemical and mineralogical composition and physical/chemical properties of natural zeolitite are reported in Malferrari et al. (2013). To obtain NH4CZ, NH_4 exchange experiments between natural zeolitite (fraction with particle size less than 3.0 mm) and swine manure were carried out in static mode (Vassileva and Voikova, 2009) in laboratory, and the findings were paralleled in large-scale application in a prototype (Coltorti et al., 2012) located in Codigoro (Ferrara, Italy) near the experimental field arranged for ZeoLIFE project (Coltorti et al., 2012; Malferrari et al., 2013). Briefly, the prototype (supplementary information SI-1) is composed by a 2.2 m (ø) x 5.3 m (h) tank for the swine manure storage (about $10m^3$). The loading of swine manure is performed using a pump that takes manure directly from the manure pool; 250 kg of natural zeolitite are introduced from the top into the vessel and mechanically stirred with swine manure for 45 minutes. After a resting time (8-20 hours), NH4CZ is discharged and recovered opening the ball valve at the bottom of the tank. A vibrating sieving system may be, optionally, inserted at the bottom of the vessel to separate the different particle size of NH4CZ, with a total daily production of 500 kg. At the end of each production cycle, NH4CZ was stored, air dried in controlled open-air conditions and then periodically characterized (supplementary information SI-2A).

Greenhouse experiments

This study was conducted at CRSA Med Ingegneria facilities, north east of Italy (WGS84: 44°28’50’’N 12°16’21’’E), in a 60 m3 greenhouse (3.3 m x 9 m x h 2 m, Figure 1A) in 2012 (spring and summer).
Maize seeds have been sowed in lysimeters (Figure 1A) measuring 24cm in diameter and 30cm in depth, with a stone layer and a drain pipe at the bottom, for water samples collection. The soil used in the experimental trials was collected in ZeoLIFE experimental field and sieved at 2 mm; it is a silty clay soil with 41.9, 38.9, and 19.2% of silt, clay and sand,

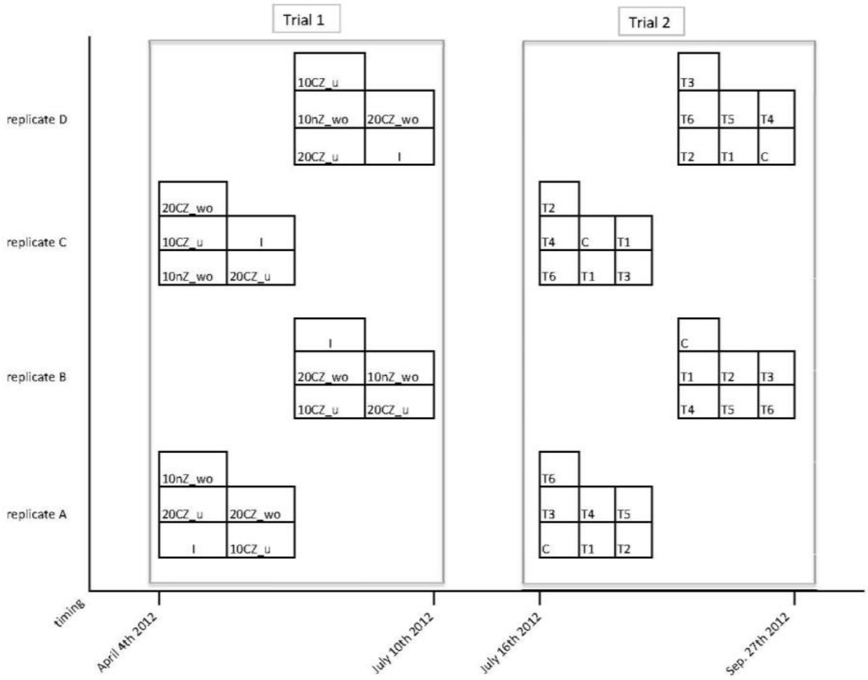
respectively (Di Giuseppe et al., 2014). Main characteristics of the soil at the beginning of the study are listed in Table 1, and are consistent with the typical composition of an agricultural soil in Ferrara district, with a medium-high nutrient content (ARPAV, 2007).



A)

Figure 1 (A) Overview of the greenhouse and lysimeters. (B) Diagram of the two trials and timing. Treatment codes are explained in Table 2 and Table 3.

B)



C)

Figure 1 (A) Overview of the greenhouse and lysimeters. (B) Diagram of the two trials and timing. Treatment codes are explained in Table 2 and Table 3.

Property	u.m	Bulk soil
Cation Exchange Capacity (CEC)	meq 100 g^{-1}	33.6
Exchangeable Ca^{2+}	mg kg^{-1}	5660
Exchangeable K^{+}	mg kg^{-1}	582
Exchangeable K (as K2O)	mg kg^{-1}	701
Exchangeable Mg^{2+}	mg kg^{-1}	401
Exchangeable Na^{+}	mg kg^{-1}	368
Total Nitrogen	mg kg^{-1}	2.7 -17.7
Soluble K	mg kg^{-1}	76.5
Soluble P (as P2O5)	mg kg^{-1}	175.3
Soluble Iron	mg kg^{-1}	62.4
Soluble Mg	mg kg^{-1}	6.2
Soluble Zn	mg kg^{-1}	1.9
Soluble B	mg kg^{-1}	1.61
Copper	mg kg^{-1}	42.8

Table 1 Main chemical and physical characteristics of the soil employed in the experiment (Di Giuseppe et al., 2014).

Two sets of experiments were performed with a randomized complete block experimental design using a complete factorial arrangement of treatments (Figure 1B). The treatments consisted of (i) two soil amendment types with NH$_4$-charged (NH4CZ) and natural (nZ) zeolitite, (ii) two soil amendment doses of 10 g kg^{-1} (dose-1) and 20 g kg^{-1} (dose-2), and (iii) different reduction of chemical fertilizer. The soil amendment doses were selected on the basis of the

literature (Ming & Allen, 2001, Leggo et al., 2006, Malekian et al., 2011), and the cost-effective of the treatment (Islam et al., 2011). Each treatment was performed in quadruplicate and four not amended soil lysimeters were used as a control. The soil amendments were broadcast applied to the soil depth of the 7L lysimeters and incorporated to the total depth prior to the planting of crops. In this study, maize was selected over other crops in view of its rapid growth cycle, responsiveness to changes in nutrient availability, and represents a typical crop in the farming system of the Region (also related to animal feeding). Three seeds of maize Cisko Class 300 were planted 4 cm deep in each lysimeter and at 26 days after sowing (DAS), maize in each lysimeter was thinned to two plants. The lysimeters were surface irrigated and scheduled with 2-day intervals and, during each irrigation event, 15% more water was applied to allow water drainage for sampling. In this study, the irrigation was performed in the same way in all the treatments, in order to avoid this limiting factor. The nitrogen source, applied once at the beginning of the tests, was urea (46% N). The reductions of urea respect to each trial control (6 and 11% in the first trial, and 30, 50 and 70% in the second trial) were established considering that the average nitrogen content in NH4CZ of 7.8 mg N g^{-1} (supplementary information SI-2B).
The aim of first trial was to find out the best zeolitite/soil ratio to be applied in the open field experiments, whereas the second trial was mainly devoted to select the best fertilization reduction after zeolitite addition.
In the first trial (Table 2), 5 treatments per 4 replicates (20 lysimeters) were conducted for 89 days of experiment. Simulating a high nitrogen fertilization of full field for corn (about 370 kg N ha^{-1}) and a depth of distribution of the fertilizer along the soil profile in lysimeters (25 cm), 248 mg kg^{-1} urea have been added to the soil for traditional farming practice, then applying a reduction of 6 and 11% according to the different treatments. In particular, for two treatments (10CZ_u and 20CZ_u) urea was added compensating for the amount of nitrogen absorbed as ammonium in NH4CZ by the prototype process (Coltorti et al., 2012; supplementary information SI-3). The addition of nZ and NH4CZ were calculated on the basis of the dry weight and the depth of plowing. More in detail, assuming a depth of homogeneous distribution of zeolitite along the soil profile equal to 40 cm (depth of plowing), dose-1 (10 g kg^{-1}) and dose-2 (20 g kg^{-1}) correspond to 5 kg m^{-2} (or 50 t ha^{-1}) and of 10 kg m^{-2} (or 100 t ha^{-1}) of zeolitite in the field, respectively. In order to evaluate the best approach and select the optimum zeolitite addition, the treatments were:

- Intensive (I): traditional farming practice with 370 kg N ha^{-1} (positive control)
- 10CZ_u: dose-1 of fine NH4-charged zeolitite, with reduction of Urea-N
- 20CZ_u: dose-2 of fine NH4-charged zeolitite, with reduction of Urea-N
- 20CZ_wo: dose-2 of fine NH4-charged zeolitite, without nitrogen addiction
- 20nZ_wo: dose-2 of fine natural zeolitite (nZ), without nitrogen addiction (negative control)

Table 2 Treatments description of the first trial in spring 2012.

	Treatments				
Type	Intensive(I)	10CZ_u	20CZ_u	20CZ_wo	20nZ_wo
Bulk soil*	TdC	TdC	TdC	TdC	TdC
NH4CZ (g kg^{-1})	0	10	20	20	0
Natural zeolitite (g kg^{-1})	0	0	0	0	20
Urea addiction (%)	100	94.4	88.9	0.0	0.0

*TdC: Codigoro soil, collected, air-dried and 2mm-sieve. Before use zeolitite (both natural and treated) was air-dried and its moisture was checked in oven at 105°C for 48h.

The second trial (Table 3) was conducted using an artificial soil, except for one treatment performed with the already used zeolitite/Codigoro soil, coming from the first trial, for simulating the second year of production. The artificial soil (Std) was composed by 1:1 Po river sand and peat of northern European origin (46% organic carbon, 0.7 % organic nitrogen, pH 4). This trial was carried out with 7 treatments per 4 replicates (total of 28 lysimeters), lasting 73 days.
In order to simulate a full range of nitrogen fertilization on maize compatible with the Nitrates Action Program of Emilia Romagna Region (NAP, 2011), 240 kg ha^{-1} of nitrogen (equivalent to about 522 kg ha^{-1} of urea) were provided as the Maximum Allowable Concentration (MAC).

The following treatments were chosen in order to evaluate the best approach and, thus, select the best nitrogen addition:

- Control (C): traditional farming practice (positive control)
- T1: dose-1 of fine NH4CZ with low reduction of Urea-N
- T2: dose-1 of fine NH4CZ with medium reduction of Urea-N
- T3: dose-1 of fine NH4CZ and ultrafine (<90µm) NH4CZ, with high reduction of Urea-N
- T4: dose-1 of fine NH4CZ with low reduction of Urea-N
- T5: dose-1 of fine NH4CZ, residual from first trial with the residual Codigoro soil, and medium reduction of Nitrogen addiction (long-term test).
- T6: minimum dose of fine NH4CZ with minimal Urea-N addiction (limit-complying test)

The treatment T1, T2 and T3, with the same content of zeolitite (10 g kg^{-1}), were supplied with a reduction of 30, 50 and 70% Urea-N compared to the Control. In particular, in T3, the zeolitite addiction was performed adding 80% of the zeolitite in coarse “fine” form (<3.0 mm), like in the other treatments, and 20% of an "ultra fine" form, obtained operating an additional sieving at <90µm using the *in-situ* sieving apparatus. This fraction has a greater specific surface area and a higher content of both ammonium and phosphorus than the coarser fraction (supplementary information SI-2B).

In the treatment T5 the soil of Codigoro was reused, sowing again the soil of the treatment 10CZ_u of the first trial, in order to evaluate possible effects of residual nitrogen. Moreover, this test was performed in order to assess the long-term effects of the use of zeolitite; in particular we want to check if the zeolitite, once the absorbed ammonium was consumed by the first crop cycle, could be recharged through the addition of chemical fertilizers to the soil.

Treatment T6 provided a minimum amount of zeolitite (6 instead of 10 g kg^{-1}) and a minimal Urea-N addiction in order to match altogether 240 kg N ha^{-1}, as MAC used in Control. The amount of NH4CZ was calculated considering its N content and a urea-like behavior, in order to comply with regulation for fertilizer distribution.

Table 3 Treatment description of the second trial in summer 2012.

	Treatments						
Type	Control (C)	T1	T2	T3	T4	T5	T6
Bulk soil*	Std	Std	Std	Std	Std	TdC	Std
NH4CZ (g kg^{-1})	0	10	10	10**	0	10***	6
Natural zeolitite (g kg^{-1})	0	0	0	0	10	0	0
Urea addiction (%)	100	70	50	30	70	50	3

* Std; artificial standard soil; TdC: Codigoro soil; ** 80% fine NH4CZ and 20% ultra fine (<90µm) NH4CZ, collected in prototype; *** residual NH4-charged zeolitite from first trial (treatment 10CZ_u)

Data collection

The leached solution from each lysimeter was collected every 20 days in order to assess the nitrogen leaching in terms of NH_4-N and NO_3-N concentration. The two trials were stopped at 97 and 73 DAS, before the influence of lysimeter volume on roots elongation. During the growth monitoring, measurements of the aerial biomass (height in cm from the base of the plant to the top of the upper leaf) were performed approximately every 20 days. At the end of each trial, all the plants were collected from each lysimeter, oven dried at 70°C until constant weight was attained, in order to assess the production in term of aerial biomass (dry weight).

Moreover, at the end of second trial (day 73), the photosynthetic activity (PN) and leaf chlorophyll content (soil-plant-analysis development, SPAD) were measured with an ADC-LCPro+ instrument (for determination of CO_2 per leaf area and time unit) and a portable SPAD meter (Model SPAD-502, Minolta crop, Ramsey, NJ), respectively. The SPAD meter measures the transmission of red light at 650 nm, at which chlorophyll absorbs light, and transmission of infrared light at 940 nm, at which no absorption occurs. On the basis of these two transmission values, the instrument calculates a SPAD value that is well correlated with chlorophyll content and used as an indirect indicator of crop N status. Joined to the evaluation of the aerial biomass, a quantitative and qualitative morphological study (relative growth rate, density/appearance of the root) was conducted.

Then, several macronutrients in the corn leaves of the second trial were measured according to international standards (ISO 5378 for N determination; EPA 3051A and ISO 11885 for other macronutrients determination). Briefly, after oven drying at 70°C for 24h and homogenizing, the leaf samples were assayed for total N (Kjeldahl method, modified as described in Cataldo et al., 1974), and after microwave-assisted mineralization (MLS 1200 Mega, Milestone), for P, S, Ca, Mg, K and Na (by inductively coupled plasma mass spectrometry, Thermofischer). In particular, leaf N-content is an important physiological parameter that indicates the plant N status (Lemaire et al, 2008).

Data analysis

Treatment significant differences were calculated at Fisher's leastsignificant difference (LSD) at p-level b 0.05 in one-way ANOVA (SAS, 2008). Duncan's multiple range tests (DMRT) was performed for multiple significance between the treatments.

EXPERIMENTAL RESULTS

First greenhouse trial

Nitrogen concentration in leachate

Results of the first trial are reported in Table 4. The initial concentration of NO_3-N in the leachate was strictly related to urea addiction, and quickly reduced in all treatments after seed germination (at 36 DAS). Moreover, the treatment with natural zeolitite and no urea-N addiction (10nZ_wo) showed a residual N content, probably deriving from previous agricultural practices on the agricultural soil used in the trial (Table 1). In this study, the phenomenon reported by Ahmed et al. (2006) where the inclusion of 1g kg-1 zeolitite have improved the soil retention of NH_4 as well as minimizing the conversion of NH_4 to NO_3 was not observed, probably due to the two different urea additions (2 g kg^{-1} in Ahmed et al. (2006) and about 0.2 g kg^{-1} in this study).

		NO_3-N (mg L^{-1})					NH_4-N (mg L^{-1})				
Treatment	DAS	23	36	54	72	89	23	36	54	72	89
Intensive (I)		90.9	83.5	45.0	4.2	1.3	2.95	0.27	0.15	0.06	0.15
10CZ_u		124.0	93.7	20.7	9.8	4.2	2.32	0.21	0.08	0.06	0.16
20CZ_u		95.9	153.3	17.7	7.6	2.2	0.54	0.23	0.07	0.04	0.15
20CZ_wo		84.1	50.9	15.6	5.4	1.6	2.20	0.15	0.07	0.04	0.12
10nZ_wo		65.3	40.3	24.9	10.1	1.3	0.17	0.17	0.07	0.04	0.14

Table 4. First trial: Trend of NO_3-N and NH_4-N content in leachate, for four treatments and control. Mean of four replicates was reported for 5 sampling times (every 15-20 days). A high variability in measurements was observed with a coefficient of variation (CV%) ranging from 11% to 57% and from 8% to 50 % for NO_3-N and NH_4-N, respectively. No significant differences were observed in NH_4-N and NO_3-N trends between treatments and control.

The maximum allowable concentration (MAC) for drinking water in Italy (Legislative Decree 31/01) and for International guideline (WHO, 1993) is 50.0 mg l^{-1} NO_3^-, corresponding to 11.3 mg l^{-1} NO_3-N. The nitrates content in all treatments was higher than MAC, with a decreasing trend in 89 DAS for all treatments, without significant differences, complying with the regulation limit in 72 DAS. It is important to notice that, as occurred for NH_4-N, in the treatment 10nZ_wo, where no urea addiction was performed, the nitrates were still present, and sometimes, over the regulation limit (from 65 mg l^{-1} to 1.3 mg l^{-1}). This could confirm the hypothesis an effect of residual N fertilization of earlier crop years, due to the source of the test soil, and suggesting an incomplete consumption of N by the crops. This residual N could allow the maize growth (1.22 cm day^{-1}) although lower than in the other treatments (up to 1.34 cm day^{-1}).

Biomass production.

At 97 DAS, the crop production was estimated collecting the emerging biomass (plant) and the root, which were weighted before and after drying (Figure 2).

For the production of aerial biomass (dry weight) measured at end of cycle, only the treatment 10nZ_wo had a production lower than the other treatments (p-level: 0.019). Even if the treatment 20CZ_wo presented a reduced production, it was not significantly different from the control (I) and the other treatments with NH4CZ (10CZ_u, 20CZ_u). In other words, the fertilization regimes containing either NH4CZ or organic fertilizer did not produce significant differences in plant biomass with respect to the conventional fertilizer. However, the integrated fertilization regimes (with urea addiction) produced differences in the plants, as the biomass of plants grown with integrated organic fertilizer (20CZ_u) was greater than this one grown with only NH4CZ (20CZ_wo).

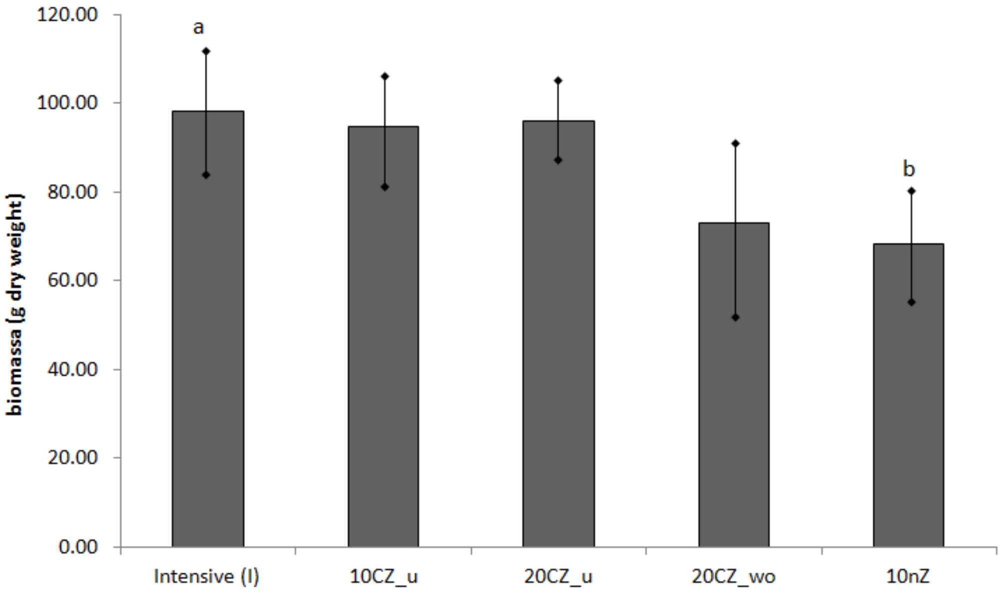


Figure 2 Effect of treatments on the production of aerial biomass at the end of the first trial (dry weight). Values represent means ± standard deviation (n = 4). Different letters indicate significant differences between treatments at the p-level < 0.05. Treatment codes are explained in Table 2.

Second greenhouse trial

Nitrogen concentration in leachate

In the second trial, the monitoring of leachate in the different treatments included the measurements of conductivity, chlorides, ammonia nitrogen and nitrates (Table 5).

As far as NO_3-N concentration is concerned, no significant differences were found in 15 DAS among treatments and control, all not complying with the regulation limit (MAC). On the other hand, in treatments T4, T5 and T6 a strong decrease occurred, reaching the control value; for the other treatments, the decrease was moderate, except for only treatment T1, always over the regulation limit. At the end of the experiment (73 DAS), the nitrates were found lower than the regulation limit in the majority of treatments (T2, T3, T4, T5 and T6) and in the control.

In particular, considering treatments in order of decreasing nitrogen input, T6 (with low zeolitite and nitrogen addiction) had the lowest nitrogen content in water as expected.

As regards the NH_4-N content, in the first 15 days of experiment, when the request of plant nutrients is not yet at the maximum, it can be observed a significant low concentration for the treatments with the highest urea reduction and in Codigoro soil (T5), compared to the control. At 73 DAS, all the treatments presented the same level of NH_4-N (average 0.94±0.30 mg l^{-1}).

Conductivity remained stable in the leachate of all treatments with the only exception of T5, where an increase, probably linked to the leaching of the chloride present in the experimental field soil, had been observed. For the whole duration of the test, the pH was maintained at constant values for all treatments (7.5± 0.2).

	NO_3-N (mg l^{-1})				NH_4-N (mg l^{-1})				conductivity (mS cm^{-1})				Cl$^-$ (mg l^{-1})			
Treatment	day 15		day 73		day 15		day 73		day 15		day 73		day 15		day 73	
C	24.9 ±	5.4	4.6 ±	0.9	1,2* ±	0,1*	0,7* ±	0,1*	1.8 ±	0.3	1.1 ±	0.4	160.1 ±	95.5	154.1 ±	74.3
T1	35.6 ±	10.0	17,5* ±	17,2*	3.1 ±	4.9	0.8 ±	0.2	2.2 ±	0.6	1.8 ±	0.5	175.7 ±	37.6	196.3 ±	76.8
T2	29.5 ±	7.6	7,1* ±	2,6*	5.3 ±	4.4	1.3 ±	0.6	1.9 ±	0.7	1.4 ±	1.0	173.4 ±	13.8	151.9 ±	110.3
T3	32.3 ±	13.0	7.5 ±	2.9	0.5 ±	0.6	1.1 ±	0.7	1.5 ±	0.5	1.3 ±	0.2	164.5 ±	39.9	128.8 ±	46.7
T4	35.2 ±	13.6	4.2 ±	0.4	4.8 ±	8.3	0.9 ±	0.5	1.5 ±	0.3	1.5 ±	0.5	111.5 ±	25.2	153.8 ±	74.8
T5	20.0 ±	5.5	4.6 ±	0.4	0.02 ±	0.02	1.1 ±	0.7	1.4 ±	0.3	2.1 ±	0.5	120.8 ±	19.9	233.4 ±	75.7
T6	28.8 ±	13.8	4.3 ±	0.4	0.11 ±	0.12	0.5 ±	0.1	1.4 ±	0.2	1.4 ±	0.5	155.1 ±	42.0	165.6 ±	119.9
MAC	11.3		11.3													

Table 5. Leaching results for the second trial: trend of NO_3-N and NH_4-N content, conductivity and chlorides in leachate for the seven treatments. Mean ± deviation standard of four replicates, except for (*) where three replicates were used. The maximum allowable concentration (MAC) for drinking water in Italian regulation (Legislative Decree 31/01) and International guideline (WHO, 1993) is 50.0 mg l^{-1} NO_3^-, corresponding to 11.3 mg l^{-1} NO_3-N.

Biomass production.

Final growth and root production of the corn grown under the different fertilization treatments are shown in Figure 3 and Figure 4.

At end of experiment, as far as the production of aerial biomass (dry weight) concerned, the differences among treatments with the same artificial standard soil were not significant (Figure 3). At the same time, there was no significant difference between artificial and Codigoro soil (T5), except for T4 with natural zeolitite, which had the lowest production.

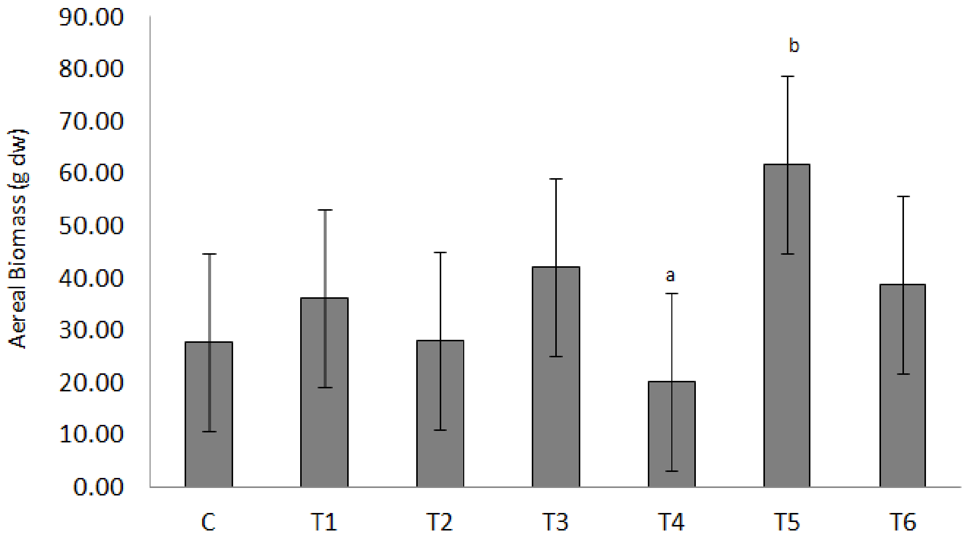


Figure 3 Effect of treatments on the production of aerial biomass at the end of the second trial (dry weight). Values represent means ± standard deviation (n = 4). Different letters indicate significant differences between treatments at the p-level < 0.05.

Moreover, the different fertilization treatments did not affect the root biomass (fresh weight) of the plants (Figure 4), at either the normal or lower dose. This parameter only differed for the treatments T5 and T2, both carried out with the 50% urea reduction and 10 NH4CZ. Furthermore, T2 with artificial soil has yielded an even greater effect compared to T5 with agricultural soil, as expected.

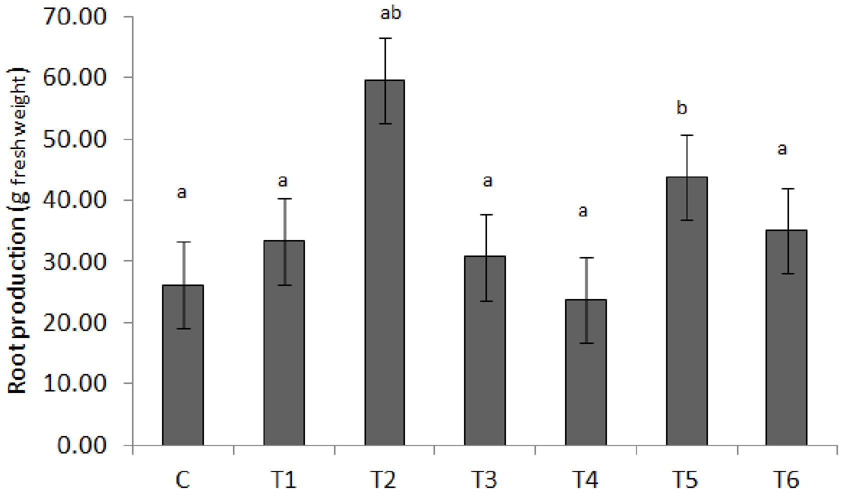


Figure 4 Effect of treatments on the production of root biomass (fresh weight) in second trial. Different letters indicate significant differences between the treatments (p-level <0.05)

In Figure 5, the assessment of the roots involved (i) the measurement of root biomass (dry weight) and (ii) the morphological analysis, considering the total length of roots, the number of primary roots and absorbent and the radical diameter. Considering these parameters, the treatment T5 showed the most root biomass (dry matter), followed by T1 and T3. Other treatments induced low total production of roots.

Figure 5 Example of radical apparatuses of one plant in the 6 treatments and the control, at the end of the second trial. The roots have been cleaned, washed and air dried in order to observe type, elongation and structure.

The treatment T5 showed an impetus in the radical development already in the earliest stages of growth, when the volume of the primary structures was defined, that was maintained in the subsequent stage of production. As far as the architecture and hierarchical organization structures are concerned, T5 showed again features fully different from other treatments, developing a reduced amount of primary roots in the first crown, but having the greatest diameter. Furthermore, it is interesting to observe that the control (C) presented a reduced development in terms of accumulated biomass and minimum root diameter, with respect to the others.

Considering the treatments with artificial soil, T4 and T6 had the lowest number of roots in the first crown and the smallest average diameters, showing a behavior similar or lower than the control. Conversely, T1, T2 and T3 showed an overall increase of the primary structures and root biomass.

Measurements of the photosynthetic activity and chlorophyll content of plants

Photosynthetic activity (PN) and chlorophyll content (SPAD) were carried out on all plants at the end of the crop cycle, before destructive measurements.

The leaves of the control C and T1 showed a greater net photosynthesis (PN), up to 30 µmol CO_2 m^{-2} s^{-1} (Figure 6), while the treatments T2 and T3 recorded values around 25. The treatments T4, T5 and T6 showed PN values significantly lower than the other ones, in particular the treatment with Codigoro soil (T5) with the lowest values ever, (just over 10).

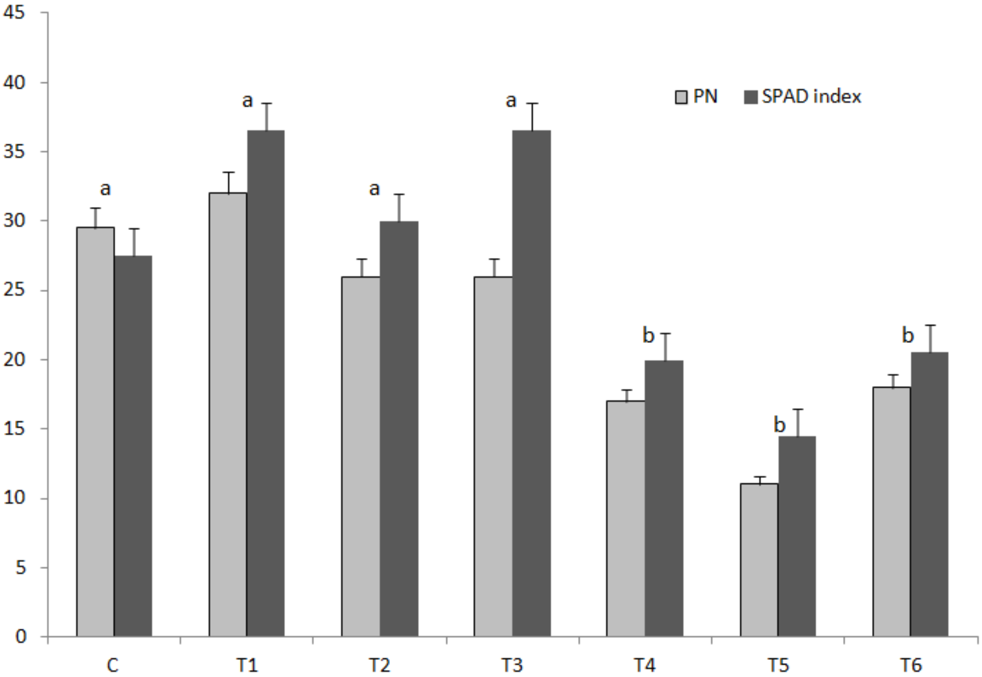


Figure 6. Crop growth evaluation in the second trial on the basis of photosynthetic activity (PN in µmol CO_2 m^{-2} s^{-1}) and SPAD index. Values represent means ± standard deviation (n = 4). Different letters indicate significant differences between treatments at the p-level < 0.05.

The SPAD index, which indicates the intensity of the green leaf area, is related to the presence of nitrogen and chlorophyll (Yang et al., 2014). Very low indices were found in T4, T5 and T6. In particular, the T5 SPAD index was found close to 15, less than half compared to T1 and T3. Moreover, T1 and T3 showed a SPAD index higher than the control, leading to suppose a positive effect of NH4CZ on N availability. In fact, during leaf senescence, the rapid drop in leaf SPAD readings is suppressed in plants subjected to higher N application (Yang et al., 2014).

The reduced transpiration and photosynthetic activity, as well as resulting in leaf chlorosis induced by T5, can be attributed to stress in plants whose root systems (as described above) had already filled the volume of the container at the time of the survey, resulting in the most developed.

It can be supposed that plants in T5 had good availability of nitrogen at the beginning of the crop cycle and the residual nitrogen which was adequate for the needs of plants until the end; it was more difficult to discriminate between the role of the nitrogen released by NH4CZ and that released by the Urea-N. Tall plants grew in T5 compared to all other treatments, even if equal or greater nitrogen was added in other treatments (eg. C, T1, T2). However, it should be considered a possible contribution of the Codigoro soil, in relation to the nutrient availability, as well as to an initial remarkable, content of macro-and micro-nutrients (as shown by chemical analysis), compared to the artificial soil, constitutionally inert from the chemical point of view.

Focusing on the group of treatments based on artificial soil, T4 and T6 had produced a smaller radical development and considerably more simplified by an architectural point of view (therefore less efficient); measures of photosynthesis and SPAD index are in agreement with this behavior, also confirmed by the reduced production of aerial biomass and radical, at least for plants in T4.

The negative effects on the physiology of plants produced by T4, could be partially explained by a "locking" of ammonia nitrogen by natural zeolitite, as reported by Ahmed et al. (2008), and not re-sold to plants in sufficient quantities during the initial step of crop cycle, probably due to Urea-N amount (-30%). It has to be noticed that the nitrogen resulting from the hydrolysis of urea was in the ammonia form and it represented the only source of this element in the artificial soil for plants of maize (very demanding in nitrogen).

The reduced performance of T6 could be explained by the lower concentration of NH4CZ and the lower amount of Urea-N added to the substrate sand-peat (up to 10-20 times less compared to the other treatments). Control, T1, T2 and T3 had maintained a good photosynthetic efficiency and chlorophyll content even in the last days of the crop cycle. However, the plants of the control C, despite the full supply of urea, showed a significantly lower production of biomass and a more simplified radical organization with respect to treatments T1, T2 and T3: this can be probably related to the presence of NH4-charged zeolitite into the latter phase of crop cycle, and their role in increasing water retention and nutrients in a naturally poor substrate.

Macronutrients in leaves.

Regarding the macronutrients in leaf at 73 DAS (Figure 7), it can be observed that the concentrations of phosphorus, potassium, sulfur, calcium, magnesium and sodium were comparable in all treatments, suggesting a good level of biomass growth, similar to the control. On the other hand, N leaf content was remarkably different in treatments T1, T2, T3, containing NH4CZ and a fertilizer reduction. Moreover, the nitrogen content (almost 2.5%) in T1 and T2 led to suppose the possibility to increase the production, while for the other treatments the nitrogen content less than 1% suggested a suffering situation, with limitation in plant growth. In fact, a typical growth maize stage presents 2.4% N leaf content at 75 DAS and 1.1% or more at 105 DAS, at the final stage (Tajul et al., 2013; Ahmed et al., 2008; Jones et al., 2012; Tejada and Benitez, 2011).

Phosphorous leaf content was not affected by the amount of fertilizer and there were no differences among the zeolitite doses assayed, with showed a comparable P leaf level (about 1,550 mg kg^{-1} dw) in line with other studies (about 1,300 mg kg^{-1} dw by Tejada and Benitez (2011) up to 2,600 mg kg^{-1} dw by Lazcano et al. (2011). On the other hand, the K leaf content was higher in all the treatments of this study (average value of 21,100 mg kg^{-1} dw) than those by Lazcano et al. (2011) and by Tejada and Benitez (2011), where a mean value of 13,500 mg kg^{-1} dw was observed. Calcium leaf content was about 7.7% in all the treatments with artificial standard soil and low urea addition (T2, T4 and T6), while the treatment with Codigoro soil and low urea addiction (T5) showed a significant low Ca leaf content, more than half of treatment T1 with the same urea addition on artificial soil (9.5%) or in the control C (10.3%). A similar trend was also observed for Mg leaf content, as the Mg:Ca ratio was about 1:3 for the control and all treatments with artificial standard soil, and 1:1.8 for T5 with Codigoro soil. Sodium and sulphur were similar in all treatments, corresponding to standard leaf content at 73 DAS.

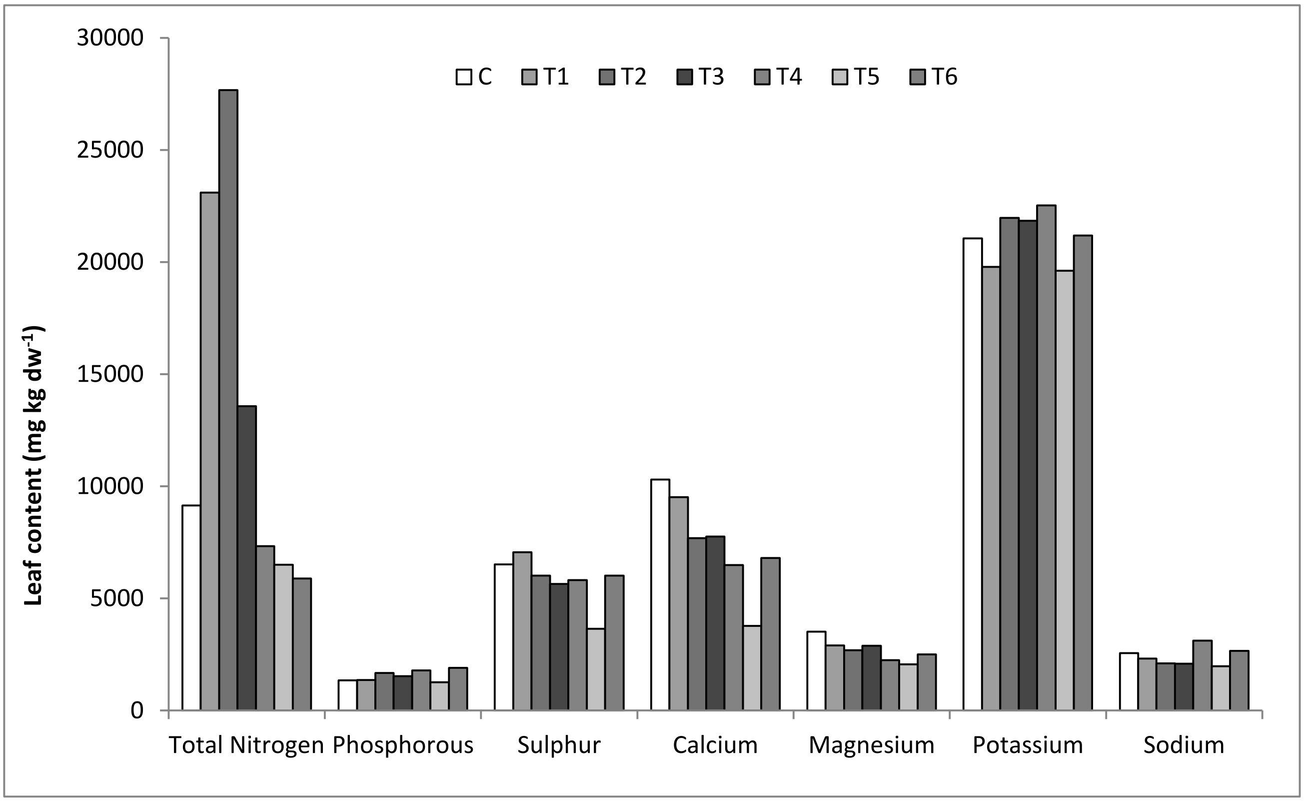


Figure 7 Analysis of macronutrients in the corn leaves at 73 DAS, after harvest in the second trial. Optimal Nitrogen content is set at 2% (20,000 mg kg^{-1}) while the sufficient level at 1% (Tajul et al., 2013; Ahmed et al., 2008; Jones et al., 2012). Calcium leaf content showed a significant difference between T5 and T1 (same urea addiction in two different bulk soil, p-level: 0.005) and between T5 and Control (p-level: 0.0004). All other compounds did not show significant difference among treatments (p-level >0.05).

Two crop years with Codigoro soil – trial 1 and 2

One of the aims of ZeoLIFE projectwas to assess the long-termeffect of zeolitite,when only one application of NH4CZ in soil is enough for improving soil texture and maintaining its capability to exchange cations with the plant roots over time. In order to simulate the effect of zeolitite on plant growth for almost 2 crop years, the treatment 10CZ_u of first trial (hence called T1-1st) was fertilized (reducing nitrogen addiction up to 50%) and sowing again in second trial (T5, hence called T1-2nd) The fertilization with urea was required due to low content of residual nitrogen in the soil, after maize production in the first trial.

The comparison between T1-1st and T1-2nd (Figure 8) showed a lower growth rate (0.92 cm day^{-1}) in second trial than in the first one (1.30 cm day^{-1}), probably due to a higher consumption of nitrogen (not present in leachate).

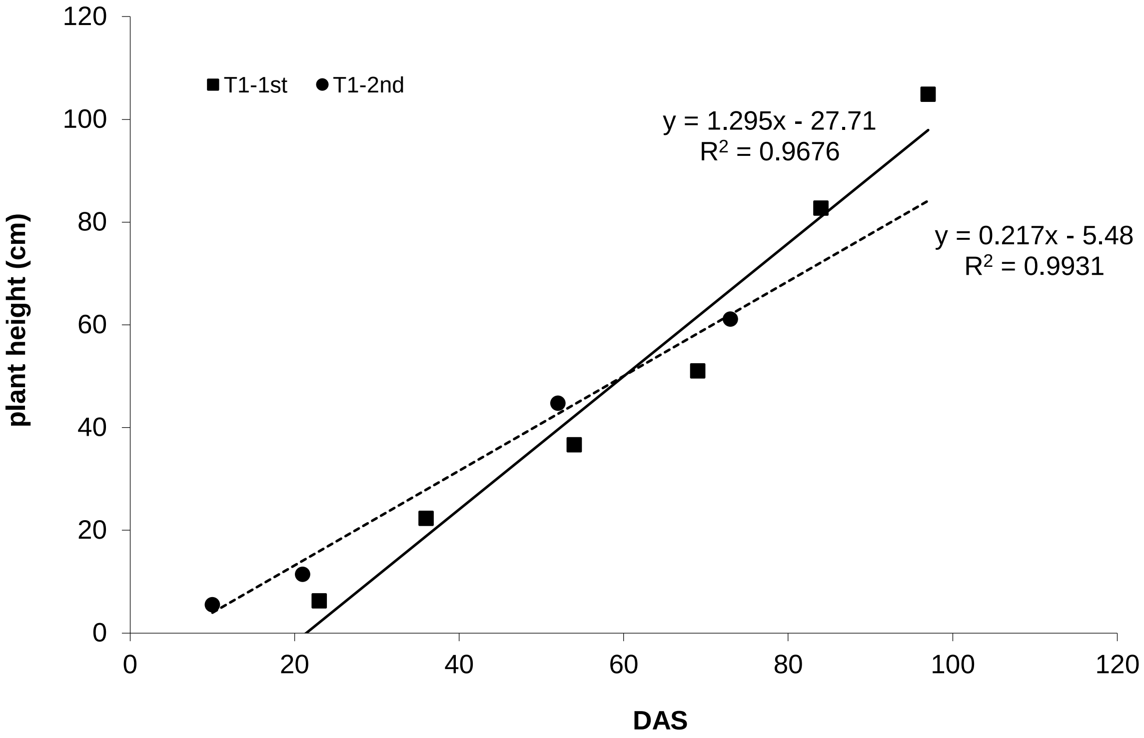


Figure 8. Growth rate of two maize productions in Codigoro soil with 10 g kg^{-1} NH4CZ and Urea-N progressive reduction (94.4% in T1-1st and 50.0% in T1-2nd). The comparison between first (square) and second trial (dot) allowed the assessment of the simulation of two crop years on the same NH4CZ addition, performed only in the first trial.

Furthermore, the comparison between first and second trial (Table 6) showed a downward trend of the final growth in terms of biomass and roots, in comparison to the control (I). Despite lower plant growth in the second trial, the N content in leachate reached the same value in both treatments, allowing to comply with the regulation limit (11 mg l^{-1}) with a significant reduction of urea (50% in T1-2nd).

Treatment	Trial	DAS	Plant height (cm)	Aerial biomass (g $_{fw}$*)	Roots (g $_{fw}$)	NO_3-N in leachate (mg l^{-1})
Intensive (I)	1	89	106.4 ± 18.7	276.6 ± 25.5	533.7 ± 256.6	1.3 ± 0.3
			±	±	±	±
T1-1st	1	89	104.9 ± 12.9	309.1 ± 63.0	430.0 ± 180.7	4.2 ± 2.8
			±	±	±	±
T1-2nd	2	73	61.1 ± 13.9	128.4 ± 13.0	186.1 ± 43.8	4.6 ± 0.4

Table 6. Production assessment of Maize crop in Codigoro soil treatments and NO_3-N content in leachate. Comparison of data collected in trial 1 and 2. The control treatment to be considered was the Intensive (I) in trial 1, with Codigoro soil and with 370 kg N ha^{-1}.*fw: fresh weight.

DISCUSSION

The main result of the first trial was that applying the dose 10 g kg^{-1} of NH4CZ and reducing urea fertilization may offer a significant advantage by reducing the leaching of NO_3-N, and maintaining the crop growth rate. The economic viability of the dose-1 was considered in the second greenhouse trial, and then in the subsequent open-field experiments of ZeoLIFE project. Anyway, the use of Codigoro soil could have influenced the results, reducing the NH4CZ effect evaluation. Therefore, in the second trial, an artificial standard soil without any N residual source was used, in order to observe the actual potential of zeolitite.

The findings of the second trial showed that the nitrates concentration complied with the regulation limit (50 mg l^{-1}) in the treatments and in the control, except for T1 where 70% urea-N addiction was applied. Probably the low Urea-N reduction could contribute to maintain high level of nitrates in leachate, also considering the low root production in the crop of this treatment.

Regarding crop production, for all fertilization treatments and zeolitite doses assayed with artificial soil, no significant differences on the production of aerial biomass were observed, while the treatment with Codigoro soil showed the taller plants. The same results were found for root biomass, which only T5 determined a significant difference compared to all other treatments. Remarkably, T2 with the same urea reduction of T5 (50%) yielded a good effect on root elongation.

As far as crop quality is concerned, the macronutrients content in leaf was performed at the end of the second trial, testified for an overall good leaf health, but N content. In fact, N content varied in dependence of the treatment: the 2.5% N leaf content in T1 and T2 led to suppose the possibility to increase the production, while for the other treatments it was less than 1%, suggesting a suffering situation, with limitation in plant growth. This was confirmed by the measurements of the photosynthetic activity and chlorophyll content (SPAD). In particular, SPAD index, related to the presence of nitrogen and chlorophyll in the leaf (Yang et al., 2014), was very low in treatments with low amount of NH4CZ or N-fertilizer (T4, T5 and T6). In treatment T5, simulating the second year of sowing on used NH4CZ, the SPAD index was close to 15 and the N leaf content less than 10%, representing a typical situation of N lack (Yang et al., 2014). In fact, the color of the leaves in T5 was yellow indicating a chlorosis, process in which leaves produce insufficient chlorophyll, even if the plants were taller than those of the control and the other treatment. As the roots resulted the most developed in T5, another reason could be attributed to stress in plants whose root systems had already filled the volume of the container. At 52 DAS, the crop growth in T5 was higher than 19.55 cm at 40 DAS found in field by Singh et al. (2004), and then drastically decreased. Anyway, in this study, the Urea-N reduction of 50% in the second crop year could be a limitation for crop growth, even if the NH4CZ was present and could still support the crop development.

When natural zeolitite was added (T4), some negative effects on plant physiology were observed and could be partially explained by a "locking" of ammonia nitrogen by nZ, as reported by Ahmed et al (2008). During the initial step of crop cycle, NH4-N probably was not ceased to plants in sufficient quantities, also as consequence of the reduced Urea-N supply (-30%).

The reduced performance of T6 could be explained by the very low amount of NH4CZ (6 g kg^{-1}) and the minimal addiction of Urea-N to the artificial soil (up to 10-20 times less compared to the other treatments). This demonstrated that the NH4CZ behavior is different with respect to chemical fertilizer one and the N content in NH4CZ should not be considered an equivalent of Urea-N. Thus, the maximum amount of NH4CZ to be added in a soil should be selected on the basis of soil type and not of the N-content limit (for example, 240 kg N ha^{-1} for Maize). These results may suggest that the employment of synthetic fertilizers foreseen for the different production regulations may be revised downwards when they are associated with the use soil conditioners such as zeolitite.

In order to achieve an overall evaluation of all parameters analyzed in the second trial, a ranking approach was carried out (Table 7). Three macro-groups of parameters were considered in order to evaluate the leaching process, the crop production and the crop quality before harvest. For each macro-group, three parameters were selected, respectively: (i) Nitrates, ammonia and chloride content in leachate for the leaching process, (ii) maize growth rate, aerial biomass and root elongation for crop production and (iii) N leaf content, SPAD and PN activity for crop quality. Considering the control as a target for treatment evaluation, each parameter was compared to the control value by calculating the treatment/control ratio, whereas the value greater than 1 as good result.

The final score for each treatment was calculated using the formula:

$$score = \sum_{1}^{3} a_i \times \sum_{1}^{3} y_i \qquad (1)$$

Where a_i the group weights, and y_i the ratio of parameters of each group.

In this study, quality of the leachate and crop quality were considered very important, so the weight was 1 and 1.5, respectively, while 0.5 weight was attributed to the crop production.

	Leaching process			Crop production			Crop quality				
Treatment	NO_3-N	NH_4-N	Cl^-	Growth rate	Aerial biomass	Roots	N leaf content	SPAD	Chlophylla content	Final score	Ranking
C	1	1	1	1	1	1	1	1	1	9.0	-
T1	0.2	4.7	0.8	1.4	1.3	1.5	2.5	1.1	1.3	15.1	+++++
T2	0.1	2.6	1.0	1.2	1.0	1.4	3.0	0.9	1.1	13.1	+++
T3	0.6	3.1	1.2	1.5	1.5	1.7	1.5	0.9	1.3	12.8	++
T4	1.1	4.1	1.0	1.0	0.7	0.9	0.8	0.6	0.7	10.7	+
T5	1.0	3.3	0.7	1.7	2.2	2.9	0.7	0.4	0.5	10.8	+
T6	1.1	7.0	0.9	1.2	1.4	1.3	0.6	0.6	0.7	13.9	++++

Table 7 Evaluation of the six treatment of the second trial (T1-T6, described in Table 3). The final score was obtained by the formula (1), where the single ratio of each parameter was weighted depending by type (weight 1 for NO_3-N, NH_4-N, Cl^{-1}; weight 0.5 for growth rate, aerial biomass and roots; weight 1.5 for N leaf content, SPAD and Chlorophyll-a content). The treatment/control ratio was calculated considering the analytical results before harvest, and all the control parameters were set to 1. When the ratio is >1, the treatment had a performance better than the control, when <1 the worst. The ranking was “+++++” for the best and “+” for the worst. T4 and T5 had very close final value so they obtained both the worst ranking (+).

The ranking allowed a first selection of best management practice compared to the traditional farming practice (Control), to be performed in the field experiment. In particular, the best treatments with NH4CZ were found T1 and T6, with two different reductions of Urea-N (-30% and -97%, respectively). This led to suppose that NH4CZ gave a good contribution in N-availability during crop growth. Also T2 resulted a feasible solution, considering the low amount of NH4CZ (50 t/ha) and the high reduction of Urea-N (- 50%). This was confirmed by the findings of T5, with the real soil and two crop years (high reduction in NO_3-N leaching, and good crop production), even if its score was low but even higher than control. Also T4 was found with a low score but even higher than control, thanks to the good effect of the natural zeolitite on NO_3-N leaching and soil texture correction. Thus, considering the low content of natural zeolitite (50 t ha^{-1}) and the reduction of 30% fertilization, the treatment T4 could be also selected for the open field activities of ZeoLIFE project.

CONCLUSIONS

The results presented here led to conclude that the addition of NH4-charged zeolitite to highly productive agricultural land had no negative consequences in terms of crop growth, crop nutrition or soil quality and may even provide high agronomic benefits with long-term effect on soil properties. The lack of negative effects seen at application rates of either 6 or 10 t ha^{-1} also suggested that the applications of NH_4-charge zeolitite may be scaled-up in open field studies. Moreover, the reduction of chemical fertilizer was feasible, even at high degree, allowing a reduction in groundwater pollution by nitrates.

These results are important in terms of satisfying the environmental risk assessment required to formulate legislation for the use of alternative fertilizer and soil correctives in agriculture.

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