International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 13s, 2025
https://www.theaspd.com/ijes.php

Nitrogen-Transforming Bacteria and N_2O Dynamics in Tropical Rice Amended with Plant Residues

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Abstract

Purpose: In tropical rice systems, when reactive nitrogen (N) exceeds crop uptake, it results in inefficient N use and elevated nitrous oxide (N_2O) emissions, leading to experimental use of plant residues with nitrification-inhibitory (NI) properties to enhance fertilizer efficiency and mitigate nitrate leaching and denitrification by promoting N retention in the soil during the study.

Methods: Five treatments, comprising inorganic fertilizer (100% NPK, T1), fresh neem leaves (T2), green manure, Sesbania (T3), used tea leaves (T4) and calcium carbide (T5), were selected and applied in well prepared rice fields for three seasons (pre-monsoon, monsoon and summer), to evaluate their efficacy towards reducing N_2O emission via microbial process.

Results: A marked reduction in N_2O emissions was observed in T4-treated fields during the pre-monsoon, while T2 recorded the lowest emissions during monsoon and summer rice. Across all ecosystems, microbial propagule densities were significantly lower in amended soils than in 100% NPK treatments, indicating reduced net N mineralization. Denitrifier populations were lowest in T4 during pre-monsoon and summer, and in T2 during monsoon. Nitrifier populations were consistently lowest at T2 across ecosystems. A significant correlation between soil NO_3^- oxidizers and N_2O emissions was observed, and the reduced populations of NH_4^+ and NO_3^- oxidizers in T2 and T4 suggest strong inhibition of ammonium oxidation, likely due to allelochemical effects.

Conclusions: The findings underscore the potential of residue-mediated biological nitrification inhibition (BNI) as a sustainable strategy to improve nitrogen use efficiency and mitigate greenhouse gas emissions across varying seasonal rice cultivation systems.

Keywords: Nitrous oxide (N_2O) emissions; Nitrification inhibitors; Tropical rice ecosystems; Denitrifiers; Nitrifiers; Nitrate leaching

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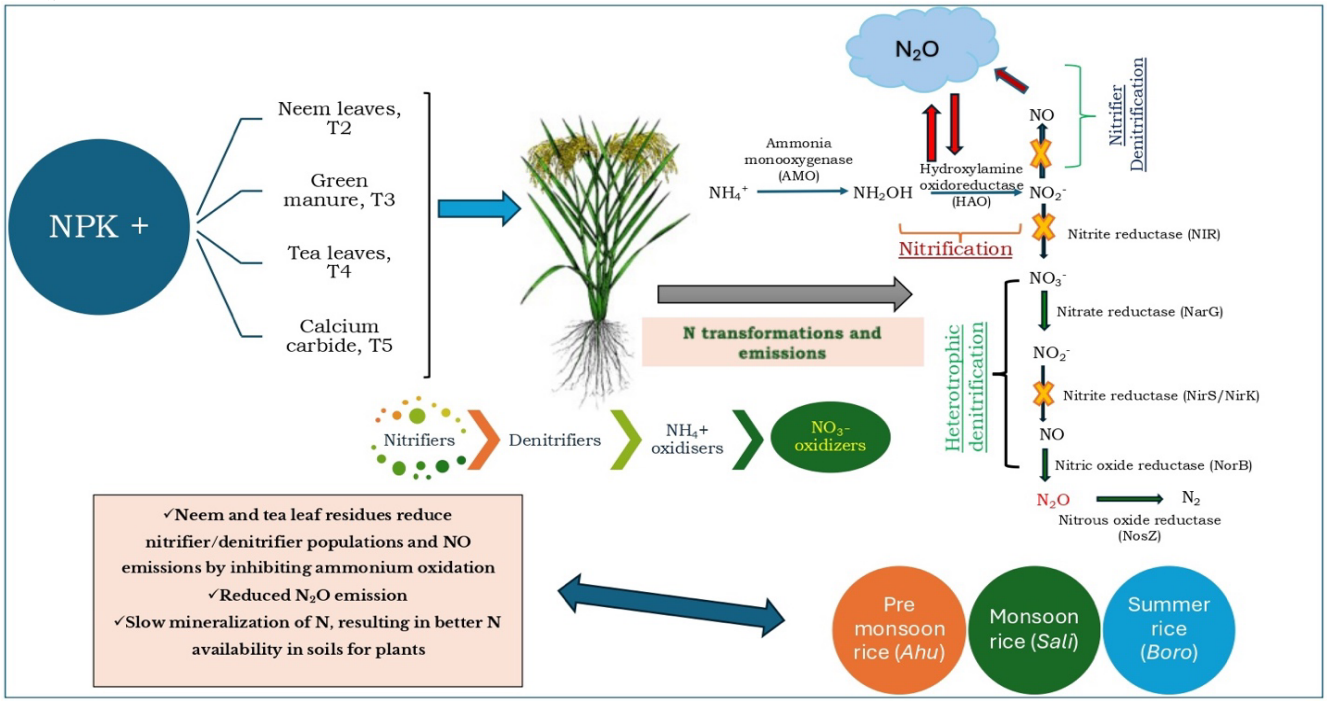
Graphical Abstract



1. INTRODUCTION

Nitrous oxide (N_2O), a potent greenhouse gas, is produced in the soils and manures by the transformation of reactive nitrogen (N) and is often boosted with the availability of excess N particularly under wet conditions (Chen et al., 1997; Zou et al., 2009). It has been established that denitrification and nitrification in managed and natural soils solely contribute about 70 % of the global N_2O emissions (Syakila and Kroeze, 2011; Braker and Conrad, 2011). However, apart from the mentioned microbial processes, dissimilatory and assimilatory nitrate reduction and chemo-denitrification also contributes to the emission of N_2O from the soils. The release of N_2O is also impacted by relative contribution and interactions of environmental and management drivers, including N supply, temperature, pH, soil moisture, soil management, crops, and the cropping systems (Pappa et al., 2011) from soil to the atmosphere. Soil nitrate causes N_2O emission directly via denitrification which reduces the CO_2 advantage of short relation coppice. Additionally, nitrate leaching indirectly contributes to N_2O emission. The concentration of soil nitrate depends on microbial activity (mineralization), nitrate deposition (fertilization) and nitrate consumption by plants and microbes. As per observation of NOAA (2024), concentration of N_2O in the atmosphere has risen from 219 ppb in preindustrial times to 337 ppb in present, accounting 80% of the world's total N_2O emissions to agricultural activities. In the upcoming decades, N_2O is projected to increase by 35–60% largely due to poor manure management and increased application of synthetic N fertilizers in the crop fields. Additionally, excessive use and inappropriate timing of N application will lead to N leaching affecting water quality, resulting in increased N_2O emission from the landscape-draining waterways (Hassan et al., 2022). N_2O from soil is a downside of the large productivity increase in agriculture, due to synthetic nitrogen fertilizer application. Inorganic fertilization is one of the key factors influencing the production and consumption of N_2O (Phillips et al., 2009) since N applied through fertilizers, manures and other N sources are not used efficiently by the crops (Galloway et al., 2003); the excess N is susceptible to loss as N_2O emissions (Bhatia et al., 2010). Soil management systems that add organic waste and incorporate carbon have been evaluated as important alternatives for increasing the capacity of atmospheric carbon sinks and mitigation of global warming (Urzedo et al., 2013).

Unlike the upland soil system, paddy fields have a unique soil profile after extended flooding. Oxidizing and reducing layers are developed in the cultivated layer during the rice growing seasons. Once applied to paddy field, ammonium nitrogen having fertilizers is nitrified in the oxidized layer, at the water-soil interface, forming NO_3^-, which moves downwards to the reduced layer and is denitrified there, producing N_2O. However, it has been proved that

denitrification processes exist not only in the upper flooded cultivated layer, but also in the underground saturated soil layer. During the rice growing season, with dry-wet alternation and rice-winter upland crop rotation N_2O generated in the underground saturated soil layer could move upwards to the atmosphere, compared with the water environment. It was concluded that N_2O was emitted mainly through rice plants in the presence of flood water, while through soil surface in the absence of flood water (Xing et al., 2009).

In recent decades focus has been given on optimization of N fertilizer utilization efficiency to minimize gaseous nitrogen loses (N_2O, NO and N_2) from the agricultural systems and increase crop productivity. An alternative practice which helps in reducing N_2O emissions without necessarily reducing N inputs or crop yields is the application of nitrification inhibitor (NI) (Li et al., 2015) in agricultural soils. The Third Assessment Report of the IPCC (2001) stated that nitrogen fertilizer management such as the use of NIs, slow-release fertilizers, and organic manure could tentatively cut N_2O emissions from nitrogen fertilizer use by 30% on a global scale. The IPCC Fourth Assessment Report (Smith et al., 2007) also considered nutrient management including NIs and slow-release fertilizers as a mitigation possibility, with the mean mitigation potential of N_2O by nutrient management estimated to be 0.07 t CO_2 eq ha^{-1} yr^{-1}. Good number of reviews on N_2O has suggested NIs and slow-release fertilizers as mitigation options for N_2O emission reduction (Smith et al., 1997; De Klein et al., 2001; Oenema et al., 2001; Dalal et al., 2003; Bolan et al., 2004; De Klein & Ledgard, 2005; Akiyama et al., 2009; Hyatt et al., 2010; Jiang et al., 2010).

Nitrification inhibitors (NIs) have been used in the field to improve the efficiency of nitrogen absorption by plants from soils and to reduce both nitrate leaching and denitrification by maintaining the N in the soil as NH_4^+ (Chen et al., 2010). This helps in reducing N_2O emissions and sustaining crop productivity in an agricultural ecosystem. Traditionally, synthetic nitrification inhibitors like calcium carbide (CaC_2), hydroquinone, dicyanamide, urea fertilizer mixed with NI nitrapyrin as chlorinated pyridine (CP) have been used in agricultural ecosystems to reduce the chances of N loss by various mechanisms, thereby enhancing N assimilation in crops (Zhang et al., 2015). At present biological nitrification inhibitors (BNI), such as plant exudates, is attracting attention. Inhibition can arise from competition between plants and microbes for available NH_4^+, but the exudation of nitrification suppressing compounds from plants (e.g. *Brachiaria humidicola*) has been proposed as a mode of nitrification inhibition. Use of nitrification inhibitors of biological origin to control N_2O emission from Indian agriculture is reported from Northern India (Patra and Chand, 2009). The production of BNI compounds by crop species and their effectiveness in lowering N_2O emission in situ are yet to be proved (Butterbach – Bahl et al., 2013). Interest in NI stems from the fact that retardation of nitrification in soil reduces the loss of N by leaching and denitrification. These inhibitors enhance the persistence of N in the soil and help to achieve more efficient use of N for crop production (Sahrawat and Ahmad, 1996). Keeping in view the importance of biological nitrification inhibitors in increasing N utilization efficiency in cropping systems experiments were conducted both in-situ and ex- situ in three seasons of tropical rice ecosystems to evaluate the efficiency of plant residues having anti-microbial properties as nitrification inhibitors by blending them with conventional N fertilizer (Urea) as per recommended dose. During the study, parameters such as N_2O production potential, nitrifier and denitrifier populations, N content in soils were observed and their relationship were worked out.

2. MATERIALS AND METHODOLOGY

2.1 Geographical location, climatic condition and soil physico-chemical characteristics of the experimental site

Field experiments were conducted in two consecutive years for three tropical rice seasons, at Central Brahmaputra Valley Zone (CBVZ) located at Brahmaputra valley of India. The experimental site is situated approximately at 26.41°N 92.49°E of Nagaon district of Assam and falls in the sub-tropical humid climatic region characterized by hot - wet summers. Winters generally starts from November till early March with a dry and cold weather. The daily rainfall and maximum, minimum air temperature were recorded from https://power.larc.nasa.gov. The soil of the experimental area is characterized by recent and old alluvium soils with sandy - loam to silt loam texture. The basic physico-chemical properties of the soil of experimental area are presented in Table 1.

Table 1. Basic physico-chemical properties of the soil of experimental area

Property		Property	
Sand (%)	60.7 ± 0.6	Bulk density (g cc^{-1})	1.2 ± 0.5
Silt (%)	20.2 ± 0.8	Available nitrogen (kg ha^{-1})	125.2 ± 0.4

Clay (%)	19.2 ± 0.9	Available phosphorus (kg ha^{-1})	32.8 ± 0.02
Soil temperature (°C)	22.4 ± 0.03	Available potassium (kg ha^{-1})	174.6 ± 3.1
Soil pH	5.7 ± 0.2	Total Carbon (mg g^{-1})	14.8 ± 0.1
Soil textural class	Sandy loam	Soil organic carbon (mg g^{-1})	9.8 ± 0.4

2.2 Experimental design

Experiments were conducted in well-prepared fields for two consecutive years by transplanting popular rice varieties during pre-monsoon (Ahu), monsoon (Sali) and summer (Boro) cropping system with various biological materials having different microbial activity inhibiting properties. Each treatment was replicated 4 times in a randomized block design in a plot size of 2m x 2m. Five treatments were selected to observe the nitrification inhibiting properties.

T1. Conventional fertilizer (NPK) - Control,
T2. Fresh neem leaves + NPK, (NL)
T3. *Sesbania aculatea* (dhaincha) + NPK, (SA)
T4. Used tea leaves + NPK, (TL)
T5. Calcium carbide (CaC2) + NPK, (CC)

2.3 Fertilizer management

Fertilizers were applied in the field by broadcasting before the last ploughing and mixed thoroughly with soil. The conventional N fertilizer in the form of Urea (N), Super Phosphate (P) and Muriate of Potash (K), (NPK) was applied in the ratio of 40:20:20 kg, 60:20:40 kg, and 60:30:30 kg N-P_2O_5-K_2O ha^{-1} for *Ahu*, *Sali* and *Boro* rice respectively, as per recommendation of the Department of Agriculture, Government of Assam, India. This treatment was taken as control (T1). Half of the total urea, full doses of super phosphate and potash were applied at the time of final puddling. The second and third doses of urea were applied during the tillering and panicle initiation stages respectively.

To observe the efficacy of nitrification inhibiting effect of the biological materials, the selected treatments were applied as follows:

- Fresh neem leaves (T2) were cut into small pieces and mixed thoroughly with the first dose of NPK (i.e., half dose of urea, full dose of P_2O_5 and K_2O) and applied @ 5-ton ha^{-1} (equivalent to 31.4 ± 0.25 kg N ha^{-1}) (on fresh weight basis) in the field before transplanting
- Similarly, used tea leaves (T3) @ 5-ton ha^{-1} (equivalent to 18.8 ± 0.56 kg N ha^{-1}) (on dry weight basis) were mixed with 1st dose NPK (i.e., half dose of urea, full dose of P_2O_5 and K_2O) and applied in the field before transplanting.
- Seeds of *Sesbania aculatea* (dhaincha) (T4) were sown in a separate field 30 days prior to rice cultivation. After 30 days the above ground plant parts were cut into pieces and incorporated uniformly as green manure @ 5-ton ha^{-1} (equivalent to 39.8 ± 2.1 kg N ha^{-1}) along with first dose of NPK (i.e., half dose of urea, full dose of P_2O_5 and K_2O)
- Calcium carbide (CaC_2) was mixed with NPK (i.e., half dose of urea, full dose of P_2O_5 and K_2O) at rate of 30 mg kg^{-1} and applied in the field before transplanting.

2.4 Microbial production potential of Nitrous oxide producing bacteria under the influence of different treatments

The production of nitrous oxide from soils was estimated following the method described by Huang et al., (2004) with slight modification. Soil samples amended with plant residues from field were collected in pre-sterilized sample collection bottle at transplanting, active tillering, panicle initiation, maturation stages and harvest. For production potential of N_2O, soil samples were passed through a 2 mm stainless sieve for use. For each treatment, 10g soils were weighed and incubated at 25°C for 24 hours in pre-sterilized 50ml airtight serum bottles. The serum bottles were covered in Teflon to avoid air leakage. Three sets of each treatment were kept under incubation for gas sampling. Bottles without soil samples for each set were set up as corresponding blank. Head space gas produced inside the serum bottles was collected with a 20ml syringe with a 3-way stop cork through a rubber cork at 0, 3, 6, 12 and 24 hours after incubation. The gas samples were analyzed in a gas chromatograph equipped with an ECD (Varian GC, CP-3800) (Mei et al., 2004). The injector, column and detector temperature in the GC were maintained at 80, 150 and 300°C respectively. N_2O fluxes were estimated by successive increase in gas concentration inside the tube at each sampling time from the initial concentration at 0 minute. The N_2O – N concentration was estimated as given by

Wang et al., 2011 as in Eq.1 and Seasonal total N_2O - N was calculated as given Ma et al., 2009 (Eq.2). The N_2O flux was calculated according to the equation of Wang et al., 2011 as follows

$$F = \Delta x * \frac{V}{A} * \rho * \frac{273}{273 + T} \quad \text{..... (1)}$$

Where F represents N_2O flux in μg N_2O-N m^{-2} h^{-1};
ρ is the density of N_2O under standardized state ($1.25x10^9$ μg N_2O-N m^{-3})
Δx is the change in concentration in ppmv N_2O inside the tube from time 0 to t min.
V is the volume of the tube
A is the area from which N_2O was emitted into the tube.
T is the chamber temperature.
Seasonal total N_2O emission is expressed as seasonal integrated flux (E_{sif}) in mg N_2O-N m^{-2} for the entire crop growth period was computed by the method given by Ma et al., 2009 by using the following formula:
Seasonal total N_2O emission (E_{sif}) =

$$\sum_{i=1}^{n} (Ri * Di) \quad \text{..... (2)}$$

Where R_i is the mean gas emission, D_i is the number of days in the sampling interval and n is the number of sampling times.

2.5 Microbial population study under different treatments applied

Soil samples were collected from the experimental field in airtight pre sterilized sample bottles and stored in freezer until further analysis. Serial dilution technique (10^3 to 10^{10}) was implied for further analysis of microbial population. Briefly, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} dilution were plated in nutrient agar media in 3 replications and incubated @ 25 - 27°C for 48 hrs for microbial population study. Total microbial colonies in the agar plates were counted and the plates with more than 300 colonies were rejected. The colonies counted are expressed as colony forming units per g of soil (CFU g^{-1}) calculated by the following equation (3):

$$\text{CFU ml}^{-1} = \frac{\text{no.of colonies per ml plated}}{\text{dilution factor}} \quad \text{..... (3)}$$

2.6 Quantification of total nitrifying and denitrifying bacteria

To quantify the heterotrophic bacterial population involved in N transformation, modified Winogradsky's medium was used. For the incubation, 5g soil samples were mixed in a modified Winogradsky's medium and incubated in a shaker at 27°C @ 120 rpm for 72 hrs. The chemical composition the first medium used for cultivation of denitrifiers (Das and Dangar, 2008) consisted of (g/L): $(NH_4)_2SO_4$ = 1.0, K_2HPO_4 = 1.0, $FeSO_4.7H_2O$ = 0.03, NaCl = 2.0, $MgSO_4.4H_2O$ = 0.5, $CaCl_2$ = 0.02 at pH adjusted to 8.5. The second medium was used for cultivation of nitrifiers with the following chemical composition: KNO_3 = 1.0, K_2HPO_4 = 1.0, $FeSO_4.7H_2O$ = 0.03, NaCl = 1.0, $MgSO_4.4H_2O$ = 0.5, $CaCl_2$ = 0.02 at pH adjusted to 8.5. The 3 ml microbial biomass pallet formed in the above two media after incubation were than plated in sterilized agar plates of the same medium with 2% glucose and incubated again for 7 days @ 25°C. After completion of incubation, the plates were flooded with sulphanilic acid reagent (equivolume mixture of sulphanilic acid, 8g/L and α-naphthyl amine, 5g/L dissolved separately in 5M acetic acid) and the color absorbing pink colonies were counted in a bacterial counter. The colonies counted are expressed as colony forming units per g of soil (CFUg^{-1}) calculated as given in Eq. 4:

$$\text{CFUg}^{1} = \frac{\text{no.of colonies per gram of soil plated}}{\text{dilution factor}} \quad \text{..... (4)}$$

Further, to probe the biochemical characterization of the nitrifiers and denitrifiers, tenfold serial dilution of the soil samples were made with sterilized distilled water and 1ml aliquots of 10^{-1}, 10^{-3}, 10^{-5} and 10^{-7} dilutions were transferred to test tubes containing 3 ml of sterilized Winogradsky's modified medium as described above and incubated for 3 weeks at 30°C. Aliquots of media without any soil suspensions were taken as control. After 3 weeks, 3 drops of freshly prepared Greiss-Ilosvay's reagent were added to the medium. The tubes showing purplish red color within a few minutes were recorded as positive for nitrifiers. Those tubes which did not show color formation were recorded negative and a pinch of zinc dust was added to these tubes. After the addition of Zn dust, the tubes which showed pink color were scored positive for denitrifiers. Most probable number were calculated by referring to the tables of Cochran (1950) (Elbanna et al., 2012).

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3. RESULTS

3.1 Nitrous oxide production potential (µg N_2O-N g^{-1})

Significant differences in N_2O emission were observed among the treatments **(Figure 1)**. In *Ahu* rice, the highest nitrous oxide production was recorded at T1, NPK (0.67 ± 0.04 µg N_2O-N g^{-1}) and lowest at T4, TL (0.53 ± 0.03 µg N_2O-N g^{-1}). In *Sali* and *Boro* rice, the highest nitrous oxide production was observed at T3, SA (0.92 ± 0.04 µg N_2O-N g^{-1}) (0.88 ± 0.02 µg N_2O-N g^{-1}) and lowest at T2, NL (0.65 ± 0.06 µg N_2O-N g^{-1}) (0.66 ± 0.02 µg N_2O-N g^{-1}) respectively.

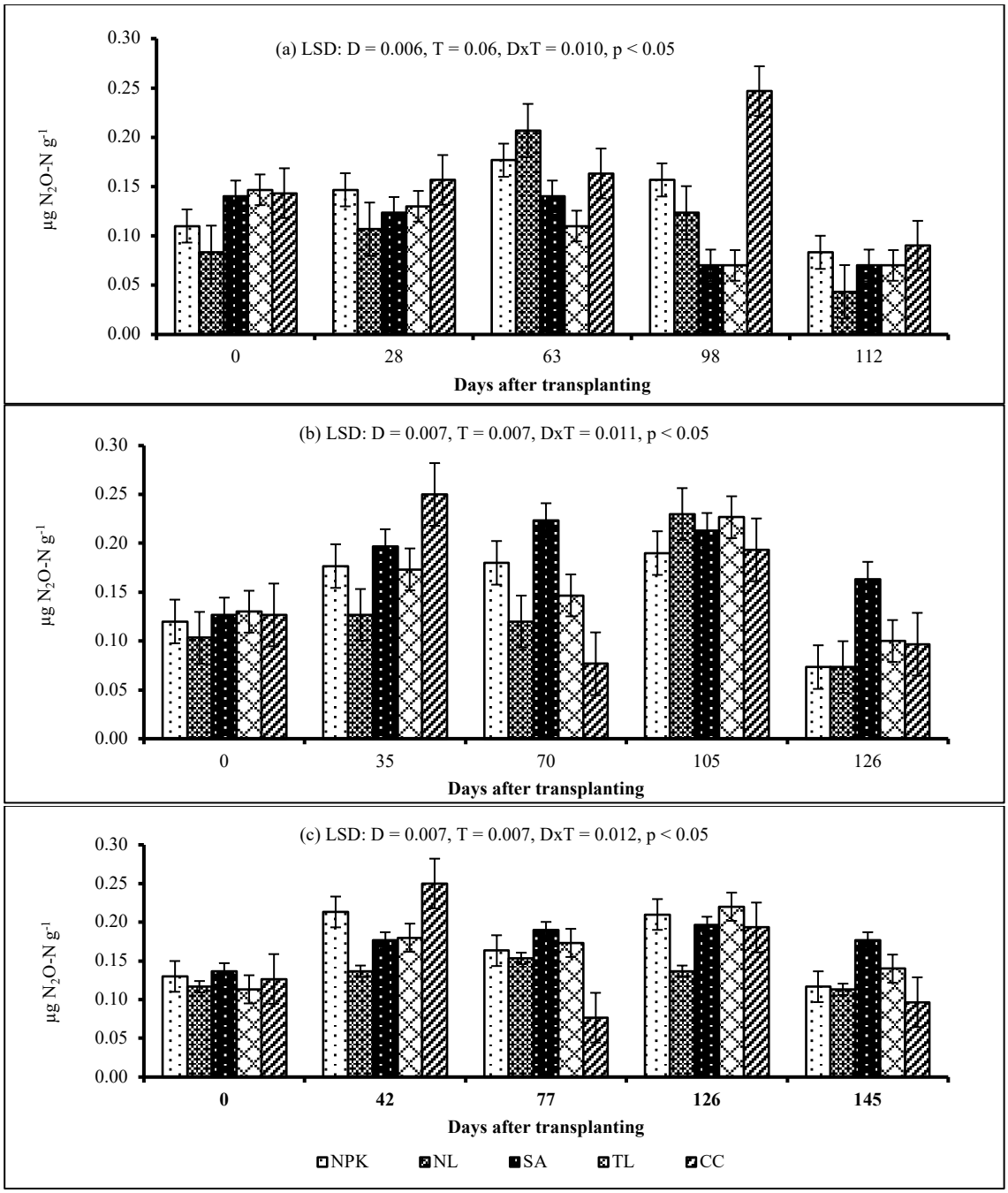

Figure 1. Variation in nitrous oxide production (µg N_2O-N g^{-1}) at different plant-based residues and inorganic fertilizer in (a) *Ahu* (pre-monsoon), (b) *Sali* (monsoon) and *Boro* (summer) rice ecosystems. *The bars indicate mean*

± S.E. (n = 3). T– treatment; D – days after transplanting; LSD – least significance difference. NPK (T1)- inorganic fertilizer; NL(T2)- fresh neem leaves, SA(T3)- Sesbania aculatea (green manure), TL (T4)- used tea leaves, CC (T5)- Calcium carbide

3.2 Microbial population (CFU g^{-1} x 10^7)

Total colony forming units were observed under the influence of different plant-based residues during transplanting (0 DAT) and maturation stage and significant ($p < 0.05$) difference in microbial population was seen under each treatment over initial base values in all the three ecosystems **(Table 2)**.

Treatments	(a) Soil microbial parameters in *Ahu* rice					
	N_2O production (µg N_2O-N g^{-1})	Microbial population (CFU g^{-1} x 10^7)	Nitrifiers (CFU ml^{-1} x 10^8)	Denitrifiers (CFU ml^{-1} x 10^8)	NH_4^+ oxidizers (MPN x 10^7)	NO_3^- oxidizers (MPN x 10^6)
	(a) Soil microbial parameters in *Ahu* rice					
NPK	0.67 (0.04) b	**60.0 (8.8) d**	**4.3 (1.2) b**	**15.7 (1.5) c**	**0.15(0.07) d**	**0.17 (0.04) b**
NL	0.56 (0.06) a	45.2 (3.1) c	2.0 (1.0) a	11.7 (1.5) a	0.11 (0.02) b	0.14 (0.01)a
SA	0.54 (0.04) a	41.5 (5.0) b	4.0 (1.0) ab	14.7 (1.5) bc	0.13 (0.05) c	**0.17 (0.06) b**
TL	0.53 (0.03) a	39.0 (4.0) b	2.7 (1.2) ab	11.0 (1.0) a	0.07 (0.03) a	**0.17 (0.04) b**
CC	**0.80 (0.06) c**	40.5 (1.9) b	3.7 (1.2) ab	12.7 (1.5) ab	0.10 (0.07) b	0.16 (0.08) ab
Basic soil	–	34.7 (5.2) a	–	–	–	–
	(b) Soil microbial parameters in *Sali* rice					
NPK	0.74 (0.05) b	**57.0 (9.7) e**	4.0 (1.0) a	**21.3 (1.5) c**	0.09 (0.04) b	**0.19 (0.03) b**
NL	0.65 (0.06) a	48.3 (5.5) c	2.7 (1.2) a	16.7 (2.1) ab	0.07 (0.03) a	0.15 (0.01)a
SA	**0.92 (0.04) c**	54.0 (12.7) d	**4.3 (1.5) a**	18.7 (1.5) bc	**0.10 (0.02) b**	0.18 (0.04) b
TL	0.78 (0.05) b	43.5 (5.9) b	3.3 (0.6) a	14.3 (1.5) a	0.07 (0.03) a	0.18 (0.03) b
CC	0.74 (0.07) b	46.7 (5.9) c	3.7 (1.5) a	16.3 (3.1) ab	0.09 (0.04) b	0.18 (0.05) b
Basic soil	–	35.7 (2.7) a	–	–	–	–
	(c) Soil microbial parameters in *Boro* rice					
NPK	0.83 (0.04) c	54.3 (8.1) d	3.33 (1.5) a	13.67 (2.1) a	0.093 (0.06)a	**0.20 (0.08) c**
NL	0.66 (0.02) a	49.7 (7.6) c	3.00 (1.0) a	12.00 (1.0) a	0.092 (0.47)a	0.14 (0.04) a
SA	0.88 (0.02) c	47.2 (5.8) b	**4.67 (2.5) a**	**14.33 (1.5) a**	**0.138 (0.06)b**	0.17 (0.04) b
TL	0.83 (0.04) c	46.7 (6.5) b	3.00 (1.0) a	12.67 (1.5) a	0.077 (0.04)a	0.13 (0.03) a
CC	0.73 (0.07) b	51.7 (16.8) b	3.33 (2.5) a	13.33 (1.5) a	0.122 (0.05)b	0.16(0.02) b
Basic soil	–	40.7 (4.1) a	–	–	–	–

Table 2. Variations in microbial population characteristics and N_2O production potential at the different treatments in *Ahu*, *Sali* and *Boro* rice ecosystem. Values represents means (n = 3) with S.D. in parenthesis. Values followed by different letters in a column represents significant differences at $p < 0.05$. *NPK (T1)- inorganic fertilizer; NL (T2)- fresh neem leaves, SA (T3)- Sesbania aculatea (green manure), TL (T4)- used tea leaves, CC (T5)- Calcium carbide.*

Under the treatments, highest number of colony forming units were observed at T1, NPK (60.0 ± 8.8 CFU g^{-1}x 10^7) (57.0 ± 9.7 CFU g^{-1}x 10^7) and (54.3 ± 8.1 CFU g^{-1}x 10^7) and lowest at T4, TL (39.0 ± 4.0 CFU g^{-1} x 10^7) (43.5 ± 5.9 CFU g^{-1} x 10^7) (46.7 ± 6.5 CFU g^{-1} x 10^7) in *Ahu, Sali* and *Boro* rice respectively. Significant increase was observed in total population from the day of transplanting upto the crop maturation stage (98, 105 and 126 DAT for each season respectively) **(Figure 2)**. Application of plant-based residues significantly decreased the total colony forming units under control.

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https://www.theaspd.com/ijes.php

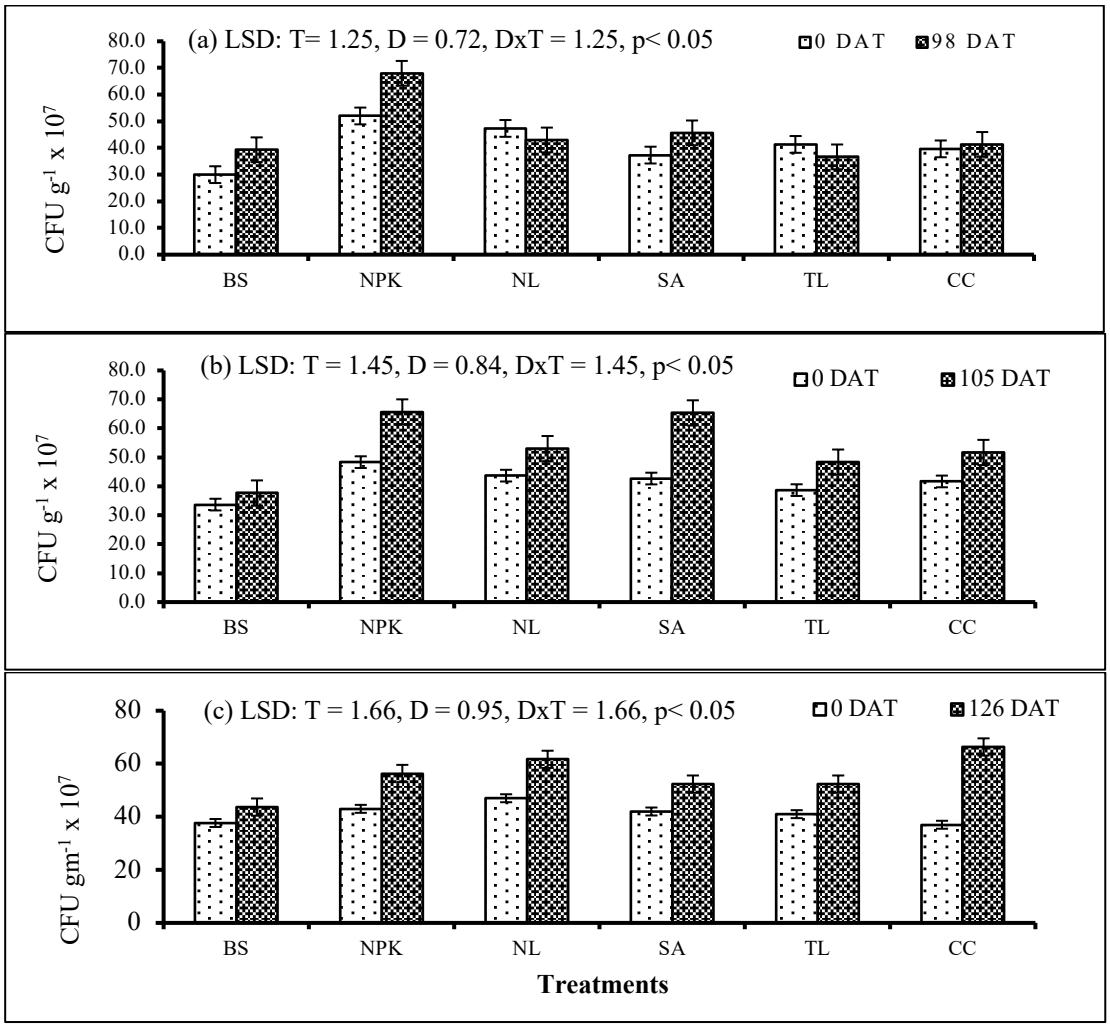

Figure 2. Variation in total colony forming units at different plant-based residues in soil planted with (a) *Ahu, (b) Sali* and *(c) Boro* rice varieties. *The bars indicate mean ± S.E. (n = 3). LSD- least significance difference; T – treatment; D/DAT – days after transplanting*

3.3 Presence of nitrifiers and denitrifiers (CFU ml^{-1} x 10^8)

Presence of heterotrophic bacteria (nitrifiers and denitrifiers) was observed under the treatments with Griess-Ilovskys reagent **(Figure 3)**. After 7 days of incubation, it was observed that the total population of denitrifiers in the soil sample was much more dominant over nitrifiers irrespective of the treatments. In *Ahu* and *Sali* rice ecosystem, the highest numbers of **denitrifiers** were recorded at T1 (15.7 ± 1.5 CFU ml^{-1} x 10^8 and 21.3 ± 1.5 CFU ml^{-1} x 10^8) and lowest at T4 (11.0 ± 1.0 CFU ml^{-1} x 10^8 and 14.3 ± 1.5 CFU ml^{-1} x 10^8) respectively. In *Boro* rice, however, the numbers of colonies in residue amended soils were not significantly different from each other. Highest number of denitrifying populations was recorded at T3 (14.33 ± 1.5 CFU ml^{-1} x 10^8) with lowest at T2 (12.0 ± 1.0 CFU ml^{-1} x 10^8). Among **nitrifiers**, the highest was recorded at T1 (4.3 ± 1.2 CFU ml^{-1} x 10^8) in *Ahu*, T2 (4.3 ± 1.5 CFU ml^{-1} x 10^8) in *Sali*, and T3 (4.7 ± 2.5 CFU ml^{-1} x 10^8) in *Boro* rice ecosystems. Irrespective of ecosystems, the lowest nitrifying CFUs were recorded at T2.

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Vol. 11 No. 13s, 2025
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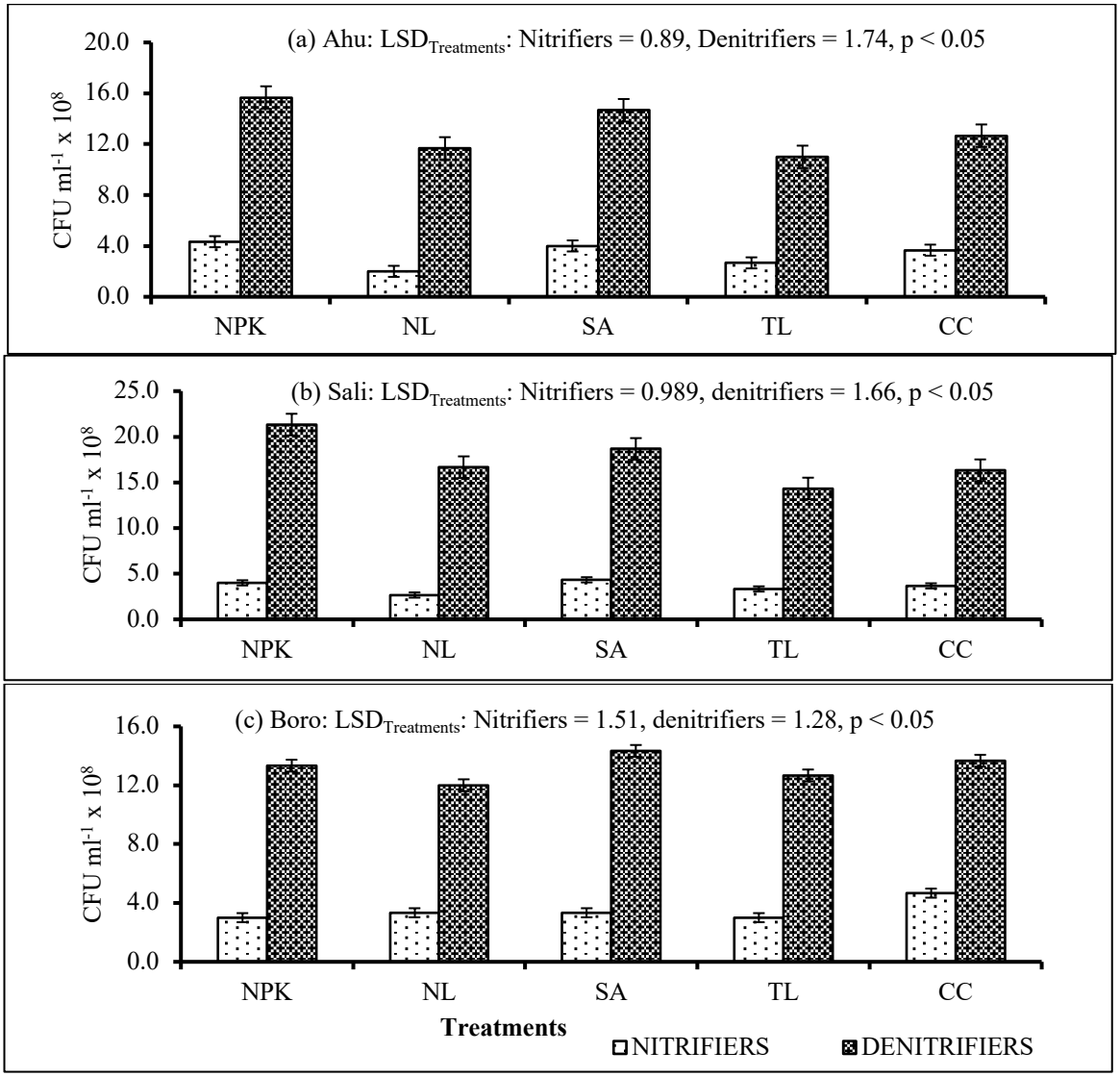

Figure 3. Variation in total colony forming units of denitrifiers and nitrifiers at different plant based residues in soil planted with. *The bars indicate mean ± S.E. (n = 3). LSD- least significance difference; T – treatment; D/DAT – days after transplanting*

3.4 Most probable number of NH_4^+ and NO_3^- oxidizers

NH_4^+ oxidizers and NO_3^- oxidizers were significantly affected using different organics in the soil (**Figure 4 and 5**). Irrespective of season, NH_4^+oxidizers increased steadily from the date of transplanting (0 DAT) towards panicle initiation up to crop maturation stage. During *Ahu* season, maximum NH_4^+oxidizers were recorded at T1 (0.15 ± 0.07) followed by T3 (0.13 ± 0.05), T2 (0.11 ± 0.16), T5 (0.10 ± 0.07) and lowest at T4 (0.07 ± 0.03) whereas maximum NO_3^- oxidizers were recorded at T1 (0.17 ± 0.04) and lowest at T2 (0.14 ± 0.01). In *Sali* rice, the maximum NH_4^+oxidizers were recorded at T3 (0.10 ± 0.02) with lowest at T4 (0.07 ± 0.03). NO_3^- oxidizers in all the treatments increased significantly after transplanting (0 DAT) to grain maturation stage (105 DAT). Maximum NO_3^-oxidizers were recorded at T1 (0.19 ± 0.03), and lowest was recorded at T2 (0.15 ± 0.01). In *Boro* rice, a similar trend of increase in the numbers was observed. Maximum NH_4^+oxidizers were recorded at T3 (0.14 ± 0.06) followed by T5 (0.12 ± 0.05), T1 (0.09 ± 0.06), T2 (0.09 ± 0.47), and lowest at T4 (0.08 ± 0.04). Maximum NO_3^- oxidizers were recorded at T1 (0.20 ± 0.08) and lowest was recorded at T4 (0.13 ± 0.03).

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 13s, 2025
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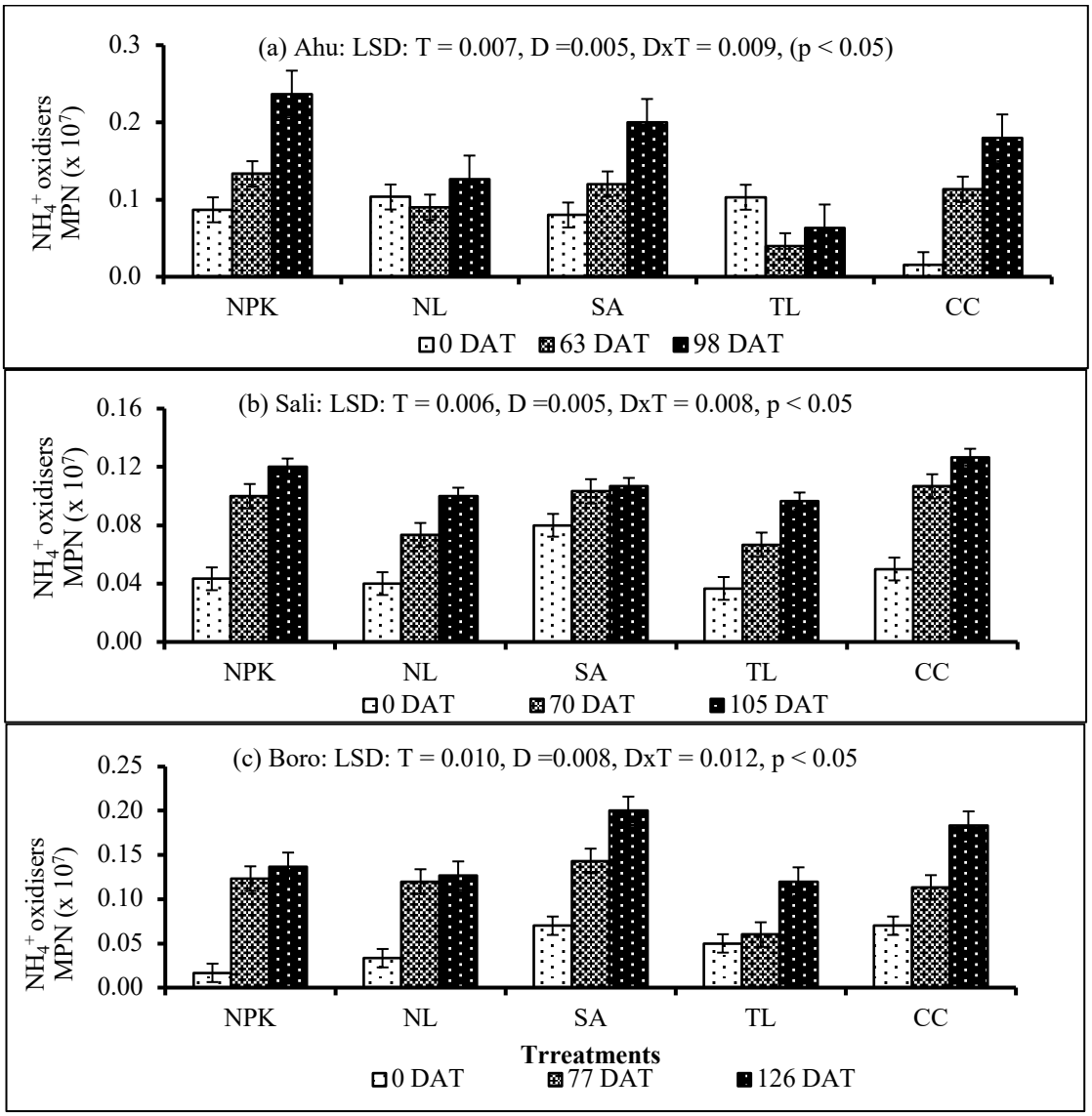
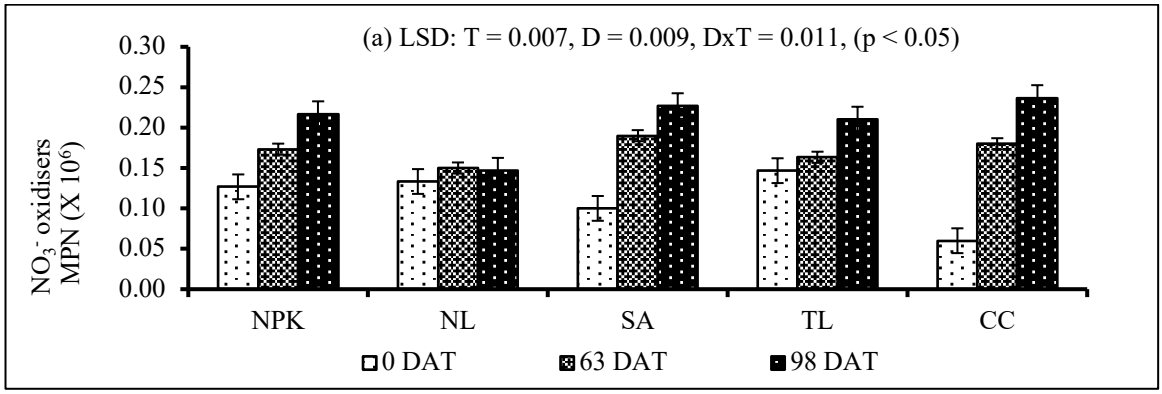


Figure 4. Variation in most probable number (MPN x 10^7) of nitrifiers (NH_4^+ oxidizers) at different plant-based residues during the three-rice cropping season. *The bars indicate mean ± S.E. (n = 3). LSD- least significance difference; T – treatment; D/DAT – days after transplanting*



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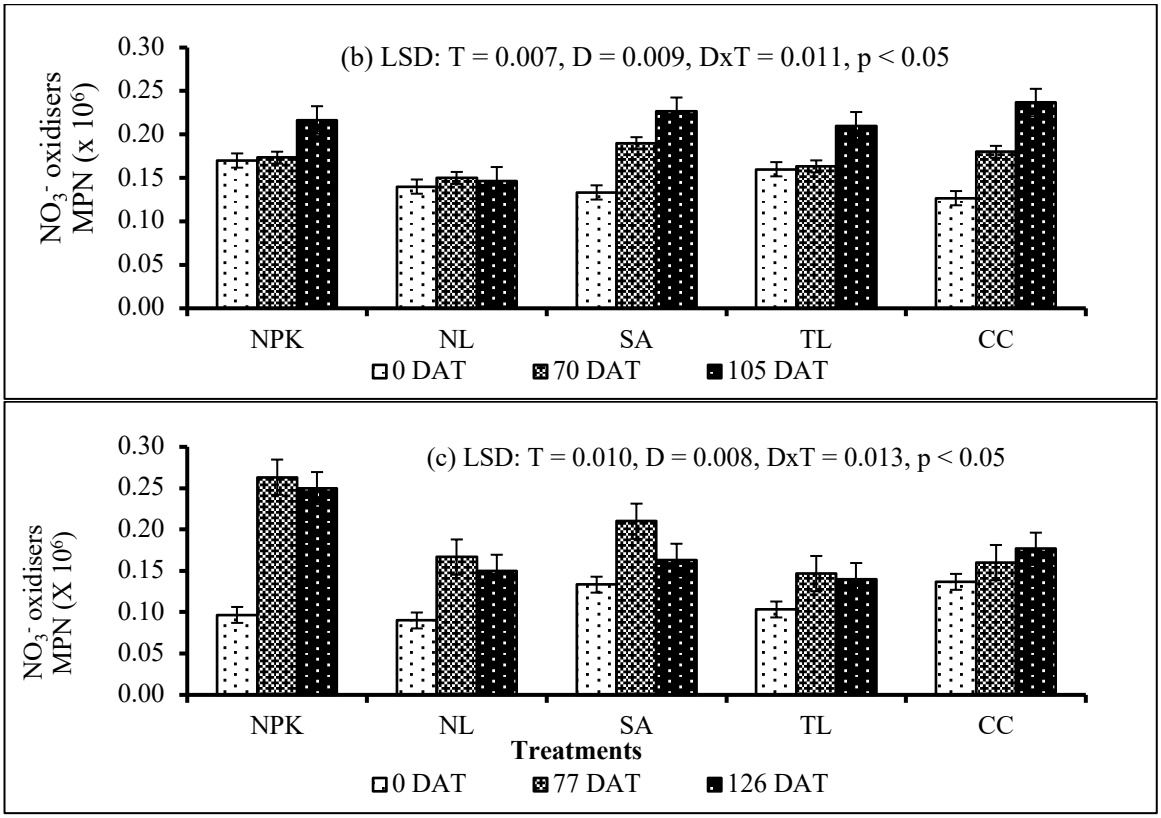

Figure 5. Variation in Most probable number (MPN x 10^6) denitrifiers (NO_3^- oxidizers) at different plant-based residues in soils planted with (a) Ahu (pre-monsoon), (b) Sali (Monsoon) and (c) Boro (Summer) rice. *The bars indicate mean ± S.E. (n = 3). LSD- least significance difference; T – treatment; D/DAT – days after transplanting*

3.5 Relationship with nitrous oxide emission (µg N_2O – N m^{-2} hr^{-1})

A significant relationship was observed between nitrous oxide production under incubation and nitrous oxide emission from the rice field **(Table 3)** at the respective days of sampling. The use of plant-based materials significantly influenced the presence of NO_3^- oxidizers particularly at T2, T3, T4 and T5 while T1 significantly influenced both NO_3^- and NH_4^+ oxidizers along with nitrous oxide emission. Correlation analysis between soil NO_3^- oxidizers and N_2O emission showed a positive relationship in all the treatments. NH_4^+ oxidizers showed significant positive correlation at NPK (r = 0.683*) only. In *Sali* rice**,** the use of plant-based materials significantly influenced the presence of NO_3^- oxidizers at T3 and T5, while both NO_3^- and NH_4^+ oxidizers were significantly influenced at T1 and T4 along with nitrous oxide emissions. Correlation analysis between soil NO_3^- oxidizers and N_2O emission showed a significant relationship at T1, T3, T4 and T5 while NH_4^+ oxidizers were significantly related in T1, T2, and T4. In *Boro* rice**,** correlation analysis between soil NO_3^- oxidizers and N_2O emission showed a positive relationship ($p<0.05$) in all the treatments while NH_4^+ oxidizers were significantly influenced at T1 (r = 0.937**) and T5 (r = 0.713*) only along with nitrous oxide emission.

Table 3. Correlation coefficients of different soil microbial population and N_2O production potential (µg N_2O-N g^{-1}) with field N_2O flux (µg N_2O – N m^{-2} hr^{-1}) values of the days in which the soil samples were collected for laboratory incubation study under different treatments. ***Correlation significant at the 0.01 level (2- tailed)* **Correlation significant at the 0.05 level (2- tailed).*

Soil microbial parameters	N_2O emission (µg N_2O – N m^{-2} hr^{-1})					N_2O emission (µg N_2O – N m^{-2} hr^{-1})					N_2O emission (µg N_2O – N m^{-2} hr^{-1})				
	Pre-monsoon (*Ahu*) rice					Monsoon (*Sali*) rice					Summer (*Boro*) rice				
	NPK	NL	SA	TL	CC	NPK	NL	SA	TL	CC	NPK	NL	SA	TL	CC

N_2O production (µg N_2O-N g^{-1})	0.901**	0.810**	0.774**	0.566*	0.919**	0.729**	0.658**	0.577*	0.710**	0.811**	0.699**	0.767**	0.716**	0.863**	0.811**
NH_4^+ oxidizers (MPN x 10^7)	0.683*	0.552	0.317	0.111	-0.081	0.693*	0.804**	0.488	0.705*	0.123	0.937**	0.603	0.521	0.552	0.713*
NO_3^- oxidizers (MPN x 10^7)	0.737**	0.805**	0.699*	0.779*	0.537*	0.819**	0.579	0.752*	0.874**	0.609*	0.795*	0.729*	0.637*	0.781*	0.722*

4. DISCUSSIONS

Nitrification inhibitors had a substantial impact on the amount of N_2O produced in soils planted with three distinct types of rice during the various seasons, according to the current study. During the rice cropping seasons, T4 (TL) applied fields in the *Ahu* rice ecosystem produced the least amount of N_2O. This contrasts with the *Sali* and *Boro* rice ecosystems, where the lowest output was observed at T2 (NL) applied fields over control (inorganic fertilizer, NPK) and calcium chloride (T5, CC), a recognized chemical inhibitor employed in the current studies. This difference in emission rate amongst the treatments resulted from the applied N inhibitors' varying capacities to slow down the nitrification process (Bhatia et al., 2010). By suppressing the activities or by blocking the hydroxylamine oxidoreductase and ammonia monooxygenase enzymatic pathways involved in ammonia oxidation in *Nitrosomas* (Subbarao et al., 2013), the nitrification inhibitors postpone the bacterial oxidation of NH_4^+ to NO_2^- in soil. This lowers the emission of N_2O -N by directly inhibiting the nitrification process. According to Majumder et al. (2000), this depression also indirectly affects the denitrification process by reducing the N_2O-N because there are insufficient amounts of substrates (NO_3-), which is consistent with our findings about NO_3- in soils (Figure 6). In support of the results obtained during the study, it is noted that, decomposition of the plant materials (NL and TL) produces inhibitory compounds such as phenolics and terpenoids (Jordan et al., 1979; Inderjit, 1996) which are released into the soil, lowering the degree of nitrification towards the maturation stages of the crop growth period (Subbarao et al., 2006). The inhibiting properties of phenolic compounds released from tea wastes are reported by Krishnapillai, (1979) and support our explanation that less N_2O production from soils treated with used tea leaves in rice ecosystem might be due inhibition of nitrification by these phenolics. Although this mechanism is not conclusive but to the best of our knowledge as it has never been reported before on N_2O emission reduction. Nitrification inhibitors work efficiently in fully or partially aerobic conditions, especially in sandy loam soils (Majumder et al., 2000) inhibiting nitrification and reducing N_2O emission from field and are in good agreement with our results of N_2O suppression by the applied plant based biological materials. Datta and Adhya (2014), reported *Nimin* and *Karanjin*, apart from being a potent inhibitor of nitrification, have potency to reduce denitrification process in rice paddies as a result of lower denitrification enzyme activity (DEA). We hypothesize that similar mechanisms have operated in the present investigation resulting in lower N_2O production at T2 (NL) and T4 (TL) applied fields.

Irrespective of ecosystems, propagule densities of microorganisms were significantly ($p < 0.05$) lower in NPK + NI amended (NL, SA, TL and CC) soils over only NPK applied soils. This decrease in colony forming units (CFUs) might be attributed to the decrease in net N mineralization in the field due to inhibition of microbial activities. In our study, addition of nitrification inhibitors might have slowed down the decomposition of the plant residues causing a deficiency of nutrients (C and N) to the microbial community to efficiently perform their metabolic activities and absorb nutrients for their growth as reported by Alden et al., (2001) and Xiao et al., (2007). Among the studied rice ecosystems, lower denitrifier population was recorded at T4 (TL) in *Ahu* and *Boro* rice while in *Sali* rice it was at T2 (NL). Irrespective of rice ecosystem, lowest nitrifier population was recorded at T2 (NL). Soil applied with inorganic fertilizers (NPK) increases the N pool in soil. This increase in N pool influences the soil microbial

indices as indicated by Gunapala and Scow, (1998). Incorporation of plant-based residues in combination with NPK in soils might have released some kind of inhibitory compounds during their decomposition at different stages of the crop growth which have altered the microbial processes of nitrification and denitrification as reported in biochar amended soil (Cayuela et al., 2013) and profoundly transform the abundance and structure of denitrification community (Guo et al., 2013), influencing the N_2O emission from soils. Significant reduction of NH_4^+ oxidizers in T2 (NL) suggest significant inhibition of ammonium oxidation to nitrite by the fresh neem leaves in the present study. Similar results have also been reported in *Nimin* treated plots (Datta and Adhya, 2014) which is in concordance with our results of reduced NH_4^+. Some types of medically bioactive phenolic compounds are structurally related to the compounds that inhibit soil nitrification (Mao et al., 2006). These phenolic compounds can be the substrates of ammonium monooxygenase (AMO) that can competitively inhibit NH_3 oxidation (Keener and Arp, 1994; McCarty, 1999) and reduce the population of NH_4^+ oxidizers in plots treated with tea leaves (T4, TL). The reduction of NH_4^+ and NO_3^- oxidizers in soils treated with plant-based NI provides indirect evidence of allelochemical effect of inhibition and further work is needed to specify and verify specific allelochemicals from these biological materials.

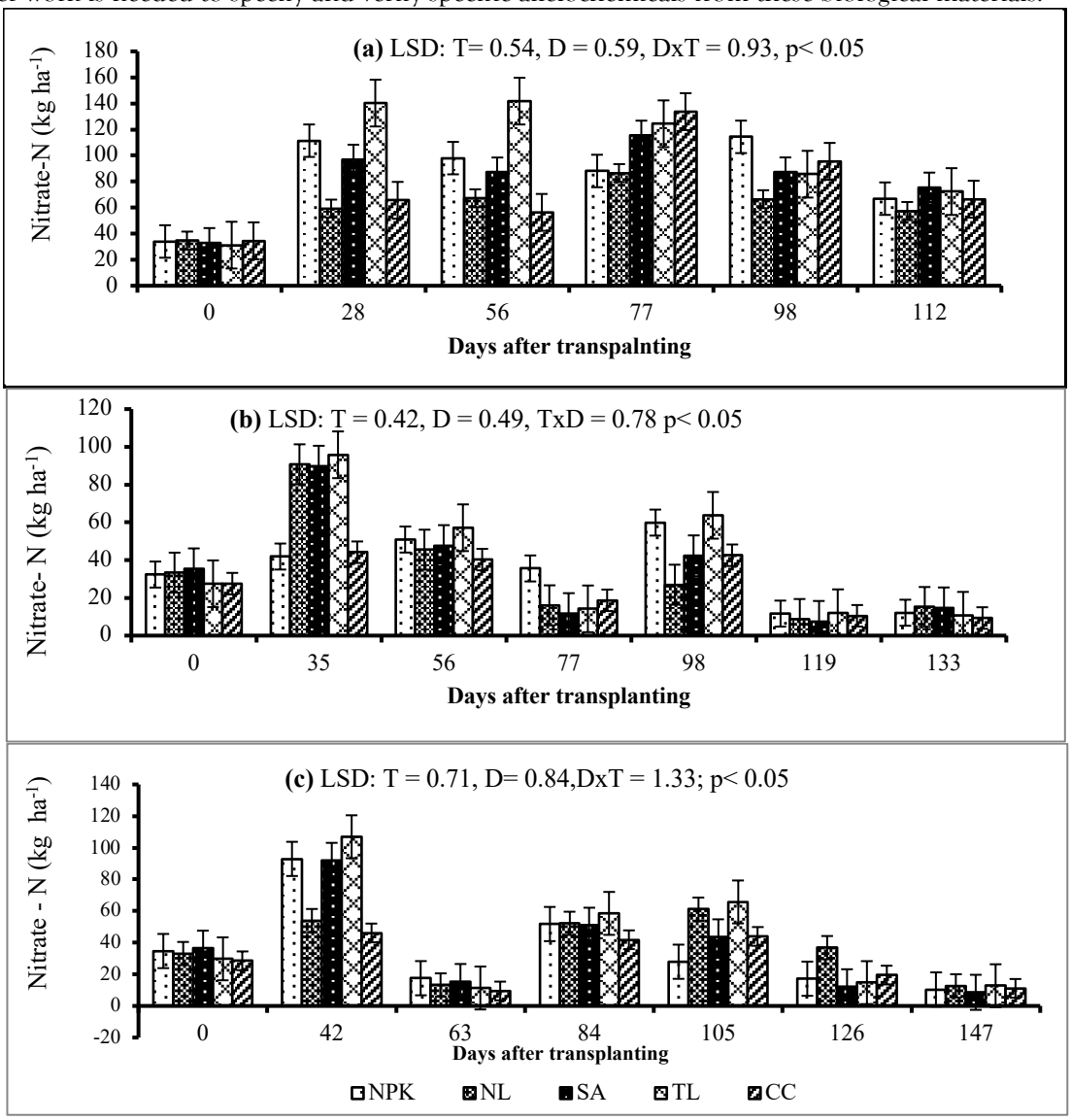

Figure 6. Total nitrate-N (kg ha^{-1}) in soils planted with (a) *Ahu* (pre-monsoon), (b) *Sali* (Monsoon) and (c) *Boro* (Summer) rice. *Values are mean ± S.E. of means (n = 3). LSD- least significance difference; T – treatment; D/DAT – days after transplanting.*

5. CONCLUSION

The present study highlights the potential of plant-derived nitrification inhibitors (NIs) such as neem leaves (NL) and tea leaves (TL) in mitigating nitrous oxide emissions across diverse rice ecosystems. Treatments with these biologically active materials consistently reduced N_2O production, ammonium and nitrate oxidizer populations, and overall microbial activity, as compared to conventional NPK and chemical inhibitors. The observed suppression of nitrification is likely attributed to the release of phenolic and terpenoid compounds, which interfere with key microbial enzymatic pathways. These findings underscore the promise of integrating natural inhibitors into sustainable nitrogen management strategies for low-emission rice production systems. In conclusion, application of fresh neem leaves (NL) at 5t ha^{-1} in combination with the recommended dose of N fertilizer (NPK) and on-site addition of used tea leaves (TL) with mineral fertilizer (NPK) can be recommended by stakeholders in tropical rice paddies.

DECLARATIONS

Funding: This work was supported by seed money grant number RGU/Ch (Acad)/ 07/ Botany (30) provided by The Assam Royal Global University.

Conflict of Interest: The authors declare that there is no conflict of interest, financial or otherwise

Availability of data and material (data transparency): All the data are available online

Ethics approval (include appropriate approvals or waivers): Not applicable

Author's contributions: The authors confirm the study, conception and design: Anushree Baruah; data collection: Jasmin Sultana and Neelav Kumar Das; analysis and interpretation of results - Daimalu Boro, Neelav Kumar Das, Jasmin Sultana and Anushree Baruah; Draft manuscript preparation: Jasmin Sultana, Anushree Baruah, Sthiti Porna Dutta, Annu Kumari. All authors have read and agreed to the published versions of the manuscript.

Consent to participate: no consent was required for participation from any individual for the study

Acknowledgments: The authors would like to acknowledge The Assam Royal Global University, Guwahati, Assam, India, for giving the support required to complete this research work.

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