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Changes in soil mineral N content and abundances of bacterial communities involved in N reactions under laboratory conditions as predictors of soil N availability to maize under field conditions

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Abstract

Proper management of soil fertility requires specific tools for predicting N availability for crops as a consequence of different fertilization strategies. More information is required, especially for organic fertilizers, depending on their mineralization rate, composition and processing (i.e., fresh or composted manure), as well the influence of soil characteristics on their effect. Laboratory soil incubations were used as a proxy for understanding plant-soil N dynamics under field conditions. Chemical and microbiological measurements as contents of mineral N, potentially mineralizable N and the abundance of key genes regulating the overall N-cycle were used as predictors of mineral N availability to maize in two contrasting pedoclimatic conditions. Our results showed that there was a good correlation between chemical and microbiological measurements from laboratory soil incubation experiments and soil-plant N dynamics of maize cropping systems. Mineralization patterns from soil incubation proved to be useful for optimizing fertilization management of maize under field conditions as long as incubation time is normalized over maize growth cycle, according to the simplified model of Growth Degree Days. Average cumulative soil mineral N values calculated over a short incubation period (42 days) showed a significant correlation (R^2=0.72, $p<0.05$) with maize N uptake. The shape and kinetic parameters of net N mineralization from medium term (112 days) soil incubation provided consistent information on the interaction between fertilizers and native fertility. The abundance of N fixation, nitrification, and denitrification genes (*nifH*, *amoA*, *nirK* and *nirS*) was sensitive to soil characteristics and N fertilization. This work provides a suitable starting point for developing a crop-based approach for using incubation data to optimize maize fertilization. However, more studies with different maize cultivars and pedoclimatic conditions are needed to generalize this approach.

Keywords: N mineralization; curve fitting; Growth Degree Days; *nifH*; *amoA*; *nirK*

Introduction

The decline in soil organic matter (SOM) content is a major threat to soil fertility in European semi-arid regions due to their specific climatic conditions (Diacono and Montemurro 2010). Therefore, proper fertility management must be utilized to build up SOM so that the soil can cope with the high mineralization rate of these environments. Among these strategies, fertilization with composted manures provides a good option in areas without manure from livestock farms with positive effects on soil chemical and physical properties as the increase in OM sequestration (Gabrielle et al. 2005; Fagnano et al. 2011; Alluvione et al. 2013), aggregate stability (Diacono and Montemurro 2010; Chen et al. 2014; Meng et al. 2014) and hydraulic conductivity (Chen et al., 2014), which are primarily due to the hydrophobic protection of stabilized SOM (Spaccini et al. 2002; Piccolo et al. 2004; Lynch et al. 2006). Moreover, compost fertilization can increase soil biological activity and microbiological diversity (Ventorino et al. 2013), as well as suppress soil-borne diseases (Ventorino et al. 2016). Although compost maturity level (Annabi et al. 2007) and its chemical composition (Miller et al. 2010) are recognized as key factors in improving soil physico-chemical characteristics, climatic variables and soil properties may explain the high variability in mineral N availability of compost amended soils (Nedvěd et al. 2008). The highest mineralization rates of compost occur in high pH soils (Huang and Cheng 2009). Soil texture is another key factor controlling N mineralization of composted manures (Saenger et al. 2014), as for example NH_4^+ is retained by ammonium fixing clays (Nyamangara et al. 1999) and this decreases nitrification.

Knowledge of the fate of N from organic fertilizers is required for optimizing N rates to obtain valuable crop performances and minimize the impacts of N surplus on the environment (Zebarth et al. 2009). Soil incubation has been used to study N patterns in manure-amended soils (Cordovil et al. 2005; Flavel and Murphy 2006), fitting mineral N data to functional models to estimate pools of mineralizable organic N (De Neve and Hofman 2002, Griffin et al. 2005). These studies have obtained useful insights into mineral N availability from fertilizers. Riberio et al. (2010) reported a positive correlation between estimated N mineralization and lettuce N uptake. Abbasi et al. (2012) found that maize biomass and N uptake were correlated. Nevertheless, all of these results are from pot experiments, and caution is required when extrapolating the results of these pot experiments to field conditions.

The N cycle includes key processes such as N-fixation, nitrification and denitrification (Ventorino et al. 2010; Weng et al. 2013), and microorganisms involved in these processes can be detected by molecular techniques through the quantification of genes encoding enzymes such as nitrogenase reductase (*nifH*), ammonia monooxygenase (*amoA*) and nitrite reductase (*nirS* and *nirK*). Among available molecular methods, quantitative real-time polymerase chain reaction (q-PCR) is

68 the most sensitive and most suitable approach for determining abundance of functional genes from soil-derived DNA and
69 RNA (Sharma et al. 2007).
70 In this work, we used data from a three-year field study (Alluvione et al. 2013) and a medium term (112 days) soil incubation
71 period to find the relationships between field and laboratory experiments in studying N availability in fertilized soils. Chemical
72 and bacterial properties, such as contents of mineral N, potentially mineralizable N and the abundance of the aforementioned
73 key genes in soil, were determined for predicting mineral N availability for crops in the field.
74 Our work aimed to answer the following questions: (i) are results from field and laboratory experiments related?; (ii) is it
75 possible to identify a simple index from laboratory incubations that can predict crop performance and optimize fertilization
76 strategy?; (iii) can the evaluation of the abundance of key genes of N-cycle be used for predicting N available for plant
77 nutrition?

Material and methods

Experimental set-up

Field trials were carried out over three consecutive years (2006-2008) under two contrasting pedoclimatic conditions to assess the effects of different types of fertility management on maize crop yield and soil C and N dynamics. A detailed description of the experimental set-up, sampling techniques, chemical analyses and data elaboration is reported by Alluvione et al. (2013). Briefly, sites were located in the Po River Valley near Torino (TO site - 44°53' N, 7°41'E, Piedmont Region, Northern Italy) and in the Sele River Plain near Napoli (NA site - 40°37' N, 14°55' E, Campania Region, Southern Italy). In this work, we report yield and soil mineral N content of the two sites concerning the following treatments: compost (COM) and urea (UREA) fertilization added to soil at the same N rate (130 kg N ha^{-1}) compared to an unfertilized control (0N). Compost was made with a park, garden, and separately collected urban waste mixture and, according to Alluvione et al (2013), had the following composition: 610 g kg^{-1} (f.m.) dry matter; 21 g kg^{-1} (d.m.) ash; 256 g kg^{-1} (d.m.) organic C; 391 soluble C / total C ratio; 12.2 g kg^{-1} (d.m.) total N. Compost was not phytotoxic at the 150 Mg ha^{-1} rate used for the plant growth assay proposed by Chukwujindu et al. (2006). Treatments were conducted in quadruplicate following a completely randomized design at the TO site, while a randomized complete block design was used at the NA site.

The soil incubation experiment aimed to investigate mineral N and bacterial properties of the two soils and to study the relationship of these two measurements under controlled laboratory conditions.

Soils were collected (0-30 cm layer) at both field sites at the end of the 2nd year of the field experiment (autumn 2007) in an area near the experimental plots so as to have soils with the same properties as those used for the field experiment but not affected by treatments. Soils were air-dried and sieved at 5 mm; the main soil characteristics are shown in Table 1. Sieving size (<5 mm) was selected to minimize soil disturbance and preserve micro-aggregates. The following three factors were arranged over a completely randomized design with three replicates: (i) 2 soils from Napoli (NA) and Torino (TO) experimental fields; (ii) 3 types of fertilization: compost (COM), urea (UREA), and an unfertilized control (0N); and (iii) 8 sampling times. Totally, 144 experimental units (2 soils x 3 fertilizations x 8 samplings x 3 replicates) were set up, each consisting of 400 g of dry soil in a 543 cm^3 pot. Urea was added to soil as a liquid solution, while compost was grinded with a hammer-mill before the application and individual soil-fertilizer mixtures were prepared for each pot. The quantities of urea (0.08 g kg^{-1} soil) and compost (4.6 g kg^{-1} soil) to be added to each experimental unit were calculated taking as reference the N field rate (130 kg N ha^{-1}) and the 0-30 cm layer with an average bulk density of 1.2 Mg m^{-3} (measured after sieving and

soil consolidation with watering). A pre-incubation period of 5 days at 28°C and at field capacity was conducted to stabilize soil microbial activity. Afterwards, a 112-day incubation was conducted in the dark at 28°C, controlling soil moisture to simulate the fluctuations of ordinary maize irrigation. According to FAO 56 paper (Allen et al. 1998), irrigation events were simulated at a threshold value of 45% of Water Holding Capacity in order to raise soil moisture to its field capacity.

Soil hydraulic parameters were determined in the laboratory by Richard's chambers (Richards 1944). Air relative humidity was kept between 60 and 70% to simulate the conditions of our field sites during the maize growth cycle.

Plant sampling and analyses

In the field experiment, total biomass was determined by hand-harvesting maize plants at the dent stage from an area of 15 m^2 per plot. Dry matter was determined after oven-drying samples at 70°C until a constant weight was reached. The N content of plant tissues was measured using a CHN elemental analyser (NA 1500 N analyser from Carlo Erba Instruments, Thermo Fisher Scientific, Waltham, MA). Maize N uptake was calculated by multiplying N concentration in the entire plant by total dry matter yield.

Soil sampling and analyses

In the field experiment, soil samples were collected from three soil layers (0-15, 15-30, 30-60 cm) before sowing (April), at flowering (June), and after harvest (October) of each cropping cycle. In the laboratory incubation experiment, three destructive soil samples per each soil x fertilization combination were randomly collected at 0, 7, 14, 28, 42, 63, 84 and 112 days for chemical analysis. Concentrations of NH_4^+-N and NO_3^--N were measured according to the Hach® method and the extracts were analysed by spectrophotometry (Hach DR 2000, Hach Company, Loveland, CO). This method was used in both field and incubation experiments with the exception of soil samples from the TO field experiment. In this case, mineral N was extracted by 1 M KCl and analysed with a continuous flow analyser (Evolution II, Alliance Analytical Inc., Menlo Park, CA).

Quantification of bacterial functional genes

Soil samples were collected from pots after 42 and 112 days of incubation as reported above. Total DNA was extracted by the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to the manufacturer's protocol.

Bacterial functional genes were quantified with qPCR. Amplification of *nifH*, *amoA*, *nirK* and *nirS* genes were conducted in a Chrom4 System Thermocycler (Bio-Rad, Milano, Italy) using the primers listed in Table 2. The PCR mixture (25 µL total volume) included 50 ng of target DNA, 1× iQ SYBR Green supermix, (Bio-Rad) and 0.2 µM of each primer. All qPCR reactions included an initial denaturing step at 95°C for 3 min followed by 40 cycles of amplification as specified in Table 2. Standard curves were generated to calculate the abundance of evaluated genes. Each target gene was amplified with primers from different type strains: *nifH* (nifH-F, 5'-AAAGGYGGWATCGGYAARTCCACCAC-3'; nifH-R, 5'-TTGTTSGCSGCRTACATSGCCATCAT-3') and *nirK* (nirK-876, 5'-ATYGGCGGVAYGGCGA-3'; nirK-5R, 5'-GCCTCGATCAGRTTRTGG-3') from *Sinorhizobium meliloti* 1021; *amoA* (amoA-1F, 5'-GGGGTTTCTACTGGTGGT-3'; amoA-2R, 5'-CCCCTCKGSAAAGCCTTCTTC-3') from *Nitrosomonas europea*; and *nirS* (nirS-cd3aF, 5'-AACGYSAAGGARACSGG-3'; nirS-R3cd, 5'-GASTTCGGRTGSGTCTTSAYGAA-3') from *Pseudomonas stutzeri* DSM5190. Amplicons were purified from agarose gels using a QIAquick gel extraction kit (Qiagen S.p.A, Milano, Italy) and cloned using TOPO-TA cloning kit dual promoter (Invitrogen, Milano, Italy) in *Escherichia coli* competent cells according to the manufacturer's protocol. Plasmid DNA containing a single copy of the target gene was purified with QIAprep Spin Miniprep Kit (QiagenS.p.A) and quantified using the QuantiFluor™ (Promega, Milano, Italy). The copy number per microliter of each cloned gene was calculated using the following formula (Chen et al. 2015): (A×B)/(C×D) where A is the concentration of the DNA template (ng μl^{-1}), B is the Avogadro number (6.023×10^{23} copies mol^{-1}), C is the average molecular weight of a DNA base pair (6.6×10^{11} ng mol^{-1}), and D is the DNA size (bp). Ten-fold serial dilutions, ranging from 10^1 to 10^8 gene copies μl^{-1}, of each plasmid DNA sample were used to generate a standard curve for each target gene (*nifH*, *amoA*, *nirK*, and *nirS*).

Data elaboration and statistical analyses

We conducted Kolmogorov–Smirnov and Levene tests to verify the assumptions of normality and homoscedasticity of the data. For field data, we used a general linear model of analysis of variance (ANOVA). Maize N uptake, soil NH_4^+-N, and NO_3^--N contents from field experiments were analysed using fertilization (0N, COM and UREA) as the main factor, while year was treated as a repeated measure. We verified the variance-covariance structure of our dataset by performing Mauchly's sphericity test. Field data were analysed separately per site to preserve homogeneity of variance. Soil mineral N content was transformed through natural logarithm (of values added to 100 to eliminate 0 values) to preserve normality of the data before conducting the ANOVA.

For laboratory incubation, we analyzed NH_4^+-N, NO_3^--N and total mineral N content data using a one-way ANOVA, which included three factors (soil x fertilization x sampling time). Next, a one-way ANOVA with one factor was used, which considered each soil x fertilization x sampling combination as a single treatment to compare soil mineral N values recorded at each sampling date. As for field data, soil mineral N content was natural logarithm transformed (of values added to 100 to eliminate 0 values) to preserve normality of the data before conducting the ANOVA.

For microbiological data, we used a one-way ANOVA with three factors (soil x fertilization x sampling time). All statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA); statistical comparisons were made at the $\alpha = 0.05$ probability level and mean values were separated using the Sidak *post-hoc* test.

According to Abbasi and Khizar (2012) and Ribeiro et al. (2010), cumulative soil mineral N values from soil incubations can be used to predict plant uptake. We tested this hypothesis by plotting cumulative soil mineral N against average maize uptake for three years of field experimentation. To plot these data, we converted soil mineral N values in kg N ha^{-1} by using a reference layer of 0-40 cm and a bulk density of 1.2 Mg m^{-3}.

Furthermore, an iterative curve fitting procedure (SigmaPlot 12.1, Systat Software, Inc., San Jose, CA) was performed on cumulative net mineral N (n-SMN), which is the difference between each soil mineral N value and the value recorded at day 0 for each treatment, with the aim of estimating the parameters of the following mineralization functions:

1) Single pool negative exponential function (Eq.1):

$$n - SMN\ (t)\ =\ N\ \times\ [1 - exp^{(-k \times t)}] \qquad \text{(Equation 1)}$$

where n-SMN (t) is the amount of N mineralized at time t, N is potentially mineralizable N and k is the decomposition coefficient.

2) Two-pool negative exponential function (Eq.2):

$$n - SMN\ (t)\ =\ N_{fast}\ \times\ [1 - exp^{(-k_{fast} \times t)}]\ +\ N_{slow}\ \times\ [1 - exp^{(-k_{slow} \times t)}] \qquad \text{(Equation 2)}$$

where n-SMN (t) production is split into two N pools differing in their decomposability. The N_{fast} pool represents N in easily decomposable organic materials (i.e., fresh residues), while N_{slow} pool includes more recalcitrant sources with a slow rate of decomposition. The k_{fast} and k_{slow} are the corresponding decomposition coefficients of the two pools.

3) Sigmoid, 3-parameter function (Eq.3) :

$$n - SMN\ (t)\ = \frac{N_s}{1+exp^{-\frac{(t-t0)}{k_s}}} \qquad \text{(Equation 3)}$$

where N_s represents potentially mineralizable N, K_s is the inflection point, t_0 is the time needed to raise SMN values to $N_s/2$, and K_s is the mineralization coefficient.

In the Results section, we reported the best performing functions according to the regression coefficient (R^2) values and to the standard error of estimate (SEE). No constraints were applied to parameters estimated by the fitting procedure, and the best performing functions were identified among those with realistic parameters for potentially mineralizable N (not higher than soil total N) and the mineralization coefficient (between 10^{-3} and 1 day^{-1}).

Results

Field experiment

Nitrogen uptake of maize in the two sites is reported in Table 3. Unfertilized plots had higher N availability in TO soil, with an average uptake that was two times greater than in NA soil (average value of 105.8 kg N ha^{-1} *vs* 181.5 kg ha^{-1} for NA and TO site, respectively). Nitrogen uptake declined at both sites over the years, but in NA soil, this decrease was more marked. In fact, taking values from the first year as a reference at NA, there was a dramatic decline from the second year (-40%), while a comparable decrease (-43%) was observed in TO soil only in the third year as a consequence of hailstorm damage. Nitrogen uptake at the TO site was significantly affected by COM and UREA fertilization in 2007 and 2008, with an average increase of +34% for fertilized plots with respect to 0N. In contrast, compost fertilization dramatically reduced N uptake at the NA site (-59% on the average), while UREA produced a 74% increase on average. Nitrogen uptake values recorded with UREA were comparable between the two sites, even though values significantly declined from the first to the third year at the NA site (-54% decrease), whereas TO soil was able to sustain N nutrition of maize with an N uptake always above 200 kg N ha^{-1}. The addition of compost did not affect N availability in TO soil with the exception of year 2008 (-29% compared to UREA fertilized soil) while it strongly reduced N uptake in NA soil (from -83% to -60% compared to the UREA fertilized soil). Soil mineral N content recorded in the 0-60 cm layer was almost three times higher in NA than in TO soil both at the pre-seeding (Fig. 1a) and the harvest (Fig. 1b) stage. At the pre-seeding stage, differences among the treatments only occurred in NA soil, with lower values recorded for 0N and COM (on average -13% with respect to UREA), while TO soil showed the same pattern at harvest. In this stage no differences were recorded for mineral N in NA soil even though the UREA effect was significant for NH_4^+-N values ($p=0.027$) with COM values -49% lower than those of UREA. Nitrate-N content followed essentially the same pattern of soil mineral N, while NH_4^+-N content was almost constant in both soils and ten times higher in NA (59 kg N ha^{-1}, average value) than in TO (6 kg N ha^{-1}) soil. The NH_4^+-N:NO_3^--N ratio was significantly higher in NA (approximately 0.45) than in TO (0.18) soil (data not shown).

Incubation experiment

Soil mineral N patterns and curve fitting

A significant soil x fertilization interaction was detected ($p<0.01$) by a one-way three factor ANOVA (soil x fertilization x sampling time), as shown in Figure 2a. As already found for maize N uptake (see Table 3), the 0N treatment had the largest difference between the two soils with soil mineral N values significantly higher in TO soil (+56%). Compost fertilization did not affect soil mineral N content in NA soil compared to soil of the 0N treatment, while a significant reduction (-18% compared to 0N) was observed for TO soil, even though values were still higher than those of the same treatment in NA soil. The UREA treatment increased mineral N content of both soils, but its effect was stronger for NA soil (+82%) than for TO (+20%) soil respectively. The NH_4^+-N: NO_3^--N ratio of the 0N treatment (Fig. 2b) was comparable to that found in the field experiment (0.35 and 0.18, in NA and TO soil, respectively). The UREA treatment significantly reduced this ratio in both soils (0.16 on average). Compost reduced this ratio in both soils, but the differences were only significant in the TO soil. Net N mineralization patterns of NA and TO soils are reported in Figures 3 and 4, showing that normalized soil mineral N (n-SMN) patterns for each soil x fertilizer combination together with the best fitting function (see Materials and methods section). For NA control soil (Fig. 3a), n-SMN values did not show variation until day 42, at which point, n-SMN values significantly increased and reached a plateau at 84 days (40 mg kg^{-1}). Compost fertilization (Fig. 3b) reduced the length of the initial 0 mg kg^{-1} constant phase by 21 days, then there was a double step increase, the first at 28 days (from 3 to 13 mg kg^{-1}) and the second between 42-112 days (from 17 to 50 mg kg^{-1}). Both 0N and COM treatments are well described by a sigmoid function (R^2 = 0.98), although compost fertilization increased the potentially mineralizable N from 39 to 51 mg kg^{-1}, reducing the initial steady state. In the UREA treatment, n-SMN rapidly increased (Fig. 3c) during the first 2 weeks (until 30 mg kg^{-1}), then a new increase was recorded between 42-63 days, reaching a maximum value of 62 mg kg^{-1}. This treatment modified the shape of the curve to a double exponential rise (R^2 of 0.98 vs 0.88-0.87 of the other models), with a recalcitrant N pool of 82 mg kg^{-1}, a mineralization rate of 0.01 mg kg^{-1} N day^{-1} and an easily mineralizable N pool of 12 mg kg^{-1} ($k=0.22$ day^{-1}), likely representing urea N. In the TO control soil (Fig. 4a), n-SMN values increased by approximately 20 mg kg^{-1} during the first week and then increased again from day 28 to the end of the experiment, reaching a maximum value of 57 mg kg^{-1}. The addition of compost (Fig. 4b) changed this pattern with a double step increase between 7 -14 days (from 2 to 22 mg kg^{-1}), and between 42-63 days (from 20 to 38 mg kg^{-1}), getting a final cumulative value of 43 mg kg^{-1}. The UREA fertilization (Fig. 4c) rapidly increased n-SMN values, producing 62 mg kg^{-1} of mineral N in the first 63 days.

Mineral N of TO soil was perfectly fitted with a single pool exponential function. Fertilization was able to modify the two parameters of the function coherently with the quality of the N input. Compost fertilization did not produce any significant variation in the kinetic parameter ($k=0.019$ and 0.021 day^{-1} for 0N and COM, respectively) while reduced the mineralizable N pool (from 65 to 47 mg kg^{-1}).

The UREA treatment did not change the mineralizable pool (almost 66 mg kg^{-1}) and slightly increased the mineralization rate (from 0.019 to 0.022 day^{-1}).

Mineral N incubation data as a proxy for field N uptake

We tested the correlation between mineral N content of lab-incubated soils and the average N uptake (3 years) of maize from our field experiment. As shown in Figure 5a, cumulative soil mineral N values at day 112 explained only 30% of the variability of the data. Because of these findings, we propose a new crop-based approach for using laboratory incubation data, starting from the hypothesis that incubation time must be normalized over the cycle of the target crop to be studied. Therefore, we converted the incubation time in maize thermal time, that is, the sum of heat units measured in Growth Degree Days (GDD) (Pampana et al. 2009). The most critical phase for maize N requirements is generally that between emergence and silking. For the maturity class used in our experiment (FAO 500), this phase corresponds to 785.5 GDD and is equal to almost 42 days of incubation at a daily constant temperature of 28°C. Therefore, we plotted the cumulative soil mineral N value at 42 days (Fig. 5b) against the average N uptake (3 years) of our field experiment and obtained a regression coefficient of approximately 0.582 and a significance close to 0.06 (d.f=4). Moreover, the performance of the regression increased significantly when average soil mineral N of the first 42 days was used and it accounted for 72% of maize N uptake ($p<0.05$) variability recorded in the field (Fig. 5c).

Functional gene analysis

A significant difference in the abundance of N-fixers was observed in the studied soils, with values of *nifH* gene abundance significantly higher in NA than in TO soil ($p < 0.05$) (Fig. 6 a). Moreover, a tendency to decrease after 112 days was recoded for both soils, even though significant variation was only detected for 0N and COM treatments in the NA soil ($p<0.05$). Compost only significantly increased the abundance of N-fixers in the NA soil at 42 days (from 7.20 x 10^4 to 2.28 x 10^5 copies g^{-1} soil, for COM and 0N, respectively). In contrast, inorganic fertilization significantly reduced *nifH* gene abundance at both sampling times in NA and TO soils, having the lowest values of dinitrogenase reductase copy numbers, ranging from 1.10 x 10^3 to 5.31 x 10^3 (Fig. 6a).

The effect of soil and fertilization on ammonia-oxidizers showed an inverse pattern with respect to N-fixers (Fig. 6b). At 42 days, a lower (but not significant) abundance of the *amoA* gene was recorded in NA soil treated with 0N and COM and a significant increase in gene copy number (1 to 2 orders of magnitude) in both UREA-treated soils (with the exception

of NA soil at day 42). This abundance was significantly higher in TO soil than in NA soil at both sampling dates with a constant difference of half an order of magnitude.

The abundance of the *nirK* gene was influenced by soil type and fertilization (Fig. 6c). At 28 days, NA soil showed significantly higher values than TO soil in 0N (2.38×10^5 *vs* 1.32×10^4) and COM (8.79×10^4 *vs* 1.32×10^4) treatments. Fertilization reduced the abundance of the *nirK* gene in NA (approximately –37% lower than 0N). By contrast, in TO soil, organic fertilization did not affect the abundance of denitrifiers (1.32×10^4 copies g^{-1} soil, on the average), while urea fertilization significantly increased *nirK* gene abundance to a value of 9.98×10^4 copies g^{-1} soil (Fig. 6c). After 112 days, an increase in *nirK* gene abundance was recorded especially in TO soil treated with 0N or COM, reaching values approximately 100-fold higher than those recorded at 42 days. In contrast, the abundance of the *nirS* gene was not affected by fertilization, soil type and incubation period because no significant differences among pots were recorded (Fig. 6d). The denitrifier community of both soils was dominated by *nirK*, whose abundance was at least 100-fold higher than that of *nirS* (approximately 2×10^2 copies g^{-1} soil) (Fig. 6 d).

Discussion

Matching Field results with incubation data

The two soils differed in native fertility, as shown by crop performance in unfertilized plots (Alluvione et al. 2013). Crop N uptake was significantly higher at the TO site, where it was slightly affected by fertilization, with an average value +85% higher than that recorded in the NA site. In contrast, crop N uptake was sensitive to fertilization at the NA site.

Low pre-seeding soil mineral N values were recorded at the TO site and corresponded to high N uptake and *vice versa* at the NA site; however, no correlation was detected between these two variables. These findings contradict those of Sylvester-Bradley et al. (2001), who showed that soil mineral N content predicted crop performance and accounted for 78% of the variation in wheat N uptake. Probably this discrepancy depends on the different environmental conditions between the pre-seeding and the cropping season. Soil mineral N content of TO site during the pre-seeding season was low due to winter leaching and soil coarse texture (Alluvione et al. 2013). On the contrary, soil mineral N values of the NA soil during the same period were higher probably due to the higher clay content that limits fall-winter N leaching (Gaines and Gaines 1994).

The sigmoid soil mineral N pattern recorded with the incubation of unfertilized NA soil suggests that net N mineralization requires 42 days to increase even under optimal conditions. Simard and N'Dayegamiye (1993) recorded the

316 same pattern in the first 40 weeks of soil incubations most likely because the initial N mineralization potential was more
317 affected by clay content (inverse proportionality) than by total C and N content (Zebarth et al. 2009). At the TO site, soil
318 mineral N values recorded at harvest in 0N plots were lower compared to pre-seeding values, suggesting that high crop N
319 uptake reduced N availability and that TO soil was able to reintegrate this N pool over the years. Moreover, despite the low
320 soil mineral N values recorded during the pre-seeding season, N uptake was always higher in TO soil than in NA soil,
321 suggesting that N mineralization was able to sustain crop requirements (even without fertilization).

322 In addition to increasing maize N uptake, UREA treatment significantly increased pre-seeding soil mineral N in the
323 NA soil, suggesting that easily available N inputs are mandatory for balancing the low N mineralization rate of this soil. This
324 finding is confirmed by incubation data where the UREA doubled the average soil mineral N of 0N treatment (from 30 to 60
325 mg kg^{-1}) and modified the mineralization curve shape from sigmoidal to exponential plot, increasing the potentially
326 mineralizable N and limiting the initial delay phase. The UREA treatment did not increase N uptake in the TO soil but lead
327 to a residual soil mineral N value that was higher than that of the control. This suggests that even a low N fertilization rate,
328 compared to recommended N dose of 250 kg N ha^{-1} in these environments, is not needed to satisfy crop requirements. The
329 low sensitivity of TO soil to UREA fertilization is also demonstrated by the mineralization curve shape not differing from
330 that of unfertilized soil. In the first year, COM fertilization dramatically reduced maize N uptake in the NA soil, probably
331 because the first addition of compost rapidly reduced N availability, pushing soil fertility to its lowest limit. Average soil
332 mineral N at the pre-seeding stage, which did not differ from that of control plots, was significantly lower than that of UREA-
333 treated soils. This pattern has also been identified by other authors in low fertility soils (Eriksen et al. 1999; Hargreaves et al.
334 2008) and confirms data on N immobilization after fertilization with composted manures, even in soils with a C:N ratio of 9
335 (Nyamangara et al. 1999).

336 Thus, this finding indicates that there was an over-exploitation of soil N stock during the cropping season and a low
337 ability of NA soil to restore soil mineral N in the absence of easily available N inputs. Alluvione et al. (2013) hypothesized
338 that the low N availability of NA soil might be due to clay-mediated protection of soil organic N limiting N mineralization
339 rate. Nevertheless, this model does not completely match with incubation data: average soil mineral N values recorded from
340 the COM treatment did not show any reduction with respect to control soil, and mineralization pattern was positively affected
341 by the fertilization with a reduction in the initial steady phase. This difference was probably due to recurrent soil inversion by
342 tillage that increases soil compaction and anoxic sites, resulting in a severe limitation of SOM and compost mineralization
343 (Holland 2004). The controlled conditions of laboratory incubation, mainly less soil compaction, allowed for better soil

aerobic conditions necessary for compost degradation than field conditions, suggesting that in NA soil, organic fertilization can have a positive effect if soil compaction is limited (i.e., adopting minimum tillage or sod seeding).

At the TO site, compost fertilization did not limit N uptake with the exception of the 2008. This difference was due to the hailstorm damage that was only compensated by the treatment with UREA (Alluvione et al. 2013). Most likely, the yield would have been the same among the treatments in the absence of this climatic event. Average soil mineral N values recorded at maize harvest were lower in COM than in UREA soil, and a similar pattern was observed during incubation: the average difference in soil mineral N was 20% and the decline of potentially mineralizable N from 65 to 47 mg kg^{-1}. The reduction in soil mineral N may be due to the reduced contribution of compost to maize nutrition (Amlinger et al. 2003) under field conditions and N immobilization by soil microflora that occurred during incubation (Nyamangara et al. 1999). In any case, maize performance was not limited due to the high SOM mineralization rate in the TO soil.

Soil laboratory incubations for predicting maize yield in the field

Soil mineral N production of NA soil (for both 0N and COM treatments) was close to zero during the first 42 days of incubation, corresponding to a maize thermal time of 785 GDD that covers the period from emergence to silking. This virtually means that during stem elongation, the mineral N content of soil was not able to sustain crop growth also considering that under field conditions, moisture and temperature could be not optimal for mineralization processes. The N mineralization pattern during the incubation of NA soil explains why crop growth was lower than expected by considering soil mineral N of the field experiment and suggests excluding the sole organic fertilization as a N source for maize under field conditions. In contrast, TO soil showed a higher attitude to produce soil mineral N, with a fast increase during the first weeks that perfectly fitted with a one pool negative exponential function. The unfertilized soil showed a cumulative soil mineral N of approximately 56 mg kg^{-1} in 756 GDD, corresponding to approximately 268 kg N ha^{-1} in the 0-40 layer (B.D. of 1.2 Mg ha^{-1}). This indicates that TO soil is able to feed maize regardless of the type of fertilization management and explains why unfertilized soil allowed a N uptake value close to 200 kg N ha^{-1} in the first two years of experiment. The significant correlation ($p = 0.06$) between cumulative soil mineral N at 42 days and maize uptake suggested that short-term incubation can be used to predict N uptake if the incubation time is synchronized with crop growth according to the concept of thermal units. Averaging values during the 42 days period increased the performance of the correlation ($p<0.05$) most likely because mean soil mineral N values can be considered as an integrated index of the different mineralization patterns occurring during the incubation period. Cumulative soil mineral N at 112 days was a poor predictor of maize N uptake, meaning that medium and

373 long-term incubation is not a wise option when the aim is to estimate N availability in crops under field conditions using soil
374 mineral N data. Nevertheless, this approach is still the best choice for iterative fitting procedures aimed at finding the best
375 functional model to describe N mineralization in different soils.
376
377 Matching microbiological indices with soil chemical data from the field and laboratory experiment
378
379 The use of PCR to quantify the abundance of the functional and bacterial genes *nifH*, *amoA*, *nirK* and *nirS* encoding enzymes
380 involved in the N-fixation, nitrification and denitrification, respectively, is a sensitive method for evaluating the effect of
381 different soil types and fertilizers on the abundance of the relative bacterial communities. These measurements provide
382 indications on the effects of soil environmental factors, such as nutrients, oxygen status, pH, pollutants, agro-chemicals,
383 moisture, texture and temperature, on the abundance of these bacterial communities (Sharma et al. 2007). Our results confirm
384 that the abundance of these bacterial communities depends on soil proprieties (Delmont et al. 2014) and fertilization type.
385 N-fixing bacteria provide an important source of N to natural ecosystems (Hayden et al. 2010). Different physical
386 soil properties, such as texture, aggregate size and clay content, can influence N fixation in arable soils (Roper and Smith
387 1991; Poly et al. 2001). The higher abundance of diazotrophic bacteria in NA than TO soil highlights a positive correlation
388 between clay texture and the abundance of the *nifH* gene, confirming what reported by Pereira et al. (2013). Furthermore,
389 diazotrophic community composition has been shown to respond to changes in the type of the added N (Orr et al. 2011).
390 Organic fertilizers can stimulate N fixation and bacterial abundance (Keeling et al. 1998; Kondo and Yasuda 2003), while
391 available N can stimulate or inhibit both (Poly et al. 2001; Tan et al. 2003). Compost fertilization positively affected the
392 abundance of N fixers in NA soil at 42 days of incubation; however, the effect decreased over the incubation period, probably
393 as result of the available SOM added to the soil (Pepe et al. 2013).
394 In contrast, high N content in response to nitrate and/or ammonium added to soil may inhibit dinitrogenase reductase
395 and thus N fixation (Lindsay et al. 2010). The higher *nifH* abundance in NA soil than in TO soil, characterised by the lower
396 mineral N content, confirms this hypothesis. Additional confirmatory evidence comes from the fact that the lowest abundance
397 of diazotrophics was in the UREA treated soils. However, Rodriguez-Blanco et al. (2015) reported that the abundance and
398 composition of diazotrophic bacterial populations can be influenced by N-fertilization as well as by plant cultivar.
399 Ammonia-oxidising bacteria are considered to conduct the rate-limiting reaction of nitrification (Kowalchuk and
400 Stephen 2001). Soil and fertilization types exerted a different effect on nitrifying populations. Abundance of ammonia-
401 oxidising bacteria was the lowest at day 42 in NA soil, suggesting a lower nitrification potential that matches well with mineral

N values of the same sampling date for the 0N treatment. Inorganic N fertilization significantly increased the abundance of the *amoA* gene both in NA soil and in TO soil, thus confirming the findings by Chinnadurai et al. (2014). This finding is consistent with the results of Kastl et al. (2015), who found that the abundance of *amoA* increased using inorganic fertilizer and that the *amoA* abundance was positively correlated with increases in fertilization rates. The higher ammonia-oxidising abundance in mineral N-fertilized than in compost-fertilized soils probably depended on the higher NH_4^+-N content of the former soil (Chu et al. 2008). Higher *amoA* gene abundance and soil mineral N accumulated in the UREA than in the organic manure-treated soil (Chu et al. 2007). The effect of inorganic N fertilization was significantly higher in TO soil than in NA soil. The effect of inorganic N fertilization also demonstrated its propensity to produce mineral N, while the high rate of NA soil nitrification was confirmed, even when a readily available source of N was applied to the soil.

Quantification of the abundance of denitrifiers may give insights on the denitrification activity and the potential N_2O fluxes (Henry et al. 2004). Real-time PCR assays, based on the amplification of *nirK* and *nirS* genes, provided a robust estimation of changes in the abundance of denitrifiers (Yin et al. 2014). Denitrifier communities of both soils were dominated by *nirK*, which suggests that in these agricultural soils, nitrification is mostly influenced by nitrite reductase encoded by this gene. This fact may be used to predict NO emissions; indeed, the abundance of the *nirK* gene was positively correlated with NO emission, while the abundance of the *nirS* gene was correlated with N_2O emission (Szukics et al. 2009; Morales et al. 2010). Moreover, according to Yin et al. (2014), inorganic and organic fertilization have no influence on the abundance of the *nirS*-denitrifier population. In contrast, the interaction between soil and fertilization types affected the abundance of *nirK*-denitrifiers. Probably, NA soil had a higher content of anoxic micro-aggregates than the TO soil due to the clay texture. This higher content of anoxic micro-aggregates may have favoured the activity of denitrifying bacteria. Organic C availability has been shown to increase denitrification rates, especially in anoxic environments of aggregate structures that protect and stabilize organic matter (Kong et al. 2010). In contrast, in TO soil, *nirK* gene abundance was probably related to N availability, which increased with UREA inputs or when soil mineral N reached a plateau.

The initial delay phase of NA soil was probably due to the reduced tendency of this soil to produce nitric N and to the increased tendency of this soil to denitrify due to the presence of anoxic sites.

Conclusions

Our experiments showed that there was a good correlation between chemical and microbiological measurements from lab-soil incubation experiments and soil-plant N dynamics of maize cropping systems. Mineralization patterns proved to be useful in optimizing the fertilization management of maize under field conditions as long as incubation time is normalized over the

maize growth cycle according to the simplified model of Growth Degree Days. According to this approach, the average cumulative soil mineral N values, calculated over a short incubation period (42 days) covering the most sensitive phase of crop N requirement, may be a good index of N availability to crops. Moreover, the shape and kinetic parameters of net N mineralization from medium term (112 days) soil incubation provided consistent information on the interaction between fertilizers and native fertility. The abundance of key genes of N cycle provides information about the soil attitude in making N available for plant nutrition as affected by soil properties (i.e. abundance of ammonium fixing clays) and N availability (mineral *vs* organic fertilization). Our work provides a prime starting point for the development of a crop-based approach for using incubation data to manage the fertility of maize. Future research should aim to validate lab-derived N availability indices with different maize cultivars (according to the FAO classification) and in different pedoclimatic conditions to extend our approach to a wide range of maize-based cropping systems.

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587 **Figure captions**

588

589 **Fig. 1** Fertilization effect on soil mineral N (SMN) contents of NA and TO soils (0-60 cm depth layer) at pre-seeding (a)
590 and harvest stage (b). Fertilization treatments: 0N, unfertilized control; COM, compost; UREA, urea. Bars with the same
591 letter are not different at $p<0.05$; n.s. = not significant. Letters are referred to the sum of ammonium-N (grey bars) and
592 nitrate-N (black bars) within the same soil

593 **Fig. 2** Fertilization effect on soil mineral N (a) and NH_4^+-N:NO_3^--N ratio (b) in incubated soils. Fertilization treatments: 0N,
594 unfertilized control; COM, compost fertilization; UREA, urea fertilization. Bars with the same letter are not different at
595 $p<0.05$. In figure 2a letters are referred to the sum of ammonium-N (grey bars) and nitrate-N (black bars)

596 **Fig. 3** Net soil mineral N (n-SMN) patterns in NA soil with different fertilizations: a) 0N, unfertilized control; b) COM,
597 compost; c) UREA, urea. Different letters indicate different measured values within each graph with $p<0.05$
598

599 **Fig. 4** Net soil mineral N (n-SMN)integrated
600 patterns in TO soil with different fertilizations: a) 0N, unfertilized control; b) COM, compost; c) UREA, urea. Different
601 letters indicate different measured values within each graph with $p<0.05$
602

603 **Fig. 5** Average maize N uptake at Napoli (NA) and Torino (TO) sites as a linear function of (a) Cumulative soil mineral N
604 (SMN) after 112 days of incubation (not significant), (b) Cumulative SMN after 42 days of incubation ($p=0.06$), (c)
605 Average SMN of the first 42 days of incubation ($p<0.05$). Fertilization treatment: 0N, unfertilized control; COM, compost;
606 UREA, urea
607

608 **Fig. 6** Soil by fertilization effect on quantification of functional genes involved in N cycling after 42 and 112 days of the
609 incubation experiment. Fertilization treatments: 0N, unfertilized control; COM, compost; UREA, urea. (a) *nifH* gene
610 abundance, nitrogen fixation; (b) *amoA* gene abundance, nitrification; (c) and (d) *nirK* and *nirS* gene abundance,
611 denitrification. Bars with different letters are statistically significantly different ($P < 0.05$); n.s. = not significant

Changes in soil mineral N and abundances of bacterial communities involved in N reactions under laboratory conditions as predictors of soil N availability to maize under field conditions

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Table 1 Main soil physical and chemical properties in the 0-30 cm layer (plowed layer) at Napoli (NA) and Torino (TO) sites (modified from Alluvione et al. 2013)

Soil texture				Chemical properties					
Site	**Sand**	**Silt**	**Clay**	**pH[a]**	**Ac.$CaCO_3$ [a]**	**OC**	**TN**	**Exct. K [b]**	**Olsen P**
		%			g kg^{-1}	g kg^{-1}	g kg^{-1}	cmol kg^{-1}	mg kg^{-1}
NA	46.5	22.3	31.2	7.4	-	7.2	0.9	1.11	31.5
TO	36	56.5	7.5	8.1	24	10.2	1	0.15	16.0

[a] pH at 1:2.5 soil:water ratio
[b] Active $CaCO_3$
[c] Exractable P

Table 2 Primers and qPCR conditions for the real-time PCR quantifications of *nifH*, *amoA*, *nirK* and *nirS* genes on soil samples collected at day 42 and 112 of laboratory incubation

Target gene(s)	Primer	Nucleotide sequences[a] (5'-3')	Reference(s)	qPCR conditions
Nitrogenase	nifH-F	AAAGGYGGWATCGGYAARTCCACCAC	Rösch et al. (2002)	95°C for 45 s, 55°C for
reductase (*nifH*)	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	Rösch et al. (2002)	45 s, 72°C for 45 s
Monooxygenase	amoA-1F	GGGGTTTCTACTGGTGGT	Rotthauwe et al. (1997)	95°C for 60 s, 55°C for
(*amoA*)	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	Rotthauwe et al. (1997)	60 s, 72°C for 60 s
Nitrite reductase	nirK-876	ATYGGCGGVAYGGCGA	Henry et al. (2004)	95°C for 15 s, 53°C for
(*nirK*)	nirK-5R	GCCTCGATCAGRTTRTGG	Braker et al. (1998)	30 s, 72°C for 30 s
Nitrite reductase	nirS-cd3aF	AACGYSAAGGARACSGG	Kandeler et al. (2006)	95°C for 45 s, 59°C for
(*nirS*)	nirS-R3cd	GASTTCGGRTGSGTCTTSAYGAA	Kandeler et al. (2006)	45 s, 72°C for 45 s

[a] Y = C or T; R = A or G; W = A or T; K = G or T; S = C or G; V = A, C, or G.

Table 3 Maize N uptake: year by fertilization effect at Naples (NA) and Turin (TO) sites. Fertilization treatments: 0N, unfertilized control; COM, compost; UREA, urea (modified from Alluvione et al., 2013)

		NA				TO			
		2006	**2007**	**2008**	**Average**	**2006**	**2007**	**2008**	**Average**
N uptake (kg N ha^{-1})	0N	178.0	57.5	81.8	**105.8**	253.5	177.2	113.9	**181.5**
	COM	42.7	40.6	46.7	**43.3**	246.9	220.0	140.6	**202.5**
	UREA	253.3	186.0	115.6	**185.0**	287.1	222.4	197.9	**235.8**
	Average	**158.0**	**94.7**	**81.4**	**111.3**	**262.5**	**206.5**	**150.8**	**206.6**
		P-level	**Lsd**			**P-level**	**Lsd**		
Effects	Treatment	0.000	-			0.016	-		
	Year	0.001	-			0.000	-		
	Interaction	0.000	23.1			0.047	28.5		

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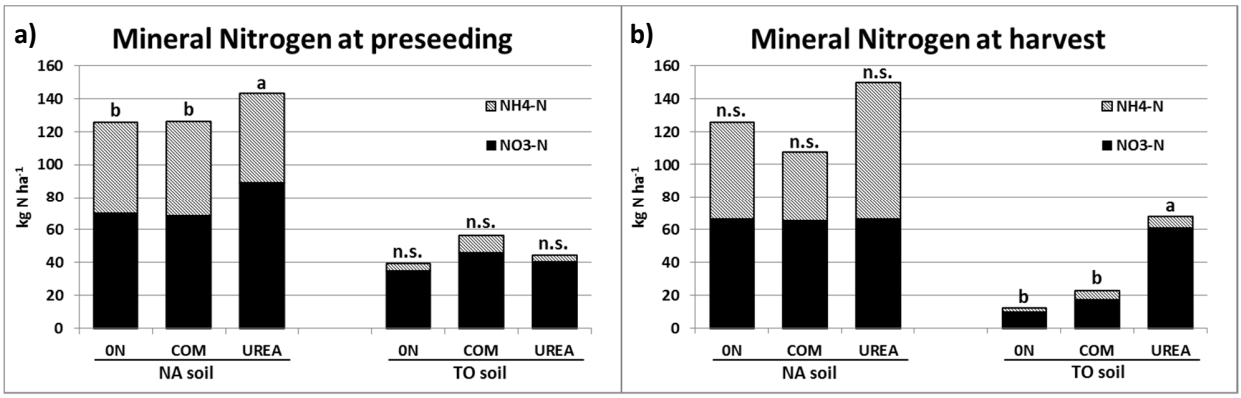
a)
Mineral Nitrogen at preseeding
kg N ha-1
NH4-N
NO3-N
b
b
a
n.s.
n.s.
n.s.
0N
COM
UREA
NA soil
0N
COM
UREA
TO soil
b)
Mineral Nitrogen at harvest
n.s.
n.s.
n.s.
b
b
a

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Table 1 Main soil physical and chemical properties in the 0-30 cm layer (plowed layer) at Napoli (NA) and Torino (TO) sites (modified from Alluvione et al. 2013)

Soil texture				Chemical properties					
Site	**Sand**	**Silt**	**Clay**	**pH[a]**	**Ac.$CaCO_3$ [a]**	**OC**	**TN**	**Exct. K [b]**	**Olsen P**
		%			g kg^{-1}	g kg^{-1}	g kg^{-1}	cmol kg^{-1}	mg kg^{-1}
NA	46.5	22.3	31.2	7.4	-	7.2	0.9	1.11	31.5
TO	36	56.5	7.5	8.1	24	10.2	1	0.15	16.0

[a] pH at 1:2.5 soil:water ratio
[b] Active $CaCO_3$
[c] Exractable P

Table 2 Primers and qPCR conditions for the real-time PCR quantifications of *nifH*, *amoA*, *nirK* and *nirS* genes on soil samples collected at day 42 and 112 of laboratory incubation

Target gene(s)	Primer	Nucleotide sequences[a] (5'-3')	Reference(s)	qPCR conditions
Nitrogenase	nifH-F	AAAGGYGGWATCGGYAARTCCACCAC	Rösch et al. (2002)	95°C for 45 s, 55°C for
reductase (*nifH*)	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	Rösch et al. (2002)	45 s, 72°C for 45 s
Monooxygenase	amoA-1F	GGGGTTTCTACTGGTGGT	Rotthauwe et al. (1997)	95°C for 60 s, 55°C for
(*amoA*)	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	Rotthauwe et al. (1997)	60 s, 72°C for 60 s
Nitrite reductase	nirK-876	ATYGGCGGVAYGGCGA	Henry et al. (2004)	95°C for 15 s, 53°C for
(*nirK*)	nirK-5R	GCCTCGATCAGRTTRTGG	Braker et al. (1998)	30 s, 72°C for 30 s
Nitrite reductase	nirS-cd3aF	AACGYSAAGGARACSGG	Kandeler et al. (2006)	95°C for 45 s, 59°C for
(*nirS*)	nirS-R3cd	GASTTCGGRTGSGTCTTSAYGAA	Kandeler et al. (2006)	45 s, 72°C for 45 s

[a] Y = C or T; R = A or G; W = A or T; K = G or T; S = C or G; V = A, C, or G.

Table 3 Maize N uptake: year by fertilization effect at Naples (NA) and Turin (TO) sites. Fertilization treatments: 0N, unfertilized control; COM, compost; UREA, urea (modified from Alluvione et al., 2013)

		NA				TO			
		2006	**2007**	**2008**	**Average**	**2006**	**2007**	**2008**	**Average**
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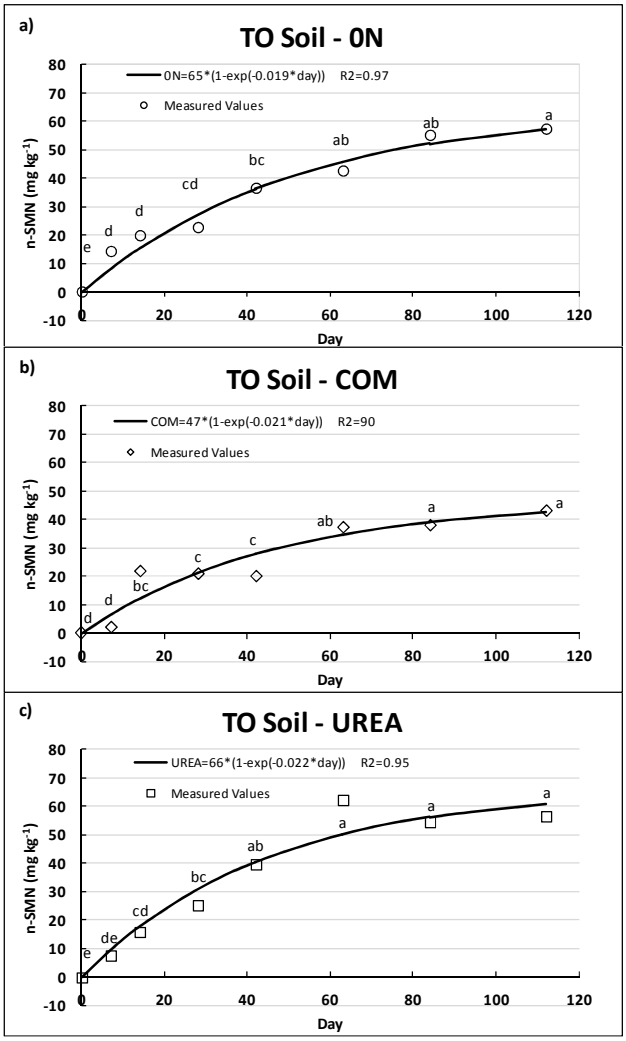
a)
TO Soil - 0N
0N=65*(1-exp(-0.019*day)) R2=0.97
Measured Values
n-SMN (mg kg-1)
Day
b)
TO Soil - COM
COM=47*(1-exp(-0.021*day)) R2=90
Measured Values
n-SMN (mg kg-1)
Day
c)
TO Soil - UREA
UREA=66*(1-exp(-0.022*day)) R2=0.95
Measured Values
n-SMN (mg kg-1)
Day

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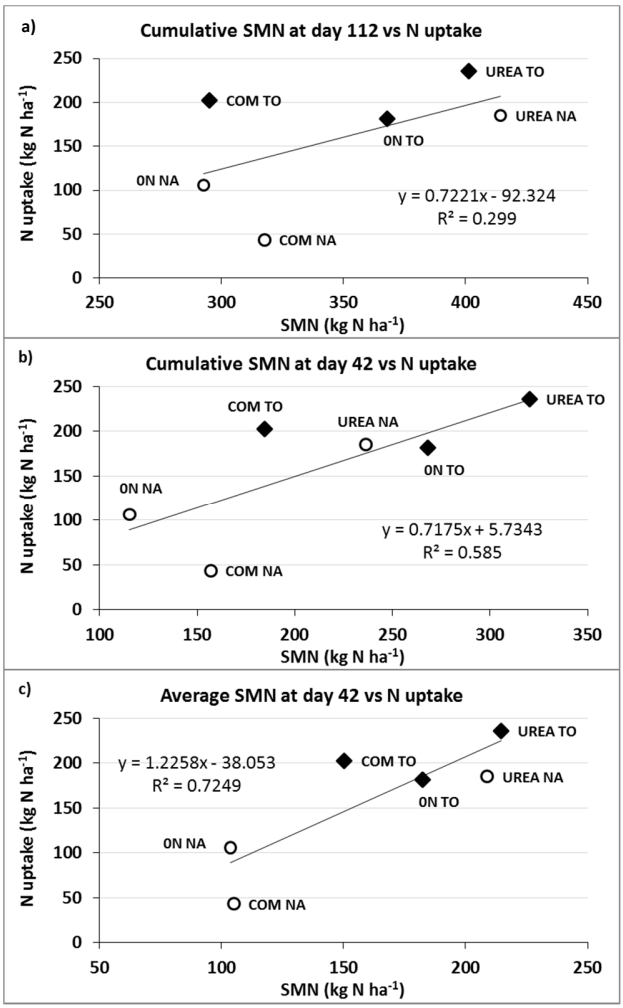
a)
Cumulative SMN at day 112 vs N uptake
N uptake (kg N ha-1)
SMN (kg N ha-1)
UREA TO
COM TO
UREA NA
0N TO
0N NA
COM NA
y = 0.7221x - 92.324
R² = 0.299
b)
Cumulative SMN at day 42 vs N uptake
N uptake (kg N ha-1)
SMN (kg N ha-1)
COM TO
UREA NA
UREA TO
0N TO
0N NA
COM NA
y = 0.7175x + 5.7343
R² = 0.585
c)
Average SMN at day 42 vs N uptake
N uptake (kg N ha-1)
SMN (kg N ha-1)
y = 1.2258x - 38.053
R² = 0.7249
UREA TO
COM TO
UREA NA
0N TO
0N NA
COM NA

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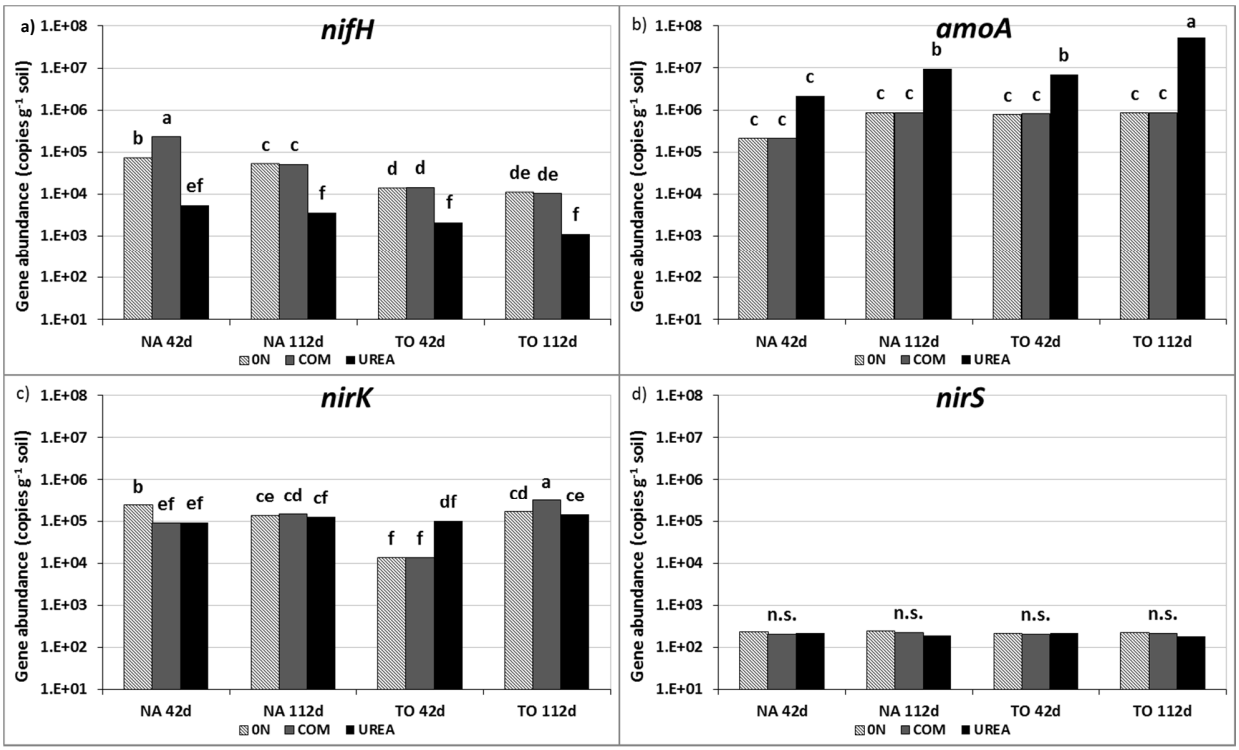
a)
nifH
b)
amoA
c)
nirK
d)
nirS
Gene abundance (copies g-1 soil)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
NA 42d
NA 112d
TO 42d
TO 112d
0N
COM
UREA
b a ef
c c f
d d f
de de f
c c c
c c b
c c b
c c a
b ef ef
ce cd cf
f f df
cd a ce
n.s.