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Do Metals Increase or Decrease Nitrous Oxide Emissions and Maize Yields from Upland Soils?

Ye Lim Park [1], Hyun Ho Lee [2], Sung Un Kim [3,4], Namgoo Kang [5,6] and Chang Oh Hong [3,4,*]

[1] Department of Applied Environmental Science, Kyung Hee University, Yongin 17104, Korea
[2] School of Civil and Environmental Engineering, Yonsei University, Seoul 03722, Korea
[3] Department of Life Science and Environmental Biochemistry, Pusan National University, Miryang 50463, Korea
[4] Life and Industry Convergence Research Institute, Pusan National University, Miryang 50463, Korea
[5] Measurment Instrumentation and Data Verification Research Team, Advanced Measurement Instrumentation Institute, Korea Research Institute of Standards and Science, Daejeon 34113, Korea
[6] Odyssey Education Program, University of Science and Technology, Daejeon 34113, Korea
* Correspondence: soilchem@pusan.ac.kr

Abstract: Metals, including copper (Cu), iron (Fe), and zinc (Zn), are associated with nitrous oxide (N_2O) production processes, such as nitrification and denitrification. This study aimed to elucidate the effects of Cu, Fe, and Zn on N_2O emissions and to determine cumulative N_2O emission and crop yields from upland soils. Metals were applied at a rate of 20 kg ha^{-1} in upland soil supporting maize (*Zea mays* L.) growth in 2018 and 2019. While the mean value of cumulative N_2O emissions across both years was 5.19 kg N_2O ha^{-1} yr^{-1} for the control soil, those of soil treated with Cu, Fe, and Zn were 3.37, 2.48, and 4.82 kg N_2O ha^{-1} yr^{-1}, respectively. Ammonium (NH_4^+) concentration in soil was highest after Fe application, and nitrate (NO_3^-) concentration was lowest. The copy number of the *amoA* gene related to NH_4^+ oxidation was lowest after Fe enhancement, implying that nitrification was inhibited. Furthermore, N_2O emission decreased with Cu addition because the copy number of the *nosZ* gene associated with N_2O reduction to N_2 was the highest. Because Cu and Fe decreased yield-scaled N_2O emission, the application of either metal could reduce N_2O emission per unit area of maize production, suggesting that both metals are beneficial soil amendments for reducing N_2O emissions while maintaining maize yield.

Keywords: denitrification; greenhouse gas; micronutrient; nitrification; water-filled pore space

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1. Introduction

Nitrous oxide (N_2O) is a major source of stratospheric NOx and contributes to ozone layer depletion. Thus, N_2O release contributes to global warming. Global annual N_2O emission is 6.7 Tg N_2O $year^{-1}$, 60% of which is attributable to agricultural soil [1]. Major efforts to mitigate greenhouse gas (GHG) generation in agricultural soil have focused on N_2O due to this contribution. The global warming potential of N_2O is 298 times greater than that of carbon dioxide (CO_2) for a 100-year time horizon [1]. In general, non-flooded upland soils are sinks for methane (CH_4) rather than sources [2].

Microbial nitrification and denitrification, including these processes in soil, produce more than two-thirds of N_2O [3,4]. Microbial enzymes involved in N_2O production and consumption often require metal cofactors, such as copper (Cu), iron (Fe), and zinc (Zn). These metals are trace elements in soils and are essential micronutrients for crop growth and reproduction. Micronutrient supply is needed when micronutrients limit crop growth, yield, and quality [5].

Notably, autotrophic ammonia-oxidizing bacteria (AOB) induce aerobic N_2O production. Ammonia (NH_3) is first oxidized to hydroxylamine (NH_2OH) in a reaction catalyzed by ammonia monooxygenase (AMO), which requires Cu and possibly additional Zn and

Fe [6]. Subsequently, NH_2OH is oxidized to nitrite (NO_2^-), a reaction catalyzed by hydroxylamine oxidoreductase (HAO) containing 24 Fe atoms in c-type cytochromes [7]. Orthologs of Cu-containing *NirK* enzymes are typically used for reducing NO_2^- to NO [8–10]. Nitric oxide (NO), a toxic intermediate, is then reduced to N_2O by an unknown enzyme, likely either *NorBC* [11,12], the tetraheme cytochrome c554 [13], or *NorS* [14].

Under anaerobic conditions, nitrate (NO_3^-) is reduced to nitrogen (N_2). Nitrous oxide is an intermediate gas in the denitrification sequence: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Nitrate reductase, which contains Fe as a cofactor, catalyzes NO_3^- conversion to NO_2^-. Nitrous oxide reductase, which is a Cu-rich enzyme that binds 12 atoms of Cu per homodimer, then reduces N_2O to N_2 [15,16]. In recent years, metal-driven N_2O production processes, including nitrification and denitrification, have attracted increased attention [17–22]. Zhu et al. reported that N_2O generation from municipal wastewater was reduced by Cu^{2+} addition due to the increased activities of nitrite and N_2O reductases [17]. However, Sharma et al. reported that Cu availability governed N_2O accumulation in wetland soils and stream sediments [18]. They observed that natural aquatic systems containing Cu at concentrations less than or equal to crustal abundances displayed incomplete N_2O reduction to N_2, which would cause N_2O accumulation and release into the atmosphere. According to Arezoo et al., the addition of FeS and FeS_2 decreased nitrate reduction and N_2O accumulation rates in agricultural peat soil [19]. Deng et al. observed that zerovalent iron played a role as an electron donor in the denitrification process and was efficient in catalyzing further N_2O reduction to N_2 in the lab-scale experiment [20]. Montoya et al. reported that total abundances of the *nosZ* denitrification gene, which is involved in N_2O reduction to N_2, were reduced by 75% on average in plots that received Zn fertilizers, thereby leading to elevated N_2O emissions [21]. In other field work, Feng et al. observed that zinc oxide nanoparticles increase N_2O emissions by increasing nitrification (AOB *amo*A) and denitrification (*nir*S) [22]. Although these studies assessed the effects of soil Cu, Fe, and Zn on N_2O production, these effects remain unclear. Notably, recent studies characterized N_2O production associated with microbial enzymes in nitrification and denitrification, but comparisons of Cu, Fe, and Zn as micronutrients on N_2O emission from upland soil on a field scale remain scarce. Therefore, an improved understanding of how these metals modulate microbial enzyme activities associated with nitrification and denitrification is still needed. Because reducing environmental pollution without compromising food security is critical [23,24], future sustainable agriculture should explore ways to lower N_2O emissions while maintaining high crop productivity. Agricultural practices are related to N_2O emission based on crop yield, referred to as yield-scaled N_2O emission (YSNE). As mentioned above, metals, including Cu, Fe, and Zn, are involved in N_2O production processes and mitigate or elevate N_2O emission [17–22]. These metals are also essential for crop growth and reproduction. For these reasons, we hypothesize that the application of metals, including Cu, Fe, and Zn, affects both N_2O production and crop yield from arable soil. In this study, we use YSNE and gene analysis associated with N_2O production processes to address cumulative N_2O emission and crop yield after supplementing upland soil with Cu, Fe, and Zn in a maize field for two years.

2. Materials and Methods

2.1. Site Description

The field experiment was conducted on upland soil at the experimental farm of Pusan National University, Miryang, Korea (35°30′08.3″ N 128°43′15.3″ E). Soil was classified as Bongsan series Typic Hapludults (fine loamy, mixed, mesic) and was well drained with a 2–3% slope. The soil pH was 6.72 and total nitrogen concentration was 1.09 g kg^{-1}. Table 1 lists the soil's specific physical and chemical properties. Precipitation and temperature data were obtained from a weather station in Miryang (Korea Meteorological Administration), located 1 km from the study site. The average values of temperature and precipitation at the study site throughout the whole year were 14.4 °C and 1216 mm, and during the maize growing season, they were 23.4 °C and 395 mm, respectively.

Table 1. Selected chemical and physical properties of the study soil (n = 3).

Parameter	Mean ± Standard Deviation
pH (1:5, H_2O)	6.72 ± 0.38
Organic matter (g kg^{-1})	26.6 ± 1.33
Total nitrogen (g kg^{-1})	1.09 ± 0.02
Inorganic nitrogen	
NH_4^+ (mg kg^{-1})	3.38 ± 0.23
NO_3^- (mg kg^{-1})	1.07 ± 0.29
Available P_2O_5 (mg kg^{-1})	113 ± 2.33
Exchangeable cation ($cmol_c$ kg^{-1})	
K	0.73 ± 0.05
Ca	4.20 ± 0.30
Mg	1.17 ± 0.07
Bulk density (g cm^{-3})	1.27 ± 0.12
Particle size distribution (%)	
Sand	43.4 ± 2.27
Silt	44.5 ± 3.15
Clay	12.1 ± 1.01
Soil texture	Sandy clay loam

2.2. Experimental Design and Field Management

Experimental plots were arranged in a randomized complete block design with three replications. Each plot was 5 × 5 m. To exclude the effect of count anions on N_2O production and crop yield, zerovalent forms of metals, including Cu, Fe, and Zn, were applied at the rates of 0 and 20 kg ha^{-1}. The metals were incorporated into soil with moldboard plows on 16 and 5 May in 2018 and 2019.

Crops may show toxicity and decreased yield when metals are absorbed in excessive amounts. In particular, safe concentrations in crops for Cu, Fe, and Zn are 2–50, 20–600, and 10–25 mg kg^{-1}, respectively [25]. Guo et al. observed that the critical concentrations of Cu added to soils that decreased maize grain yield by 10% were 711 mg kg^{-1} for calcareous soil with a pH of 8.9 and 23 mg kg^{-1} for acidic soil with a pH of 5.3 [26]. Depending on the site and the cultivars of rice used, reported critical concentrations of Fe in soil can range from 20 to 2500 mg kg^{-1} [27]. Takkar and Mann reported that maize grown for 60 days in soil treated with 50 mg kg^{-1} of Zn showed toxicity [28]. To date, South Korea has not established recommended application rates for Cu, Fe, and Zn. Crops did not show signs of toxicity after the application of up to 20 mg kg^{-1} of Cu, Fe, and Zn in previous studies [26–28]. An application rate of 20 mg kg^{-1} is allocated with 38.1 kg ha^{-1} (bulk density of the studied soil = 1.27 g cm^{-3} and soil depth = 15 cm). Therefore, 20 kg ha^{-1} was selected as the application rate in the present study.

In 2018 (Year 1), maize (*Zea mays* L.) seed was sown on May 22. The seed spacing within and between rows was 25 and 60 cm, respectively. Inorganic fertilizers, including urea, fused phosphate, and potassium sulfate, were applied to all plot surfaces at a rate of 186–35–74 kg ha^{-1} (N–P_2O_5–K_2O) on the same day soon after seeding. Precipitation was insufficient for seed germination from 22 May to 20 June (Figure 1C). The total precipitation for this period was 62 mm. The germination rate on 20 June was <10% due to severe drought. Therefore, 1-month-old maize seedlings were transplanted onto all plots on 25 July. Additional inorganic fertilizer was applied at a rate of 93–17.5–37 kg ha^{-1} (N–P_2O_5–K_2O). In 2019 (Year 2), maize seedlings were again transplanted into all plots on 11 May. Nitrogen fertilizer was applied as a split application at two development stages: 50% as basal fertilization at transplanting (11 March 2019) and 50% at the 7/8 leaf stage (5 July 2019). Plots were irrigated after fertilizer application when rainfall was insufficient in both years. Maize was harvested on 4 October and 23 July in 2018 and 2019, respectively.

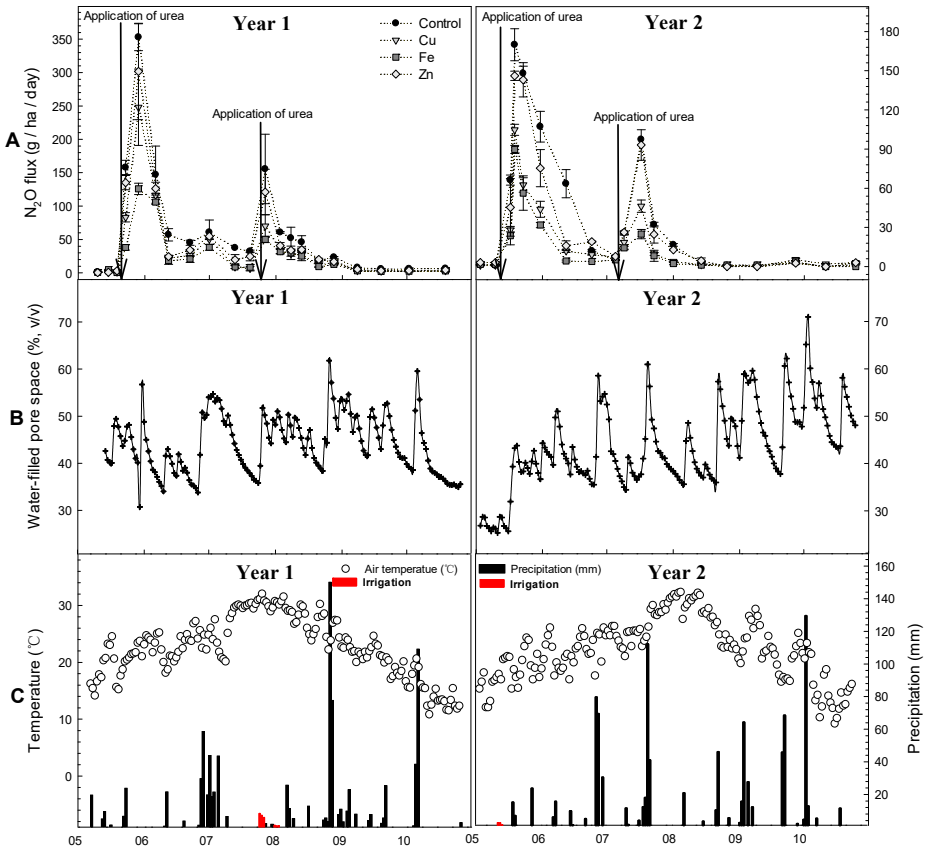

Figure 1. Daily N_2O emission (**A**), water-filled pore space (WFPS) (**B**), air temperature, and precipitation (**C**) during the growing seasons in Years 1 and 2.

2.3. Measurement of N_2O Emission

Soil-to-atmosphere N_2O fluxes were measured once every week during the growing season (May through October) and once every 2 weeks during the fallow season (November through May). Nitrous oxide flux from soils amended with metals was monitored for two years, including the growing and fallow seasons. However, N_2O fluxes only during the growing season from May to October in Years 1 and 2 are shown in Figure 1A. Gas sampling frequency increased 2–3 times per week in the following weeks after nitrogen fertilization and rainfall events. A static closed chamber method was used to measure N_2O flux [29]. The chamber collar comprised a polyvinyl chloride (PVC) tube (diameter = 18 cm and height = 23 cm) spiked into the plot center. Collars remained in place during both the growing and fallowing seasons and were left uncovered. All plants that grew in the collar were removed during the experiments. Collars were closed before gas sampling using a PVC lid (diameter = 20.2 cm and height = 17 cm) with a built-in vent tube and rubber stopper. Gas samples were collected using a 30 mL syringe from the headspace (1500 cm^3) in the chamber at 0, 20, and 40 min after lid placement between 10:00 and 12:00 h throughout the year. Samples were transferred to 12 mL evacuated glass vials (Exetainer® 12 mL vial-evacuated 838 W, Labco, Wales, UK) sealed with butyl rubber septa. Gas samples were analyzed using a gas chromatograph–mass spectrometer (GC-MS QP2020, Shimadzu, Japan) equipped with packed columns of Porapak Q. The carrier gas was helium with a flow rate of 4.25 mL min^{-1} through the column. The GC-MS was calibrated by the certified standard gases of N_2O and then the concentrations of N_2O in the samples were determined based on the calibration curve.

Gas fluxes were calculated as follows:

$$F = \rho \times \left(\frac{V}{A}\right) \times \left(\frac{\Delta C}{\Delta T}\right) \times \left(\frac{273}{T + 273}\right) \times k \times a$$

where F = N_2O flux (g ha^{-1} day^{-1}); ρ= density of N_2O (g m^{-3}); V = volume of the chamber (m^3); A = base area of the chamber (m^2); $\Delta C/\Delta T$ = the change in gas concentration inside the chamber as a function of time (g m^{-3} min^{-1}); T = temperature in the chamber (°C); and 273 = a correction factor between Celsius and Kelvin. In addition, k (min day^{-1}) is the time conversion coefficient, and a (10,000 m^2 ha^{-1}) is the area conversion coefficient. The air temperature in the chamber was measured at every gas sampling. Cumulative N_2O fluxes were calculated by multiplying the mean value of N_2O fluxes (g ha^{-1} day^{-1}) (Ri) by the length of the period (Di) and integrating the results over the monitoring period:

$$\text{Cumulative } N_2O \text{ emission } \left(\text{kg ha}^{-1}\ \text{yr}^{-1}\right) = \sum_i^n (R_i \times D_i)$$

Yield-scaled N_2O emission was calculated as follows:

$$\text{YSNE } \left(\text{kg Mg}^{-1}\right) = \frac{\textit{cumulative } N_2O \textit{ emission } (\text{kg } N_2O \text{ ha} - 1 \text{ yr} - 1)}{\textit{dried maize grain yield } (\text{Mg ha} - 1 \text{ yr} - 1)}$$

The harvested maize yield was based on the dry weight of the ear.

2.4. Physical and Chemical Analyses

Water-filled pore space (WFPS, %) was calculated every day for two years using the following equation:

$$WFPS\ (\%) = \left[\frac{\textit{volumetric water content}}{1 - \left(\frac{\textit{bulk density}}{2.65}\right)}\right] \times 100$$

Volumetric water content was measured at a 5 cm soil depth with a 5TE moisture sensor (Decagon, USA) every 3 h. The means of all values were used as the daily volumetric moisture content (m3 m^{-3}). Bulk density of soil samples collected at 0–15 cm in each plot was measured every month. The values were used in the above equation. Samples were collected using a fixed-volume core (94.64 cm^3) and dried at 105 °C. The particle density was assumed to be 2.65 g cm^{-3}.

Soil samples were collected at 0–15 cm in each plot at harvest time to analyze ammonium (NH_4^+) and nitrate (NO_3^-); 5 g of air-dried soil was extracted with 30 mL of 2 M KCl solution. After shaking for 30 min, the mixture was filtered through Whatman No. 2 filters NH_4^+ and NO_3^- concentrations in the extracts were analyzed using the indophenol blue [30] and brucine methods [31], respectively.

2.5. Total DNA Extraction and Real-Time PCR

Soil samples for DNA analysis were collected in a 2 mL tube and stored at −75 °C at harvest time (23 July 2019) in Year 2. Total DNA was extracted from ca. 250 mg of frozen soil using a DNeasy® PowerSoil® DNA isolation kit (Qiagen) following the manufacturer's instructions. DNA was stored in a deep freezer until use. Soil DNA concentration and purity were measured using NanoDrop (ALLSHENG, China), and the size was checked by electrophoresis on 1.2% agarose.

Total bacterial abundance was assessed using the 16S rRNA gene [32] with real-time PCR. Nitrifying communities were assessed using the expression of the *amoA* gene from AOB and the *hao* gene from NH_2OH-oxidizing bacteria [33,34]. Denitrifying communities were quantified by the expression of *narG*, *nirS*, *norB*, and *nosZ* [35–38].

Real-time PCR assays were performed on a CFX96 Touch System (Bio-Rad Laboratories, Inc., CA, USA). Primer sequence information for nitrifying and denitrifying microbes was collected from previous studies (Table 2). qPCR reactions were conducted in triplicate using real-time PCR reaction plates sealed with clear film. Each 20 µL reaction contained 10 µL 2x AMPIGENE qPCR Green Mix Lo-ROX (Enzo Life Sciences, NY, USA), 1 µL forward and reverse primers (10 µM), 1.0 µL of genomic DNA, and 7.0 µL PCR-grade water.

The reaction process followed the manufacturer's instructions as initiation for one cycle at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 25 s, and elongation at 72 °C for 30 s; and final elongation at 72 °C for 5 min. Standard curves were obtained using serial dilutions ranging from 109 to 101 copies μL^{-1} of plasmids containing target gene sequences. The abundance of targeted genes was expressed per nanogram of DNA instead of per gram of soil to minimize bias related to soil DNA extraction efficiency [39]. All qPCR reactions were conducted at least in triplicate. Microbes were quantified by comparison with the standard curve.

Table 2. Primer sets for qPCR.

Primer	Sequence (5′→3′)	Size (bp)	Reference
16s rRNA-1097F 16s rRNA-1242R	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	146	[32]
*amo*A-1F *amo*A-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	[33]
hao-1F *hao*-3R	TGCGTGGAAGTGCTCAC AGAGTAAGGAGTCTCGGGCAAA	992	[34]
*nar*G-2F *nar*G-2R	TA(CT) GT(GC) GGG CAG GA(AG) AAA CTG CGTAGAAGAAGCTGGTGCTGTT	110	[35]
*nir*S-1F *nir*S-6R	CCTAYTGGCCGCCRCART CGTTGAACTTRCCGGT	890	[36]
*cnor*B-2F *cnor*B-6R	GACAAGNNNTACTGGTGGT GAANCCCCANACNCCNGC	389	[37]
*nos*Z-1F *nos*Z-2R	WCSYTGTTCMTCGACAGCCAG CAKRTGCAKSGCRTGGCAGAA	700	[38]

2.6. Statistical Analysis

All statistical analyses used R Studio (version 3.4.4, R Core Team, 2018, Vienna, Austria) with the "Agricolae" package. The mean values of cumulative N_2O emission, inorganic N, YSNE, maize ear yields, and the copy numbers of the targeted genes were assessed by pair-wise comparisons. An analysis of variance was used to evaluate differences between parameters. A least significant difference test was performed to separate mean effects only when the F-test result was significant at $p < 0.05$.

3. Results and Discussion

3.1. N_2O Flux

The first and second N_2O peaks appeared soon after urea application in both years (Figure 1A). Urea was applied on 22 May and 25 July in Year 1 and 11 May and 5 July in Year 2. The peaks of N_2O flux occurred 2–3 days after application. Daily N_2O flux is related to soil WFPS, whose change is an important factor affecting N_2O emissions from arable soil [3,40,41]. Notably, N_2O can be emitted by nitrification with a soil WFPS of 35–60% that requires NH_4^+ as an inorganic N substrate for aerobic respiration, whereas a soil WFPS above 60% (O_2 is limited) induces a switch from aerobic to anaerobic respiration. Therefore, NO_3^- is an alternative electron acceptor used by microorganisms associated with denitrification that produces N_2O [40,42–44]. Soil WFPS increased by irrigation after fertilization but did not increase by rainfall events in both years [Figure 1B,C]. The daily WFPS values at the first and second N_2O peaks were 47.8% and 49.8% in Year 1 and 43.3% and 37.7% in Year 2, respectively [Figure 1B]. All N_2O peaks in both years appeared when daily WFPS was <60%, implying that nitrification was the predominant process for N_2O production.

3.2. Cumulative N_2O Emission

Metals significantly affected cumulative N_2O emissions (Table 3). Cumulative N_2O emissions among metals differed significantly in both Years 1 and 2 (Table 4). The order of cumulative N_2O emission values was Fe < Cu < Zn < control in both years. The

mean value of cumulative N_2O emissions across Years 1 and 2 did not differ significantly between Cu and Fe applications. However, cumulative N_2O emission after Fe treatment was significantly lower than that with Cu in both years. Zinc was ineffective in reducing cumulative N_2O emissions because its supplementation had no significant effect compared to the control.

Table 3. ANOVA and *p*-value of cumulative N_2O emissions, ammonium (NH_4^+), and nitrate (NO_3^-) concentration at harvest, copy numbers of 16S rRNA, *amo*A, *hao*, *nar*G, *nir*S, *cnor*B, and *nos*Z genes, maize grain yield, and yield-scaled N_2O emissions.

Parameter	Source of Variation		
	Metal (M)	**Year (Y)**	**M × Y**
df	3	1	3
Cumulative N_2O emissions	<0.001	<0.01	<0.001
NH_4^+	<0.001	NS	NS
NO_3^-	<0.001	NS	NS
16S rRNA	<0.001	-	-
*amo*A	<0.01	-	-
hao	NS [§]	-	-
*nar*G	NS	-	-
*nir*S	NS	-	-
*cnor*B	NS	-	-
*nos*Z	<0.001	-	-
Maize grain yield	NS	NS	NS
Yield-scaled N_2O emission	<0.01	<0.05	<0.01

[§] NS, not significant.

Table 4. Cumulative N_2O emission, maize grain yield, and yield-scaled N_2O emission from soils amended with different metals in Years 1 and 2.

Metal	Cumulative N_2O Emission (kg ha^{-1} yr^{-1})		
	Year 1	**Year 2**	**Year Mean [§§]**
Control	6.13 [a]	4.25 [a]	5.19 [a]
Cu	3.93 [b]	2.81 [b]	3.37 [b]
Fe	2.97 [c]	1.99 [c]	2.48 [b]
Zn	5.69 [a]	3.96 [a]	4.82 [a]
Metal mean [§]	4.68 [A]	3.25 [B]	
Metal	**Maize Grain Yield (Mg ha^{-1})**		
	Year 1	**Year 2**	**Year Mean [§§]**
Control	5.77 [a]	5.83 [a]	5.80 [a]
Cu	5.85 [a]	5.85 [a]	5.85 [a]
Fe	5.06 [a]	5.06 [a]	5.06 [a]
Zn	5.87 [a]	5.87 [a]	5.87 [a]
Metal mean [§]	5.64 [A]	5.65 [A]	
Metal	**Yield-Scaled N_2O Emission (kg Mg^{-1})**		
	Year 1	**Year 2**	**Year Mean [§§]**
Control	1.22 [a]	1.03 [a]	1.12 [a]
Cu	0.68 [c]	0.41 [b]	0.55 [b]
Fe	0.57 [c]	0.31 [b]	0.44 [b]
Zn	0.95 [b]	0.61 [b]	0.78 [ab]
Metal mean [§]	0.86 [A]	0.59 [B]	

[§] Metal mean: mean value across control, Cu, Fe, and Zn. [§§] Year mean: mean value across Years 1 and 2. Different lower- and upper-case letters denote significance at $p < 0.05$ in comparison within column and row, respectively.

Metal application significantly affected NH_4^+ and NO_3^- concentrations in soil at harvest (Table 3). Mean values of cumulative N_2O emission across years from lowest

to highest were opposite to the order of NH_4^+ soil concentrations but were consistent with NO_3^- levels [Figure 2A,B]. Mean NH_4^+ levels varied as control < Zn < Cu < Fe. Conversely, NO_3^- concentrations were Fe < Cu < Zn < control. Differences in N_2O emissions were reflected in changes in soil NH_4^+ and NO_3^- concentrations at harvest. While cumulative N_2O emissions were negatively correlated with NH_4^+ concentrations and positively correlated with NO_3^- concentrations in both years [Figure 3A,B], N_2O production was highest in the control soils, where the least NH_4^+ and the most NO_3^- were observed. Nitrification processes were active after N fertilizers were added to control plots. In contrast, NH_4^+ concentrations at harvest were highest, and NO_3^- concentrations were lowest in plots treated with Fe in both years.

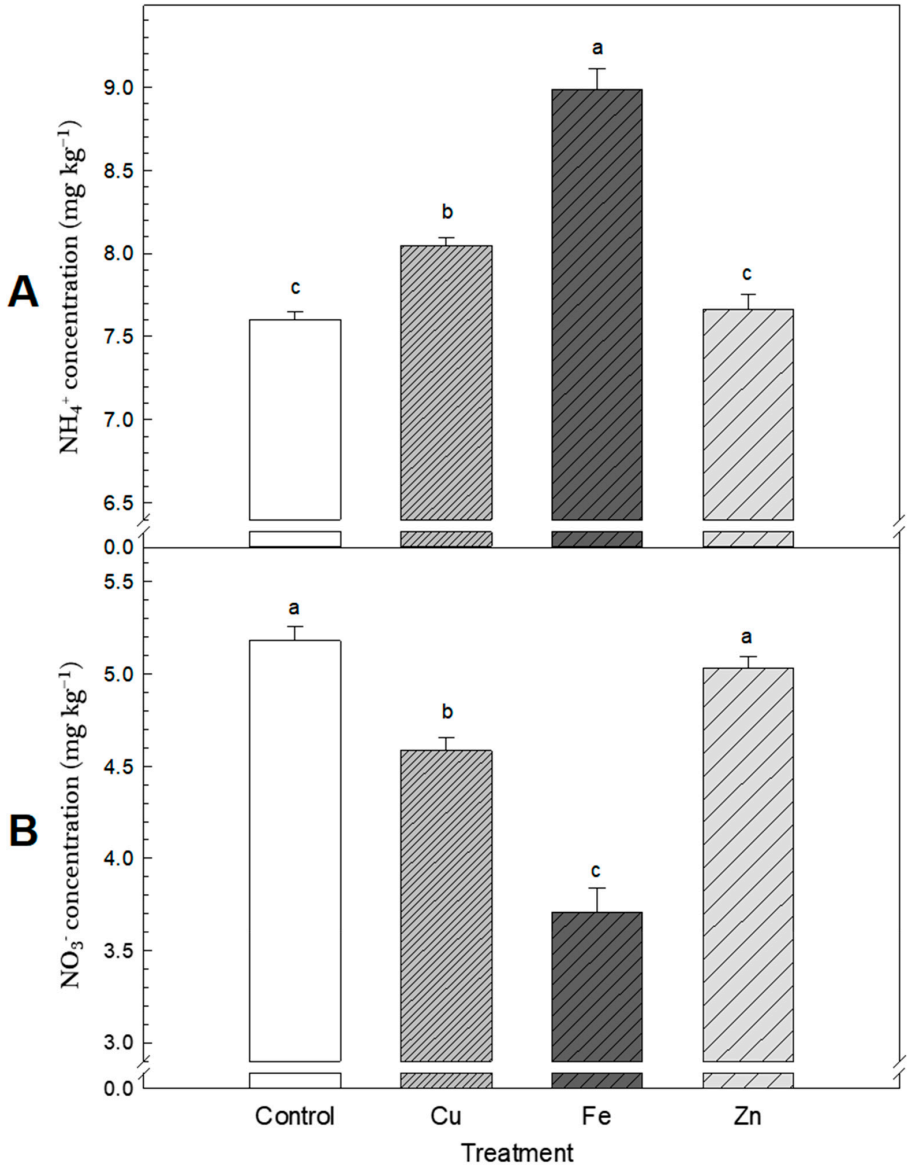

Figure 2. Mean values of ammonium (**A**) and nitrate (**B**) in upland soils amended with different metals at harvest across both years. Different lowercase letters denote significant difference at $p < 0.05$ level.

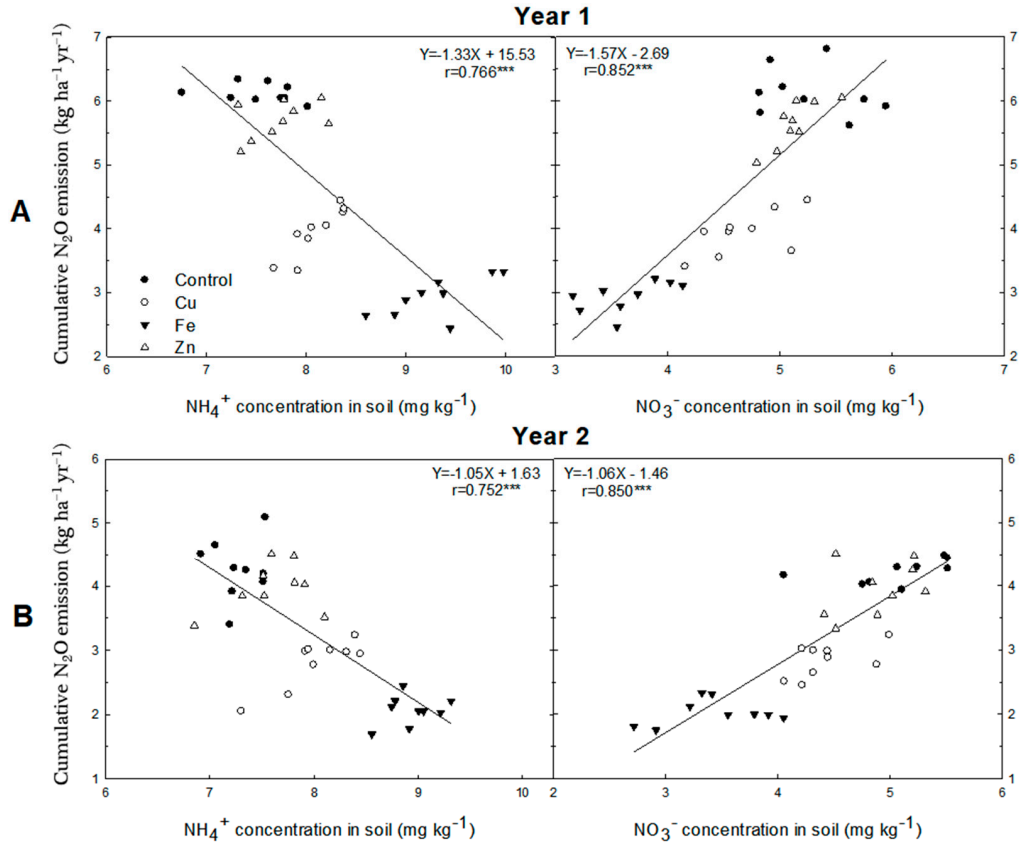

Figure 3. Relationships between cumulative nitrous oxide (N_2O) emissions and ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in soil at harvest in Year 1 (**A**) and Year 2 (**B**). *** Significant at $p < 0.001$ level.

We hypothesized that Fe impeded NH_4^+ conversion to NO_3^-, causing decreased N_2O production. The total abundance of bacteria was assessed using the 16S rRNA gene copy number to test this hypothesis. Nitrifying bacteria (measured by the relative abundance of bacterial *amoA* and *hao* genes) and denitrifying bacteria (measured by the relative abundance of the *narG*, *nirS*, *norB*, and *nosZ* genes) were quantified (Figure 4). While significant differences were observed in the copy numbers of *amoA* and *nosZ* in bacteria after metal supplementation, significant increases were not observed for *hao*, *narG*, *nirS*, and *norB*. Average gene copy numbers after metal treatments were approximately 40% higher than in the control plots. Metal application to soil promoted microbial activity. Interestingly, the copy number of the *amoA* gene that catalyzes NH_3 oxidation to NH_2OH in the first step of nitrification significantly decreased after Fe application, but that of *hao* significantly increased. However, the *narG*, *nirS*, and *norB* copy numbers showed no such increase. Fe negatively affected *amoA* abundance resulting in decreased N_2O production by inhibiting NH_3 oxidation to NH_2OH. The abundance of AOB that requires NH_3 as a substrate for oxidation could decrease when NH_3 is limited. Zerovalent iron is oxidized and converted into ferrous ion (Fe^{3+}) [45] as follows:

$$Fe \rightarrow Fe^{3+} + 3e^-$$

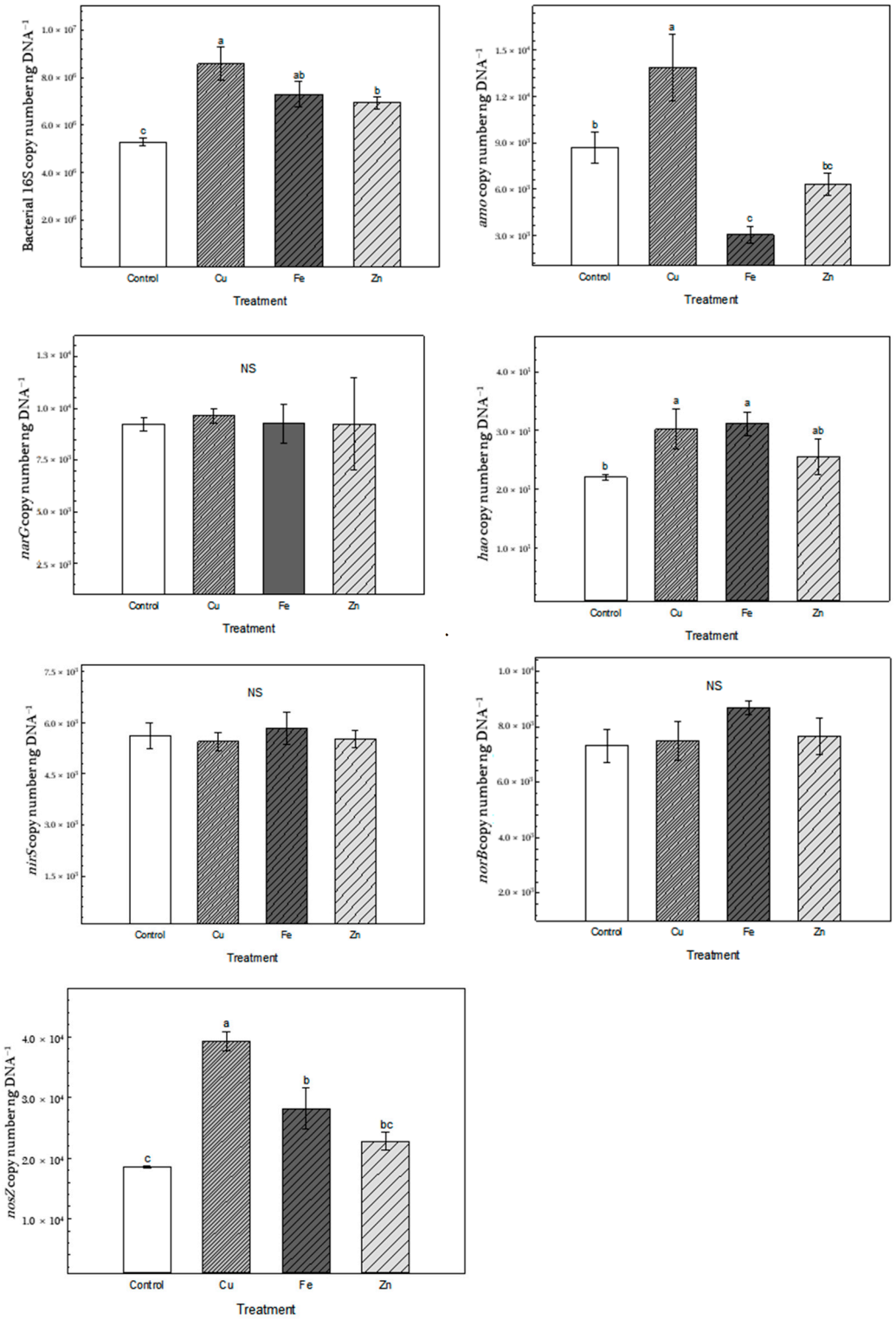

Figure 4. Copy numbers of the *amoA* gene from AOB and *hao*, *narG*, *nirS*, *norB*, and *nosZ* genes in soil amended with different metals at harvest in Year 2. Different lowercase letters denote significant difference at $p < 0.05$ level.

One mole of Fe^{3+} produces three moles of H^+ in the following reaction [43]:

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$$

Zerovalent copper and zinc are oxidized, and one mole of each metal produces two moles of H^+ [45] as follows:

$$Cu \rightarrow Cu^{2+} + 2e^-, \text{ then } Cu^{2+} + 2H_2O \rightarrow Cu(OH)_2 + 2H^+$$

$$Zn \rightarrow Zn^{2+} + 2e^-, \text{ then } Zn^{2+} + 2H_2O \rightarrow Zn(OH)_2 + 2H^+$$

An inverse reaction is predominantly promoted by H^+ concentration:

$$NH_4^+ \leftrightarrows NH_3 + H^+$$

More H^+ is produced by Fe than by Cu and Zn. Thus, more NH_4^+ was accumulated by the oxidation of Fe than by Cu or Zn oxidation [Figure 2A]. Subsequently, AOB abundance and N_2O production decreased. According to Arezoo et al., the addition of FeS and FeS_2 decreased nitrate reduction and N_2O accumulation rates in agricultural peat soil [18]. However, zerovalent Fe did not decrease the gene copy numbers of *nar*G, *nir*S, *nor*B, and *nos*Z involved in denitrification in the current study (Figure 4). The reason for the apparent impediment of denitrification by added iron sulfides in the study of Arezoo et al. was uncertain, but previous studies found that hydrogen sulfide strongly inhibited NO_3^- reduction in cultures of *Desulfovibrio desulfuricans* [46].

For nitrification to occur, optimum conditions in terms of population of nitrifying organisms, pH, temperature, oxygen, moisture, and substrate concentration and availability are most important. At the ecosystem level, several physical, environmental, and chemical factors interact in a complex manner to influence the nitrification process [47]. Soil pH is the major factor regulating the nitrification process in soils. Nitrification occurs in soil at pH ranging between 5.5 and about 10.0, with the optimum around 8.5 [47]. The pH of the studied soil was 6.75, lower than the optimum pH value for nitrification (Table 1). As mentioned above, Fe oxidation produced more H^+ and decreased the soil pH compared to those of other metals. Nitrification has long been known to generally follow a bell-shaped temperature response curve with an optimum at 30–35 °C. In this study, the mean daily temperature maintained the optimum temperature (30–35 °C) in August of both Years 1 and 2 (Figure 1). Soil moisture and aeration or soil oxygen levels are inversely related to nitrification. Oxygen content in the soil is reduced at higher soil moisture, as most pore spaces are occupied by water, and higher soil moisture also restricts diffusion of atmospheric air into the soil. Thus, optimum conditions for both moisture and aeration are critical for nitrification to occur in the soil. Nitrification is the predominant source of N_2O when WFPS is <60% [48–50]. Daily WFPS was below 60% most of the time during the growing season of maize in both years, except October in Year 2 (Figure 1). Because the experiment was not conducted under controlled conditions, determining the influence of individual environmental factors on nitrification among treatments in this field study was challenging. The decrease in cumulative N_2O emission after Cu supplementation might hypothetically be due to Cu promotion of N_2O reduction to N_2 and then decreased N_2O emission through denitrification. The *amoA* gene copy number significantly increased with Cu addition (Figure 4). Because AMO requires Cu for NH_4^+ oxidation to NH_2OH, Cu application increased *amoA* abundance [6]. Furthermore, the *hao* gene copy number significantly increased, but that of the *norB* gene that catalyzes NO reduction to N_2O remained unchanged. Decreased N_2O production through nitrification with Cu is difficult to explain. This decrease is easier to attribute to denitrification. The *nosZ* gene copy numbers significantly increased with Cu supplementation and were the highest among soil treatments (Figure 4). Thus, N_2O emissions decreased because N_2O reduction to N_2 became more active. Similar results were observed by Zhu et al., who reported that N_2O generation from municipal wastewater was reduced by Cu^{2+} addition due to increased activities of nitrite and N_2O reductases [17].

In another study, Montoya et al. reported that total abundances of the *nos*Z denitrification gene, which is involved in N_2O reduction to N_2, were reduced by 75% on average in

the plots that received Zn fertilizers, inducing elevated N_2O emissions [21]. In the current study, Zn did not increase cumulative N_2O emission (Table 4), and the gene copy number of *nos*Z did not decrease compared to the control (Figure 4). Conflicting results might be due to different sources of Zn between both studies. Montoya et al. used soluble Zn sources such as $ZnSO_4$ and Zn applied with a mixture of chelating compounds (DTPA, HEDTA, and EDTA) in their study, but an insoluble Zn source, zerovalent Zn, was used in this study. Different Zn sources might differently affect total abundances of the *nos*Z gene and cumulative N_2O emission.

3.3. Yield-Scaled N_2O Emission

Metals did not affect maize grain yields (Table 3). The mean values of grain yield across both years did not differ significantly among the treatments (Table 4). The maize grain yield ranged from 5.06 to 5.87 Mg ha^{-1}. These values were similar to the values of maize grain yield (4.1–7.9 Mg ha^{-1}) reported by several researchers who used different cultivation techniques, N fertilization rates, and cropping systems in upland soils [51–53].

Metals significantly affected YSNE (Table 3). The YSNE values after Cu and Fe supplementation were significantly lower than those of the controls in both Years 1 and 2 (Table 4). The YSNE value was lowest for Fe addition, although it was not significantly different from Cu in Year 1 and from Cu and Zn in Year 2. The order of YSNE values was Fe < Cu < Zn < control in both years. This order is similar to the order of cumulative N_2O emissions (Table 4) because maize ear yield did not differ significantly among treatments (Table 4).

The YSNE value ranged from 0.31 to 1.22 kg Mg^{-1}, slightly lower than the value (1.36–2.95 kg Mg^{-1}) reported by Halvorson et al. [54]. These authors measured N_2O emissions and crop yield from irrigated maize fields in the United States. The higher YSNE values reported by these authors were likely due to greater N_2O emissions with irrigation, despite the similar rate of N fertilizer application (246 and 202 kg N ha^{-1} for Years 1 and 2, respectively) compared with the present study (279 and 186 kg N ha^{-1} for Years 1 and 2, respectively). Irrigation maintained a relatively higher WFPS in the former study. The WFPS value ranged from 40% to 76%, higher than the WFPS range (25–72%) in the present study. WFPS can be an indicator of aerobic and anaerobic microorganism activity [55]. The higher the WFPS, the more air in the pores is replaced by water, thereby removing O_2. More N_2O is produced through denitrification when O_2 is limited.

Expressing N_2O emissions on a yield-scaled basis provides information for evaluating overall greenhouse gas impacts. YSNE reflects kg cumulative N_2O per Mg of maize grain produced. The lower YSNE value after Cu and Fe treatment compared to the control indicates that metals might reduce N_2O emission per unit of grain production. For example, based on the current findings, if the same amounts of grain were produced from the control and treated plots, the control plots would emit 53% and 62% more N_2O compared to the Cu- and Fe-treated plots, respectively, assuming the same fertilizer regime.

Furthermore, 1 M NH_4OAc extractable Cu, Fe, and Zn concentrations in soil were measured to evaluate the plant availability of those metals by application for two consecutive years (Figure 5). Plant-available Cu, Fe, and Zn concentrations in soil increased significantly compared to the control. This implies that long-term application of these metals may increase metal uptake in grains and plant tissues and decrease crop yield and quality. Shahid et al. observed that plant-available Cu, Fe, and Zn concentrations in soil and those metal concentration in rice plants increased with the long-term application of animal manure for 41 years in paddy soil [56]. However, total concentrations of Cu, Fe, and Zn in soil did not significantly increase with metal application compared to the control at harvest time in Year 2 and did not exceed safe levels of Cu and Zn established by the Korean Soil Environmental Conservation Act. (Figure 5). The current study was conducted for a relatively short-term experiment period (2 years). In the future, further research on evaluating Cu, Fe, and Zn accumulation in soil and plant tissue with long-term application of these metals should be conducted.

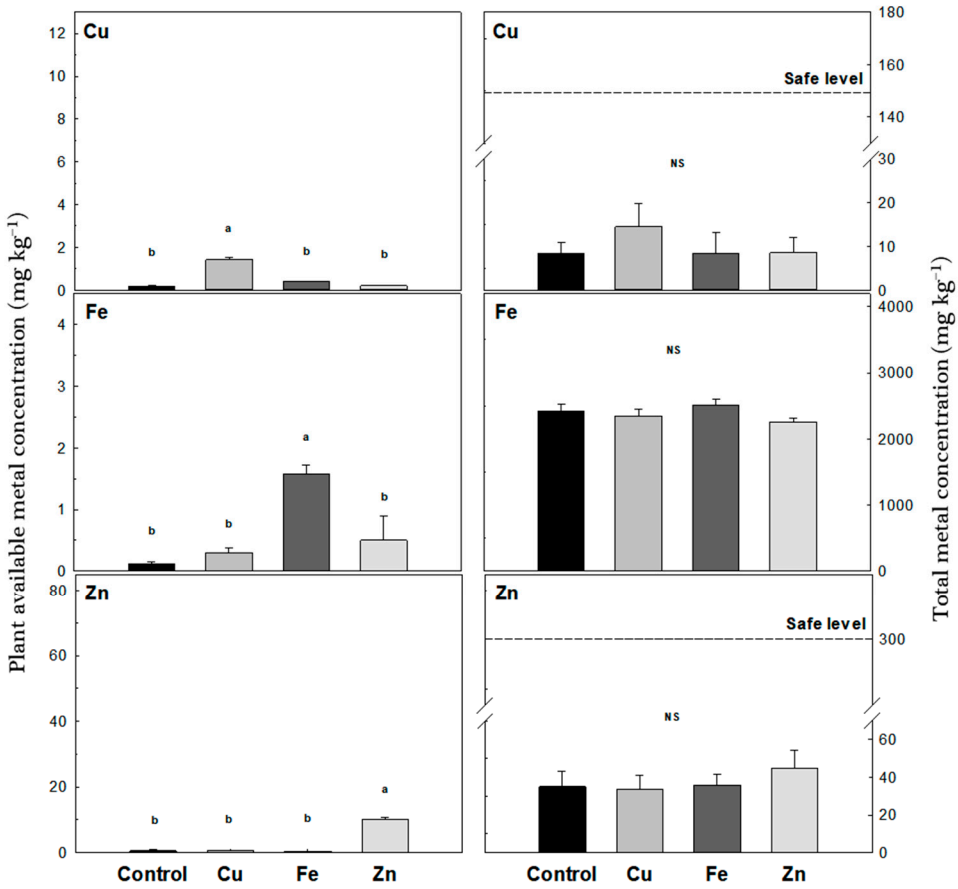

Figure 5. Concentration of plant-available metals and total metals in soils amended with different metals at harvest time in Year 2 (Dashed lines denote safe levels of Cu and Zn established by the Korean Soil Environmental Conservation Act). Different lowercase letters denote significant difference at $p < 0.05$ level.

In addition, only 20 kg ha^{-1} of metal application rate was applied in this study. The effects of lower and higher application rates than 20 kg ha^{-1} on N_2O emission and maize yield need to be determined. Lower application rates of metals, such as Cu and Fe, might not be effective in reducing cumulative N_2O emission from arable soil. In contrast, higher application rates of metals might be more effective to decrease N_2O emission, but long-term application of higher rates of metals might result in metal accumulation in soil and plants, as mentioned above. Subsequently, crop yield and quality decrease. Zhu et al. observed that N_2O production decreased with increasing addition rates of Cu^{2+} in municipal wastewater, and Cu^{2+} addition (10–100 μg L^{-1}) reduced N_2O generation by 54.5–73.2% [17]. Determining the effect of different application rates of metals on N_2O emission and crop yield remains challenging, which requires further study.

The redox state of soil related to water content is one of the important factors influencing N_2O emission. Nitrous oxide production is associated with soil WFPS values. Nitrification is the predominant process for N_2O emission from soils with <60% WFPS, whereas denitrification is the predominant process of N_2O emission from soil with >60% WFPS [48–50]. When the soil WFPS value is approximately 60%, N_2O production increases considerably due to simultaneous nitrification and denitrification [57]. When the soil WFPS value increases by >70%, soil environmental conditions favor denitrification, and N_2 is emitted instead of N_2O [58]. The current study was conducted in upland soil with WFPS ranging from 25.4% to 70.9% (Figure 1). Zerovalent Cu and Fe were well oxidized and converted to ionic forms under oxidation conditions, such as upland soil, and both metals in ionic forms were involved in N_2O production processes. However, the oxidation reaction

of zerovalent metals is poor in water-logged soil, such as paddy soil or wet soil, in high rainfall areas. Both metals contribute less to the mitigation of N_2O emission in those soils. Other factors, such as application timing of metals, application rate of nitrogen fertilizer, and temperature, also influence the effect of metals on N_2O emission. Further research on different climate, soil water content, application rate and timing of metals, and application rate of nitrogen fertilizer should be conducted.

4. Conclusions

Our maize field study revealed that Fe and Cu as micronutrients are effective in decreasing YSNE, implying that their application could reduce N_2O emission per unit of maize grain production. Both metals could be used as soil amendments to reduce N_2O emission while maintaining yield from upland soils. However, further research on application rates and timing in different soil environments should be conducted.

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