Nitrogen dynamics in soils fertilized with digestate and mineral fertilizers: a full field approach.

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Abstract

Highly stabilized digestate from sewage sludge and digestate-derived ammonium sulphate (RFs), were used in a comparison with synthetic mineral fertilizers (SF) to crop maize in a three-year plot trial in open fields. RFs and SF were dosed to ensure the same amount of mineral N (ammonia-N). In doing so, plots fertilized with digestate received much more N (+185 kg ha^{-1} of organic N) because digestate also contained organic N. The fate of nitrogen was studied by measuring mineral and organic N in soil at different depths, ammonia and N_2O emissions, and N uptake in crops. Soil analyses indicated that at one-meter depth there was no significant difference in nitrate content

between RF, SF and Unfertilized plots during crop season indicating that more N dosed with digestate did not lead to extra nitrate leaching. Ammonia emissions and N content in plants and grains measured were also similar for both RF and SF. Measuring denitrification activity by using gene makers resulted in a higher denitrification activity for RF than SF. Nevertheless, N_2O measurements showed that SF emitted more N_2O than RF (although it was not statistically different) (7.59 ± 3.2 kgN ha^{-1} for RF and 10.3 ± 6.8 kgN ha^{-1} for SF), suggesting that probably the addition of organic matter with digestate to RF, increased the denitrification efficiency so that N_2 production was favoured. Soil analyses, although were not able detecting N differences between SF and Rf after three years of cropping, revealed a statistical increasing of total carbon, suggesting that dosing digestate lead to carbon (and maybe N) accumulation in soil. Data seem to suggest that N_2O/N_2 emission and organic N accumulation in soil can explain the fate of the extra N dosed (organic-N) in RF plots.

Keywords: Digestate; Nitrate leaching; Nitrous oxide emissions; Nitro-denitrification activities; N-cycle related microorganisms.

1. Introduction

Modern intensive agriculture relies on the use of high amounts of mineral fertilizers with particular attention to nitrogen (N) that is an essential nutrient for plants. In the last decades, the use of synthetic N fertilizers has led to an unprecedented increase in worldwide agricultural production, which more than tripled between 1961 and 2020 (FAOSTAT). However, the production and use of synthetic N fertilizers has a strong ecological impact. In fact, their production, through the Haber-Bosch process, requires large amounts of energy and therefore the emission of carbon dioxide (CO_2). It is estimated that annually the production of N fertilizers requires about 2-3% of the energy consumed globally and is responsible for 1.2% of the CO_2 emitted (Gaidajis and Kakanis, 2021; Smith et al., 2020). Moreover the production and use of these fertilizers is also responsible for serious emissions of other greenhouse gases (GHG), in particular nitrous oxide (N_2O) (Hasler et al., 2015).

A solution to limit energy consumption and greenhouse gas emissions can be the use of organic waste and animal slurries. In fact, these products contain significant amounts of nutrients and organic matter, which could be potentially recovered and used for the production of fertilizers, in order to substitute synthetic products, to develop a circular economy reducing the problem of managing these substances, which are often dispersed into the environment (Herrera et al., 2022; Rockström et al., 2009; Steffen et al., 2015; Toop et al., 2017). In fact, animal manures and other organic by-products have been traditionally applied to land as fertilizers for growing crops and improving the physical and chemical properties of soil. However, the incorrect use of these by-products as fertilizers in agriculture can lead to environmental problems due to over-dosage or wrong timing in the dosage of phosphorus (P) and nitrogen (N), which can be run off and/or leached, reaching water bodies (Rockström et al., 2009; Steffen et al., 2015; Toop et al., 2017). Moreover, gaseous forms of nitrogen, such as ammonia (NH_3) and nitrous oxide (N_2O), if released into the atmosphere, are responsible for air pollution (ammonia) and an increase in the greenhouse effect (N_2O) (Cameron et al., 2013; Delgado, 2002; Reay et al., 2012). As consequence of that, chemical, physical, and biological technologies have been proposed to transform organic wastes into fertilizers and to increase these fertilizer performances and their safety (Sigurnjak et al., 2019). Among these technologies, anaerobic digestion (AD) has gained increased attention in recent decades as an effective technology to convert untreated organic wastes into useful products, with the added benefit of producing renewable energy (Pigoli et al., 2021; Herrera et al., 2022). AD is an anaerobic, microbial-driven process, which converts organic matter (OM) into biogas and digestate. Biogas produced is composed mainly of methane (~60%) and carbon dioxide (~40%), and it can be used to produce heat and electricity (Sheets et al., 2015). In addition, the AD process leaves as a by-product a nutrient-rich organic sludge, called digestate, which has been proved to be a valuable fertilizer and soil amendment. Digestates contain a variable amount of mineral nitrogen (ammonia), which is readily available to plants, so it can be used to substitute for mineral synthetic fertilizers. On the other hand, it also contains an organic N fraction which contributes to plant nutrition and/or soil N, needing to be better elucidated. In fact, organic N

contained in the digestates, if not well stabilized, can lead to a risk of nitrogen losses into the environment as nitrate because of uncontrolled nitrification (Nkoa, 2014). On the other hand, it has been shown that by extending the duration of the AD process, the organic N contained in the digestate is highly stabilized (Pigoli et al., 2021), because labile organic N is mineralized to ammonia under controlled conditions (Möller and Müller, 2012; Nyang'au et al., 2022), leading to a low mineralization rate and reducing nitrate leaching.

This work aims to evaluate the effects of the use of well-stabilized digestate and derived-digestate N fertilizers to substitute synthetic mineral fertilization on the soil N-cycle. To do so a full field plots experiment was carried out for three cropping seasons (2018 – 2019 – 2020), comparing the use of recovered fertilizers (digestate and digestate-derived ammonium sulphate) with conventional synthetic nitrogen fertilization (urea and commercial ammonium fertilizers) on a maize crop. Mineral N forms within the soil at different depths, soil emissions and the dynamics of nitrification and denitrification in soils were monitored during the experiment.

2. Material and Methods

2.1 Experimental site and setup

The experiments were carried out over three consecutive agronomic seasons (2018 - 2019 - 2020) on a maize crop, with experimental plots in triplicate and using a randomized scheme. The experimental field was located in the Po Valley (northern Italy). All the experiments compared the use of two different fertilization strategies: fertilization with recovered fertilizers (RF) (slurry-like digestate from sewage sludge in pre-sowing, and digestate-derived ammonium sulphate in topdressing) versus fertilization with synthetic fertilizers (SF) (solid granular urea in pre-sowing and commercial ammonia sulphate in topdressing), in addition to an unfertilized (U) control (Table S1).

For RF plots, distribution and dosing of digestate was carried out at the pre-sowing stage, assuming a nitrogen efficiency of 0.5, in compliance with Italian legislation, and in order to meet agronomic requirements for maize (Regione Lombardia, 2020). A tank car joined to a rigid multi-anchor-

subsoiler coupled with a Retrofit Variable-Rate Control (VRT control) was used for digestate spreading through injection at 15 cm depth. Distribution of digestate-derived ammonium sulphate was carried out in topdressing through fertigation. For SF plots, distribution of granular urea occurred at pre-sowing time, through distribution on the soil surface, as is common practice in Northern Italy. At topdressing, commercial granular ammonium sulphate was spread onto the soil surface.

Fertilization procedure followed the normal procedure adopted in Italy, i.e. surface distribution of urea and digestate injection. Although urea contains a ureic-N form, superficial distribution can lead to higher ammonia emission than after its burial, but it represents the standard procedure in Italy. On the other hand, digestate injection is suggested because the presence of ammonia can lead to high N losses. In any case, fertilizers were dosed to bring similar amounts of mineral N (ammonia nitrogen, $N\text{-}NH_4^+$), i.e. equal amounts of readily available N for plants. The quantities of nitrogen dosed and the chronological list of agronomic operations are reported in Table 1 and Table S1.

The main chemical characteristics of the soil exploited, before the start of experimentation (2018) and after three years (2021) are reported in Table 2.

A full characterization of the digestate and ammonium sulphate used, and a description of the anaerobic digestion process are reported in Pigoli et al. (2021).

2.2 Fertilizer sampling and analysis

The digestate and digestate-derived ammonium sulphate used in this work were sampled directly from the tank car immediately before the spreading; and analysed immediately.

Determination of pH was performed in aqueous solution using a 1:2.5 sample/water ratio. Total Organic Carbon (TOC) and nitrogen content, in terms of Total Kjeldahl Nitrogen (TKN) and Total Ammonia Nitrogen (TAN) were determined in compliance with standard procedures of the American Public Health Association and analytical methods for wastewater sludges (APHA, 1992; IRSA CNR, 1994). Inductively coupled plasma mass spectrometry (Varian, Fort Collins, USA), preceded by acid digestion (EPA, 1998) was used for the determination of phosphorus and heavy metals content.

Biochemical methane production (BMP) was determined following the biological method, according to the European regulations for fertilizers (EU, 2019). All the analyses were carried out in triplicate. The main characteristics of digestate and digestate-derived ammonium sulphate are shown in Tables S2 and S3.

2.3 Soil sampling and analysis

Soils were sampled eleven times during two agronomic seasons in 2019 and 2020 (Table 1). Samplings were carried out taking from each plot, samples at increasing depth: 0-25 cm, 25-50 cm, 50-75 cm and 75-100 cm); each sample was formed by three subsamples. The samples were then immediately transported to the laboratory and stored at both 4° C for subsequent chemical analysis and at -20 °C for DNA extraction. All samples were analysed within a short time from the sampling date.

For chemical analysis, samples were air dried, sieved to 2 mm and then ground to 0.5 mm. Soil pH was determined in aqueous solution using a 1:2.5 sample/water ratio (McLean, 1982) and soil texture by the pipette method (Gee and Bauder, 1986). Cation Exchange Capacity (CEC) was determined by saturating the samples with $BaCl_2$ (Rhoades, 1982). Total organic carbon (TOC) was determined by the Walkley and Black method (Olsen et al., 1982), and Total nitrogen, ammonia nitrogen and nitrate nitrogen were determined by the Kjeldahl method with Devarda's alloy (Faithfull, 2002). All the analyses were carried out in triplicate.

A chronological diagram including all management practices and soil sampling dates is shown in Table 1.

2.4 Determination of the timing of nitrogen transformation in soil

The timing of transformation in soil of the nitrogen dosed by fertilization was estimated by preparing microcosms in the laboratory. In February 2019, 1 kg of soil was sampled randomly from each of the experimental plots used in this work, and for each of them a microcosm consisting of a cylindrical jar

with a diameter of 20 cm closed on the bottom and without a lid, was created. Urea or digestate were added to microcosms corresponding to the SF and RF plots, respectively, and dosed in order to add the same amount of nitrogen used in the field in pre-sowing fertilization. The water content of the soils was brought to 60% of the maximum water capacity and kept constant throughout the experiment. After preparation, the microcosms were incubated in a thermostatic chamber, varying the temperature during the experiment. The temperatures were chosen based on the average temperatures of the soil (10 cm depth) measured near the experimental fields in the period April - July (average for the 10 years from 2008 to 2018) (data provided by ERSAF Lombardia, personal communication).

On a weekly basis, for 112 days, the soil of each of the microcosms was analysed, determining the concentration of ammonium and nitrate, using the same methods already described for the soil analyses in the previous paragraph.

2.5 Ammonia and nitrous oxide emissions measurement

The ammonia emission data reported in this work were collected as previously reported by Zilio et al. (2021). For all the experiments, the ammonia emitted from the experimental plots was measured in the hours following the pre-sowing injection/spreading. All the digestate injections took place at the same hour (h. 11:00), and the first sampling was always carried out 10 hours later (21:00).

The experiments were repeated for three consecutive years on the same experimental plots. In particular, the soil used showed a neutral pH (7 ± 0.4), it was rich in silt (44% ± 2.1) and it was relatively poor in clay (10% ± 0.5). The amounts of ammonia nitrogen dosed at pre-sowing were kept almost unchanged for all the three years tested, i.e. 200 - 229 and 185 kg N ha^{-1} for RF and SF, respectively (Table 1). The concentration of NH_3 was monitored by the exposure of ALPHA passive samplers (Riva et al., 2016; Tang et al., 2001). For each plot, one monitoring point was set up at the center of the plot, with the ALPHA samplers exhibited in sets of three. Samplers height was 0.70 m, within the range suggested by Sommer et al. (2005). To obtain background environmental

concentration values, an additional sampling point was placed at a distance of about 1,000 meters away from the fertilized fields and other possible point sources of NH_3, as suggested by Carozzi et al. (2013). Each sampler located in the plot was replaced a minimum of twice a day near sunrise and sunset, to be able to monitor the variation of atmospheric turbulence which has a direct effect on the dispersion of pollutants. During the application day and the following day, the substitution was done when the vehicles entered the field, for fertilization and for incorporation. The study of atmospheric turbulence was carried out by using an ultrasonic anemometer (10 Hz) positioned in the plots near to the samplers.

By processing the NH_3 concentration information, an analysis of the dispersion of NH_3 in the atmosphere was performed through the application of the dispersion model (WindTrax, Thunderbeach Scientific, CA). The obtained dispersion coefficient (D; s m^{-1}) was used to determine the flow (S; µg NH_3 m^{-2} s^{-1}) emitted from the fertilized surface, on the basis of the concentrations measured in each plot (C; µg m^{-3}) and environmental (C_{bgd}; µg m^{-3}), according to the following equation:

$$S = \left(C - C_{bgd}\right) \times D^{-1}$$

The ammonia emission factor (EF%) was obtained from the ratio between the released N-NH_3 (kg ha^{-1}) and the calculated amount of ammonia nitrogen (N-NH_4; kg ha^{-1}) spread onto the soil with fertilizations.

Determination of nitrous oxide (N_2O) fluxes was performed from 28 May 2020 (pre-sowing) to 17 March 2021, through the use of non-steady-state chambers as reported in literature (Bertora et al., 2018; Gregorich et al., 2005).

Chambers were supported by anchors inserted in soil down to 20 cm depth, in order to ensure the isolation of the soil column; chambers were set up in triplicate for each of the treatments (Recovered fertilizers, Synthetic fertilizers and Unfertilized control) (Peyron et al., 2016; Tang et al., 2001).

Air flows were sampled at predefined times during the monitoring period; collected air samples were analysed in a laboratory for their concentrations of N_2O, by gas chromatography, as reported by Piccini and colleagues (Piccini et al., 2017).

Emissive flow of the gas from the soil was estimated using the following general equation:

$$F = H \times dC/dt$$

where *F* is the flow, *H* is the ratio between air volume and soil surface isolated from the chamber, corresponding to the height of the chamber (m), and *t* is the closing time of the chamber.

The dC/dt ration was calculated by linear regression between concentrations and sampling times, if the increase in gas concentrations in the chamber was linear; HM model was applied in case of non-linear accumulation trend (Peyron et al., 2016). Cumulative emissions were estimated through linear interpolation between sampling days.

2.6 Soil microorganisms' quantification by qPCR

The consistency of N related microbial populations in soil was assessed targeting 5 key genes (*amoA* archaea, *amoA* eubacteria, *nifH*, *nirK*, *nosZ*) by qPCR. The *amoA* genes in both archaea and eubacteria are responsible for ammonia oxidation, the rate-limiting step of nitrification, and *nifH* gene encodes the iron protein of nitrogenase, the enzyme that catalyses N_2 fixation in eubacteria. The *nirK* gene encodes for bacterial nitrite reductase activity, converting nitrite into nitrous oxide, while *nosZ* encodes for bacterial nitrous oxide reductase enzyme, involved in the terminal step of denitrification. Determination was carried out on the same soils sampled in parallel for chemical characterization, for seasons 2019 and 2020, following the method already used in Zilio et al., (2020). Primers' sequences and references are reported in Table S4.

2.7 Statistical analysis

The statistical analyses were carried out using IBM SPSS® 23 software. Unless otherwise specified, the significance limit value p was set at 0.05 for all the analyses carried out. Plots and graphs were obtained through the use of Microsoft EXCEL 2016.

3. Results and Discussion

3.1 Nitrogen in soil

Soil N content was assessed during the second and third years of experimentation (crop seasons 2019 and 2020) by monitoring the concentrations of nitrate ($N\text{-}NO_3^-$) (Figure 1 and Table S5) and ammonia ($N\text{-}NH_4^+$) (Figure S1 and Table S6) in experimental soils at different soil depths.

The surface layer (0-25 cm) is the one most affected by N fertilization (Chen et al., 2016; Janzen et al., 1990; Zilio et al., 2020) and therefore it is interesting to start the discussion by focusing on it.

For the surface soil layer the data showed a high variability in the $N\text{-}NO_3^-$ concentrations over time (Figure 1a). Nitrate content was similar in plots before pre-sowing fertilization (on 4 April 2019): 12.14 ± 3.48 mg kg^{-1} dw, 12.69 ± 3.8 mg kg^{-1} dw and 9.63 ± 0.2 mg kg^{-1} dw, respectively for Unfertilized, SF and RF. Then, after about two months from sowing, the NO_3^- content in fertilized soils significantly increased, to 41.82 ± 11.6 mg kg^{-1} dw and 45.57 ± 6.81 mg kg^{-1} dw, respectively for SF and RF, indicating that ammonia-N of fertilizers was converted into nitrate. These figures agreed with tests carried out in the laboratory to estimate the mineralization and nitrification of fertilizers under simulated full field conditions, which showed a decrease of ammonium concentration that coincided with nitrate increasing (Figure S2) confirming, also, previous findings (Tambone and Adani, 2017).

After the topdressing fertilization (on 1 August 2019, 91 days after sowing), the nitrate concentration remained rather stable for the SF plots (38.17 ± 5.67 mg kg^{-1} dw), while it increased in the RF plots (58.66 ± 6.56 mg kg^{-1} dw). In this case, topdressing fertilization did not seem to contribute to total nitrate soil content, presumably because only two days had passed between fertilization and soil sampling, suggesting that higher nitrate content in RF than SF was due, probably, to a higher

nitrification process because of the higher N dosed at pre-sowing stage with RF than SF (Table 1). Nitrate concentrations in the following period dropped dramatically, consistent with the presence of developed plants that absorbed large quantities of mineral N, removing it from the soil (Ciampitti and Vyn, 2011; Shinano et al., 1994). Very low nitrate contents were registered at harvest (on 24 September 2019, 145 days after sowing), to 3.11 ± 0.53 mg kg^{-1} dw, 3.08 ± 0.88 mg kg^{-1} dw and 0.41 ± 0.12 mg kg^{-1} dw, respectively for Unfertilized, SF and RF. In the same period, ammonium concentrations in soils also largely dropped (Figures S1).

The data collected during the 2020 crop season confirmed the trends of the previous year. The concentrations of nitrate in the experimental soils at the beginning of the season (on 16 May 2020) before pre-sowing fertilization were similar to those of 2019, i.e. 12.34 ± 1.3 mg kg^{-1} dw, 15.04 ± 0.79 mg kg^{-1} dw and 10.17 ± 2.04 mg kg^{-1} dw, respectively for Unfertilized, SF and RF (Figure 1). Then, nitrate concentrations in the fertilized experimental soils significantly increased reaching values similar to those observed in 2019, i.e. 53.38 ± 5.7 mg kg^{-1} dw for SF and 42.48 ± 7.28 mg kg^{-1} dw for RF (Figure 1) to remain thereafter essentially stable until plant harvesting (on 5 November 2020, 146 days after sowing), when strong nitrate reductions were registered, i.e. 3.22 ± 0.3 mg kg^{-1} dw, 6.02 ± 0.53 mg kg^{-1} dw and 7.2 ± 1.4 mg kg^{-1} dw. Ammonium concentrations were mainly stable during the crop season and no differences were found between fertilized and unfertilized plots, as reported for the 2019 cropping season (Figure S1).

Observing the soil layers below the surface (25-50 cm and 50-75 cm layers) (Figure 1) during the two monitored crop seasons, the nitrate concentrations remained considerably lower than those measured for the 0-25 cm layers. This trend was confirmed for the 75-100 cm depth layer, for which low nitrate contents were registered and no differences between SF and RF plots were observed. In fact, at the 75-100 cm depth layer, the concentrations of nitrates remained in the range 0-8.32 ± 1.37 mg kg^{-1} dw (average of 5.94 ± 1 mg kg^{-1} dw) for Unfertilized plots, 0-13.02 ± 1.97 mg kg^{-1} dw (average of 7.73 ± 1.5 mg kg^{-1} dw) for SF plots and 0-12.69 ± 1.4 mg kg^{-1} dw (average of 7.78 ± 1.8 mg kg^{-1} dw) for

RF plots. These figures were similar to nitrate contents reported in the literature for undisturbed soils at the same depth, i.e. 9.6 mg kg^{-1} dw (Ryden et al., 1984).

Low nitrate (and ammonia) contents recorded at 75-100 cm for fertilized plots were similar to the nitrate concentration observed for the non-fertilized soils, suggesting that N added as fertilizers did not leach during the cropping season. This fact was more surprising when considering that RF plots received about 460 kg ha^{-1} of N, i.e., double the amount of N dosed to SF plots. These results suggest a question related to the fate of N added with fertilizers, above all considering the extra N dosed with digestate in RF plots.

Data previously reported (Zilio et al., 2021) for the same plots under study, excluded more N loss under gaseous form (NH_3) for RF than SF, despite the different method of application (surface for SF, injection + fertigation for RF) . In fact, total ammonia losses measured were similar for SF and RF plots, i.e. 24.8 ± 8.3 kg N ha^{-1} (corresponding to 13.4 ± 4.5% of the TAN) for SF and 25.6 ± 9.4 kgN ha^{-1} (corresponding to 11.6 ± 4% of the TAN) for RF (Table S7). At the same time, one can exclude a higher N uptake by corn cultivated using RF because the average grain production, the average grain N content and the N content in plant tissues were similar for RF and SF plots (Table S7). Lastly, no N accumulation in soil occurred for plots fertilized with RF in comparison with those fertilized with SF, in the results from soil analyses (Table 2). Therefore, excluding for RF plots more nitrate leaching, N accumulation in crops and/or ammonia emission than for SF, N evolution as N_2O (and N_2) needs to be investigated.

3.2 N_2O emissions

During the 2020 crop season N_2O emissions were monitored from the experimental plots for a total of 293 days, i.e., from 28 May 2020 to 17 March 2021 (Table S8). In the first days of measurements, the emissions were low for all the experimental plots (Figure 2). However, two-three weeks after spreading a sudden increase of the emissions in SF and RF plots was observed. A delay in the N_2O emission peak after fertilization has been observed in many works (Akiyama and Tsuruta, 2003; Dalal et al., 2003; Roy et al., 2014; Signor and Cerri, 2013; Velthof and Mosquera, 2011).

Starting from mid-June 2020, the N_2O emissions from SF and RF soils began to increase very fast, peaking about one month after fertilization, to remain consistent until mid-July, after which they dramatically decreased (Figure 2). N_2O emission did not coincide with high nitrate presence in the soil, probably because in the first weeks after sowing, plants which were at an early stage of development (before the second leaf), did not compete for nitrate with denitrifying bacteria (Ciampitti and Vyn, 2011), leading to N_2O (and N_2) production (June 2020) (Figure 2).

Then, in early July, the amounts of N_2O emitted were progressively reduced until they reached very low values for the rest of the season (Figure 2), even though in mid-July 2020 (see section 3.1) the nitrate soil content for both SF and RF remained quite high (Figure 1 and S1). Probably at that time the presence of more developed plants (6-7 leaves), competing with nitrifying and denitrifying microorganisms for nitrate (see section 3.3) caused the decrease of N_2O emission. Indeed, as reported in the literature, the short life cycle of soil microorganisms and unidirectional N flux from soil to roots facilitates the relocation of N from microorganisms to roots. This enables plants to become winners in the competition for N in soil (Kuzyakov and Xu, 2013).

Low N_2O emissions were also measured during the winter periods (December and January) for both the RF and SF plots, while the Unfertilized plots did not show any emissions. This fact can be explained by the continuous soil freezing and thawing responsible for the releases of ammonium and nitrate linked to the organic substance, making it available again to the denitrifying microorganisms, in the absence of plant competition (Müller et al., 2003).

Overall, the amount of total N_2O emitted from SF plots during the 293 days of monitoring was 10.3 ± 6.8 kg N ha^{-1}, RF plots emitted 7.59 ± 3.2 kg N ha^{-1}, and Unfertilized plots 1.71 ± 1.1 N ha^{-1} (Table S9). The total emissions were slightly lower for RF than SF although it was not statistically different, because of the high standard deviation (full field scale N_2O measurements over a long time are not simple to perform, leading to high variability). As a percentage of total N dosed, N_2O emissions were very low, i.e. SF plots lost 3.75 ± 2.5% of the total nitrogen dosed during the 2020 season, while 1.65 ± 0.7% was lost from RF. These values are in line with those reported in literature for the maize crop

(van Groenigen et al., 2004; Zhang et al., 2012). Therefore, these values were not able to explain the fate of the extra N dosed (185 kg Ha^{-1}) with digestate, unless one assumes that it could have been lost as N_2 because of complete denitrification. To better understand this aspect, nitrification-denitrification dynamics needed to be investigated.

3.3 Nitrification-denitrification processes in the soils studied

To verify whether the RF soils may have converted a greater amount of nitrogen into gaseous N_2O (and N_2) during the 2020 cropping season, the abundance of the Ncycle-related microbial populations in soil was quantified by measuring the number of gene copies for key marker genes involved in three fundamental processes (N fixation, N nitrification and N denitrification). The measurements, carried out at the same four soil depths already analysed in the previous paragraphs (0-25, 25-50, 50-75 and 75-100 cm from the surface) (Table S10), showed that most of the microbial activity in soils was concentrated in the first 50 cm, and significantly decreased with depth (Figure S3), as already described for similar soils and geographical areas (Zilio et al., 2020).

During the 2020 cropping season, the N-fixing populations (quantified using the *nifH* marker gene) were lower in Unfertilized plots than in fertilized ones at the beginning of the season (16 May 2020), maintaining this trend until late July. After that, at the post-topdressing sampling (7 August2020, 56 days after sowing) the *nifH* marker gene abundance strongly increased to become much higher than that measured earlier for both SF and RF plots (Figure 3 and Table S10). This trend can be explained by considering that after 60 days from sowing, maize plants absorb high amounts of nitrogen, stimulating the nitrogen fixation activity in unfertilized soils. On the other hand, in SF and RF soils, no significant increases or decreases were observed for these microorganisms during the crop season, since it is well known that the presence of N in soil depresses N fixation (Bahulikar et al., 2021; Tanaka et al., 2006).

More interesting was the monitoring of nitrifying and denitrifying activities. Soil nitrifying populations were quantified using the *amoA* marker gene, distinguishing archaea (*amoA_arc*) and

bacteria (*amoA_eub*). In both cases, the nitrifying populations were concentrated near the surface, decreasing progressively with increasing depth (Figure S3), as already reported (Zilio et al., 2020). During the whole season, the abundance of nitrifying archaea populations (ammonia oxidizing archaea: AOA) was always higher than that of their corresponding eubacterial population (ammonia oxidizing bacteria: AOB), probably because of soil pH, which remained neutral or sub-acid for the entire duration of the experiment (Table S11) favouring archaea (Prosser and Nicol, 2012). The abundance of the AOA and AOB populations measured indirectly with their respective gene markers, was similar in all experimental plots before pre-sowing fertilization (16 May 2020) and remained unchanged after fertilization (18 June 2020, 6 days after sowing), and until mid July (14 July 2020) (Figure 3). Then, after topdressing fertilization (7 August 2020) their numbers grew, reaching the maximum abundance measured in the cropping season (Figure 3) and then decreased, until they returned to very low levels at harvest (5 November 2020, 146 days after sowing). As reported in the previous paragraphs, the nitrate concentrations in RF and SF soils also followed a similar pattern, progressively increasing from pre-sowing fertilization (end of May), reaching a peak in mid-July, i.e. two weeks before pre-sowing fertilization (14 July 2020, 47 days after fertilization). The abundance of nitrifying populations and the nitrate concentration in the same soils were in fact correlated, as shown by the Pearson correlation analysis reported in Table 3, obtained using gene copy numbers and soil chemical data for the year 2020. These data were in agreement, also, with previously reported results (Zilio et al., 2020).

Denitrifying microbial populations were also studied, using two genes. For the microorganisms involved in the conversion of nitrate to nitrous oxide, the *nirK* gene was used as a marker, while for those involved in the conversion of nitrous oxide to N_2, the *nosZ* gene was used. At the beginning of the cropping season (16 May 2020), both *nirK* and *nosZ* populations had a similar abundance in all experimental plots (Figure 3) and they correlated well with each other ($r = 0.87^{**}$; $p < 0.01$). About 3 weeks after pre-sowing fertilization (18 June2020) both *nirK* and *nosZ* populations increased in the SF and RF plots, with a strong increase for RF, probably because since both higher N (Barrett et al.,

2016) and organic matter were added with digestate, the organic matter was able to enhance soil denitrification ability, which is typically due to heterotrophic microbial guilds (Burford and Bremner, 1975). Unfortunately, there was not any correlation of gene markers and TOC content, probably because TOC difference could not be detected. Maybe dissolved C and N content detections work better, and they should be considered for further work.

This proliferation in fertilized soils overlapped with the period of maximum N_2O emissions detected (Figure 2), and therefore provided direct evidence to interpret its origin. In particular, it can be deduced that the nitrification process led to the increase of nitrate in fertilized soil which was not removed by plants because they were at an early stage of development. Nitrate availability could be expected to have stimulated denitrifying bacteria (both *nir*k and *nos*Z increased) leading to N_2O/N_2 emissions. Subsequently, plant competition for nitrate could have limited denitrifying bacteria activity (*nir*k and *nos*Z decreased) reducing N_2O (and N_2) emissions, as registered for full field plots. These results seem to indicate that dosing extra N (and organic matter) with digestate strongly stimulated soil denitrification activity producing N_2O/N_2 and thereby explaining the low nitrate leaching observed when plants were less developed and therefore not able to take up nitrate, i.e. the non-differing nitrate content observed at 75-100 cm between SF and Unfertilized soil plots. Then plant development would likely have limited nitrate leaching reducing, also, denitrifying activities.

On the other hand, N_2 emissions were not assessed and N_2O emissions measured for RF were not statistically different from SF, and in any case N_2O losses were too low to explain the fate of the extra N dosed with digestate. However, literature reports that, on average, 25% of the total N lost by denitrification from agricultural soils is in the N_2O form, and the remaining 75% in N_2 form, with a strong variability due to various factors, including the availability of organic carbon in the soil. The presence of carbon in particular increases the denitrification efficiency, raising the percentage of N_2 produced and consequently decreasing the share of N_2O (Barrett et al., 2016; Jarvis et al., 1996; Mathieu et al., 2006). These data can be useful to interpret what was observed in this work. The digestate used, in fact, being particularly rich in organic carbon (TOC of 304 ± 34 g kg-1 dw (Table

S1), brought a high dose of carbon to the RF soils (as already reported in Zilio et al., 2022). The greater availability of carbon may have increased the ratio of N_2:N_2O produced by the RF soils, which would therefore have converted more nitrogen into N_2 in RF plots compared to the SF soils, explaining in part the fate of extra N in RF.

Soil N accumulation should also be considered, even though soil data did not indicate such an increase (Table 2). The digestate used showed high biological stability, as suggested by a residual potential producible biogas (BMP) figure of 89 ± 17 L biogas kg^{-1} DM, which was much lower than the values previously reported for energy crop digestates, i.e. 229 ± 31 L biogas kg^{-1} DM, and for green composts, i.e. 144 ± 3.8 and 201 ± 20 L biogas kg^{-1} DM, as extensively discussed in Pigoli et al. (2021). It can be also postulated that three experimental years were not enough to see analytically-detectable differences in soil N content, i.e., taking into consideration the total extra N added to RF compared to SF (i.e. +185 kg Ha^{-1} x 3y), it can be calculated that it represented only about 10-11% of total soil N (assuming that all extra N was preserved), from which the evolved N_2O/N_2 must be subtracted. At the same time, in Table 2, it can be seen that total organic carbon of RF was statistically higher than SF, suggesting OM (and N) accumulation. The experimental plots are still being maintained and presumably in the future, more data will be available about both C and N accumulation in soil.

These results indicate that the extra N dosed with digestate stimulated soil denitrification activities, leading to N losses as N_2O but above all as N_2. Probably, also, because of high biological stability characterizing digestate, N was accumulated in soil, as the organic carbon increase in RF plots indicated.

As suggested by one reviewer, a complete N mass balance could help in tracking N in plots differently fertilized, i.e. digestate vs. synthetic mineral fertilizers. Unfortunately, not all data were available in performing the N balance because it was not easy getting those data at full scale and for a long period (technical and economic reasons). Nitrate measurements were carried out to detect their concentration in soil during the crop season but total nitrate leached was not detected. Soil N detection did not give

clear differences, possibly because the three-year experiment was too short to obtain statistically significant differences. N_2O emissions were measured at intervals during the year and not continually, and N_2 cannot be measured. Above all this last point is important, as the impossibility of monitoring N_2 did not allow in any case the performing of a complete N balance.

Taking into consideration all that has been discussed, it can be concluded that when using digestate, the extra N dosed as organic-N did not lead to any increase of nitrate leaching in comparison with urea, as well as to any increase of N_2O emission. Probably, the presence of organic matter improved denitrification efficiency, such as suggested by gene markers, leading to N_2 emission. However, probably part of the organic-N dosed was stored in the soil because of its high biological stability. Although this interpretation leaves uncertainty (absence of a complete N balance), it offers a new key reading of the effect of dosing digestate in soil, with particular reference to the importance of the N biological stability of digestate to assure low nitrate leaching, a finding which needs to be better studied and confirmed in the future.

4. Conclusion

Digestate can be used to substitute N mineral fertilizers for agricultural crops; nevertheless, its N fate in soil needs to be better investigated since it involves dosing a complex matrix containing both mineral and organic nitrogen. In this work, it is shown that digestate was able to substitute mineral N and that although much more N (organic N) was dosed with digestate compared to the amount dosed with synthetical mineral fertilizers, no effect on nitrate leaching was found. This result was consistent with the explanation that digestate stimulated N denitrification. At the same time, organic carbon accumulation in plots fertilized with digestate seemed to suggest that part of the digestate-N was stored in the soil, although N soil analyses did not reveal it, possibly because an increase of such a kind might be too slight to emerge in a three-year experiment. More data are needed to confirm the fact that using well stabilized digestate did not lead to extra N leaching but it favoured organic matter and N accumulation in soil, revealing both nutritional and amendment properties.

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Table 1. Chronological list of agronomic operations, soil samplings and analyses carried out on soil during the experimentation.

Date	Sampling	Analyses carried out	Agronomic operation	Days from sowing
23/03/2018	Pre sowing 2018	Chemical characterization	Pre sowing spreading	-
03/04/2019	Pre sowing 2019	Nitrate and ammonium		-29
16/04/2019			Pre sowing spreading	-16
02/05/2019			Sowing	0
28/06/2019	Pre topdressing 2019	Nitrate and ammonium		57
30/07/2019			Topdressing fertilization	89
01/08/2019	Post topdressing 2019	Nitrate and ammonium		91
23/09/2019			Harvest	144
24/09/2019	Harvest 2019	Nitrate and ammonium		145
16/05/2020	Pre sowing 2020	Nitrate, ammonium, and DNA		-27
28/05/2020			Pre sowing spreading	-15
12/06/2020			Sowing	0
18/06/2020	Post sowing 2020	Nitrate, ammonium, and DNA		6
14/07/2020	Pre topdressing 2020	Nitrate, ammonium, and DNA		32
31/07/2020			Topdressing fertilization	49
07/08/2020	Post topdressing 2020	Nitrate, ammonium, and DNA		56
28/10/2020			Harvest	138
05/11/2020	Harvest 2020	Nitrate, ammonium, and DNA		146
12/01/2021	Three years after experiment start	Chemical characterization		

Table 2. Main characteristics of the soils exploited in this work, sampled on March 2018 (before starting experiments), and on January 2021 (after three years of experiment) (mean ± SD, n=3). The data refer to the soil layer between 0 and 25 cm deep from the surface. Letters are referred to One-way ANOVA ($p<0.05$; Tukey post-test).

Parameter	**Unit**	**March 2018**	**January 2021**		
			Unfertilized	**Synthetic fertilizer**	**Recovered fertilizer**
pH	pH unit	7 ± 0.7(a)	7.14 ± 0.2 (a)	7.06 ± 0.1 (a)	7.05 ± 0.2 (a)
Sand	%	45 ± 2			
Silt	%	44 ± 2			
Clay	%	10 ± 0.5			
CEC	C (mol kg^{-1})	24.2 ± 2.1 (ab)	23.8 ± 0.4 (a)	26.8 ± 0.8 (b)	22.3 ± 0.9 (a)
Organic carbon	g kg^{-1} dw[a]	10.3 ± 0.6 (a)	11.9 ± 0.2 (ab)	11.3 ± 0.4 (a)	12.3 ± 0.4 (b)
Total nitrogen	g kg^{-1} dw	1.27 ± 0.1 (a)	1.3 ± 0 (a)	1.41 ± 0 (b)	1.42 ± 0.9 (b)
Ratio C:N		8.13 ± 0.9 (ab)	9.22 ± 0 (b)	8.01 ± 0.1 (a)	8.65 ± 0.4 (ab)
$P_{avaialbe}$	mg kg^{-1} dw	43.6 ± 2.6 (a)	46.4 ± 0 (a)	60.1 ± 16 (a)	58.9 ± 16 (a)

[a]dw = dry weight

Table 3. Correlation matrix (Pearson correlation) based on the gene copy numbers and the main chemical parameters detected in the experimental soils at the depth layer 0-25 cm from surface (data of year 2020, n = 5). Statistically significant correlations have been highlighted in bold *: the correlation is significant at p <0.05; **: the correlation is significant at p <0.01. All the data used for the correlation analyses are reported in the tables S6, S7, S8, S10, S11 and S12.

	amoA archaea [a]	*amoA* eubacteria	*nifH*	*nirK*	*nosZ*	NO_3^- (mg kg^{-1} dw [b])	NH_4^+ (mg kg^{-1} dw)	pH	TOC (mg kg^{-1} dw)	Ntot (mg kg^{-1} dw)
amoA archaea	1	**0.339****	0.032	0.215	**0.256***	**0.275***	0.057	-0.176	-0.052	0.008
amoA eubacteria	**0.339****	1	**-0.352****	0.180	**0.317****	**0.338****	0.116	-0.178	0.012	0.054
nifH	0.032	**-0.352****	1	0.200	0.111	0.002	-0.054	0.185	-0.140	-0.071
nirK	0.215	0.180	0.200	1	**0.870****	-0.019	-0.133	0.036	0.083	**-0.294****
nosZ	**0.256***	**0.317****	0.111	**0.870****	1	0.078	-0.064	-0.022	0.140	**-0.249***
NO_3^- (mg kg^{-1} dw [b])	**0.275***	**0.338****	0.002	-0.019	0.078	1	**0.655****	**-0.241***	**-0.269***	0.178
NH_4^+ (mg kg^{-1} dw)	0.057	0.116	-0.054	-0.133	-0.064	**0.655****	1	**-0.431****	**-0.279***	0.168
pH	-0.176	-0.178	0.185	0.036	-0.022	**-0.241***	**-0.431****	1	0.152	-0.065
TOC (mg kg^{-1} dw)	-0.052	0.012	-0.140	0.083	0.140	**-0.269***	**-0.279***	0.152	1	**0.252***
Ntot (mg kg^{-1} dw)	0.008	0.054	-0.071	**-0.294****	**-0.249***	0.178	0.168	-0.065	**0.252***	1

[a] *amoA* from archaea and eubacteria, *nifH*, *nirK* and *nosZ* genes are expressed in gene copies g $soil^{-1}$ dw

[b] dw: dry weight

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Figure Captions

Figure 1. Average concentration (n=3) of nitrate nitrogen ($N\text{-}NO_3^-$) in experimental soils in four layers with increasing depth, starting from the surface up to a depth of one meter (a: 0-25 cm from surface; b: 25-50 cm; c: 50-75 cm; d: 75-100 cm) during the 2019 - 2020 crop seasons. Error bars show Standard deviation. Letters are referred to One-way ANOVA ($p<0.05$; Tukey post-test), comparing the three values for each sampling.

Figure 2. Daily nitrous oxide emissions from the experimental plots from 28/05/2020 (pre-sowing) to 17/03/2021.

Figure 3. Number of gene copies (mean, n=6) detected in experimental soils for the genes *nifH*, *amoA* from archaea (*amoA* arc), *amoA* from eubacteria (*amoA* eub), *nirK* and *nosZ* during the crop season 2020 (average of soil layers 0-25 and 25-50 cm depth from surface). Error bars show the standard deviation. Letters are referred to One-way ANOVA ($p<0.05$; Tukey post-test), comparing the three values for each sampling.

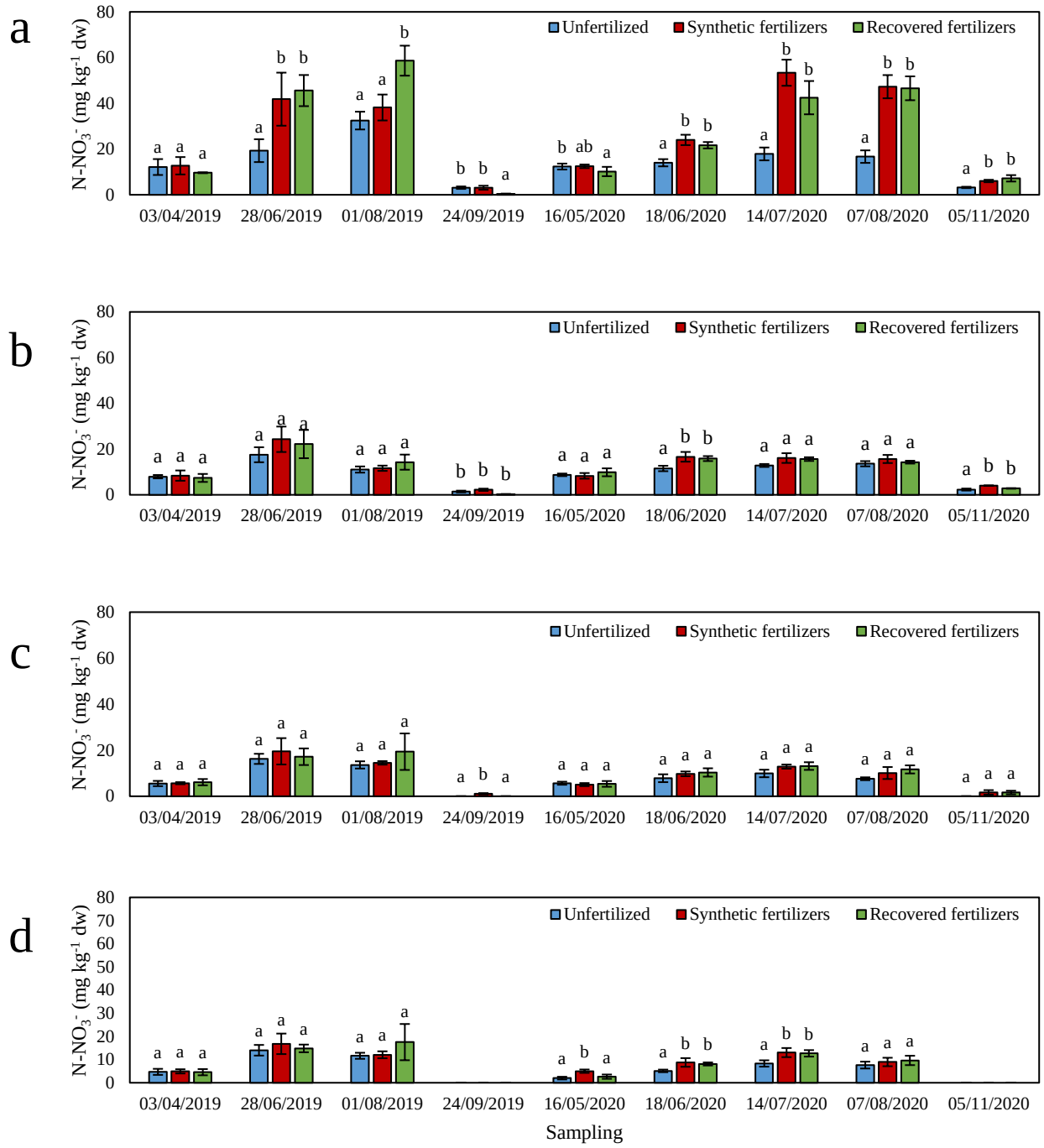
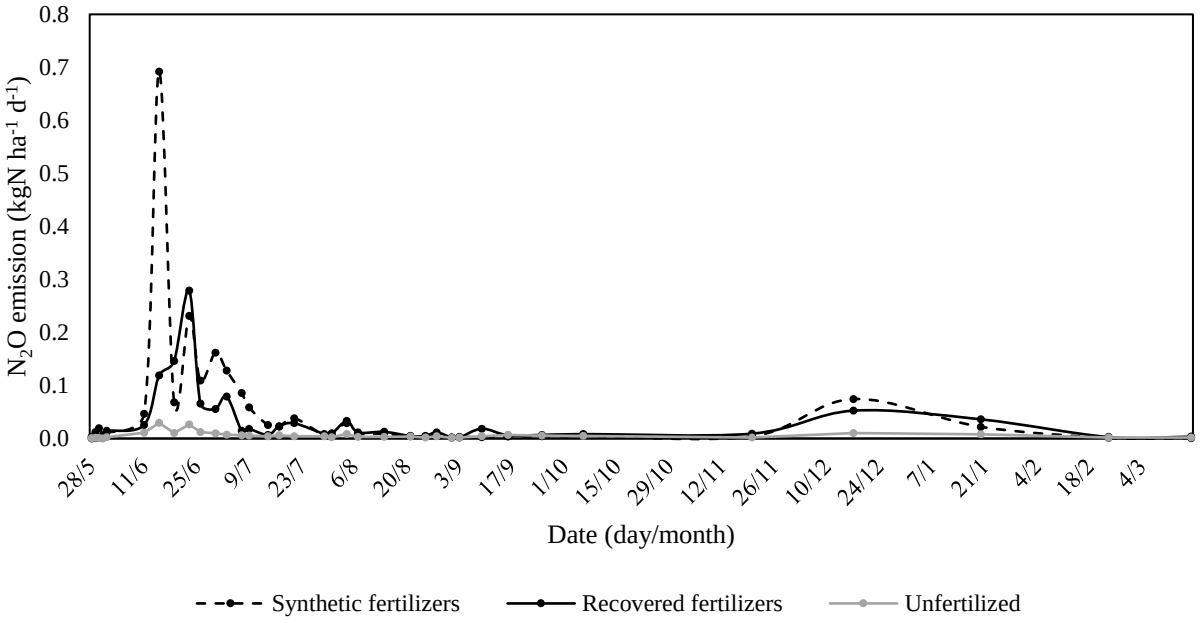

a
b
c
d
Unfertilized
Synthetic fertilizers
Recovered fertilizers
N-NO3- (mg kg-1 dw)
03/04/2019
28/06/2019
01/08/2019
24/09/2019
16/05/2020
18/06/2020
14/07/2020
07/08/2020
05/11/2020
Sampling

Figure 1.



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36 **Figure 2.**

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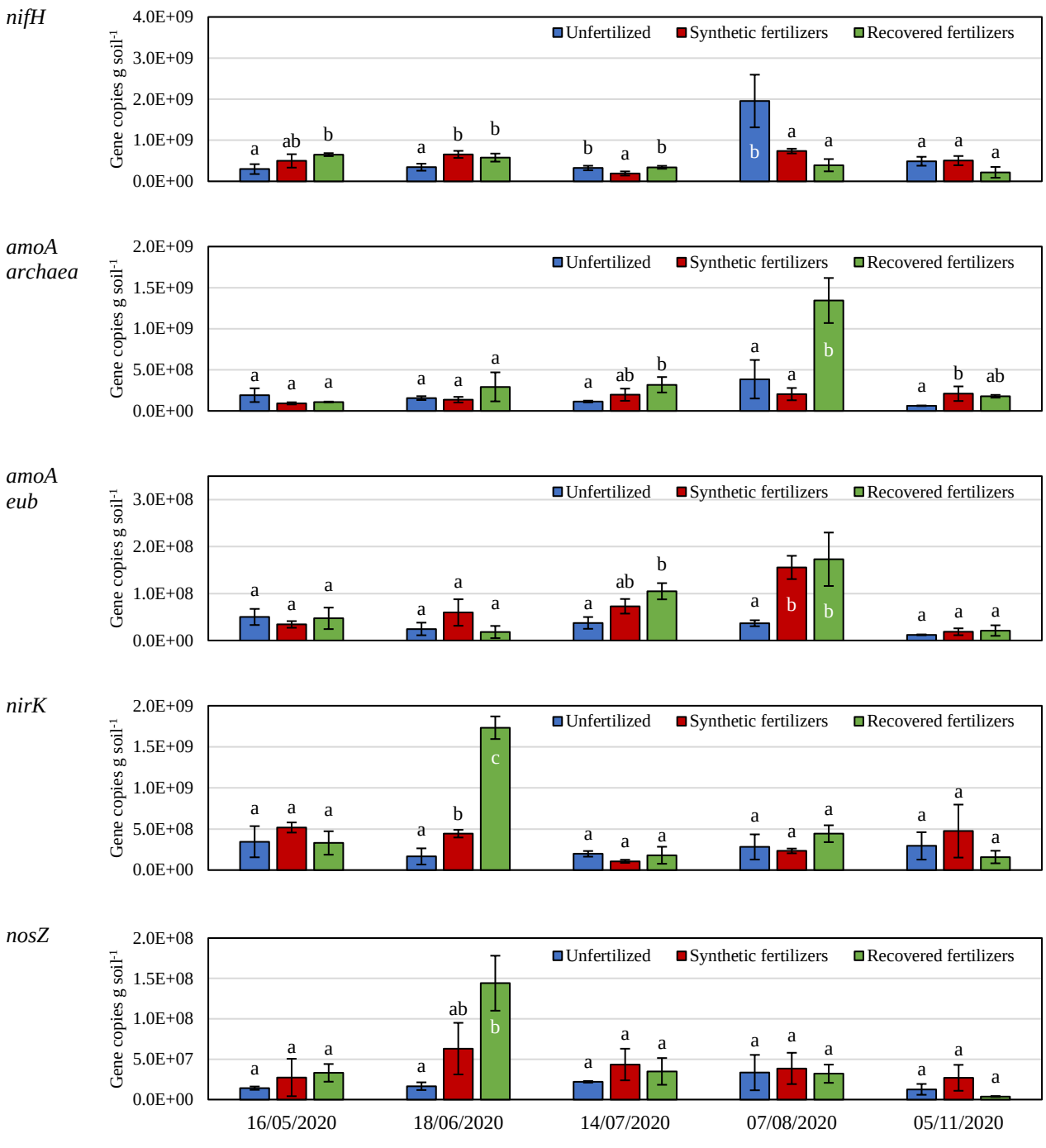
nifH
Gene copies g soil-1
4.0E+09
3.0E+09
2.0E+09
1.0E+09
0.0E+00
Unfertilized
Synthetic fertilizers
Recovered fertilizers
amoA archaea
2.0E+09
1.5E+09
1.0E+09
5.0E+08
amoA eub
3.0E+08
2.0E+08
1.0E+08
nirK
nosZ
2.0E+08
1.5E+08
1.0E+08
5.0E+07
16/05/2020
18/06/2020
14/07/2020
07/08/2020
05/11/2020

Figure. 3