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The Effect of Copper and Carbon on the Emission of the Potent Greenhouse Gas, N_2O.

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Abstract: *Owing to the dependence on denitrification dependent element copper, in regulating N_2O:N_2 molar ratios, the effects of soil copper concentration in limiting (Treatments not amended with Copper) and abundant (40 mg/kg Cu SO_4) conditions were analysed in combination with a carbon source (50 µg/g glucose ($C_6H_{12}O_6$)). In line with our hypothesis, final N_2O:N2 ratios observed on day 1 (0.5, 0.42) for Insch and Brechin soils respectively, when compared to the carbon treatment (0.8, 0.61), indicate a positive potential for carbon and copper amended soils to reduce N_2O emissions from non-toxic agricultural soils.Findings from this current research will serve as a baseline study for further research on reduction of N_2O emissions from agricultural soils*

Keywords: *carbon, copper, denitrification, greenhouse gas, nitrous oxide, N_2O:N_2 molar ratio*

I. Introduction

Nitrous oxide (N_2O) emissions can be brought about by a variety of processes and are largely directly or indirectly biological, with soils representing a large proportion of N2O production (Lassey and Harvey 2007; Thomson and Giannopoulos et al. 2012). In recent years, anthropogenic N2O sources, notably the Haber-Bosch process have doubled natural rates of terrestrial nitrogen fixation (Canfield and Glazer et al. 2010), leading to a large proportion of N2O emissions from agricultural soils. (Skiba and Smith 2000; Thistlethwaite and Macgarthy 2010; Skiba and Jones et al. 2012). N2O emissions are of global concern, primarily due to its radiative forcing capacity, role in the depletion of the stratospheric ozone and its characteristic of having an almost 300 fold greater potential for global warming than carbon di oxide (Ravishankara and Daniel et al. 2009; 1PCC 2007).

Soil N2O production is primarily brought about by microbial processes (Zumft, 1997) such as the reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to di nitrogen gas (N_2), dissimilatory reduction of $NO3^-$ to ammonia (NH_4^+), and the biological oxidation of ammonia (NH_4^+ or NH_3) to NO_2^- (Baggs, 2008). Other N_2O emitting soil microbial processes would include nitrifier denitrification, and coupled denitrification processes. (Wrage and Velthof et al. 2001).

Regarded as the most significant N_2O emitting soil process (Baggs, 2008), the denitrification process involves the stepwise reduction of NO_3^- or NO_2^- to NO, N_2O or N_2, by facultative anaerobes, with N2O developed as an intermediate (Henault and Grossel et al. 2012; Thomson and Giannopoulos et al. 2012) .Denitrifiers, which are predominantly bacteria, are capable of utilizing available NO_3^- for respiration in oxygen limiting conditions. The different reactions in the denitrification process as well as the specific reduction enzymes and genes involved, are shown in **Figure 1**.

Owing to its dependence on soil abiotic factors, amounts of N_2O produced by denitrification, vary within soils of different types, pH, as well as concentrations of carbon and copper (Morley and Baggs 2010; Felgate and Giannopoulos et al. 2012; Herold and Baggs et al. 2012). Soil pH for instance is capable of increasing N_2O: N_2 molar ratios (Bakken and Bergaust et al. 2012; Herold and Baggs et al. 2012), as a result of the inhibition of the nitrous oxide reductase at low pH levels (Knowles 1982). Also, under aerobic conditions, the nitrous oxide reductase deactivates due to oxygen intolerance, thus significantly increasing N_2O production (Knowles 1982; Graham and Van Es et al. 2013).

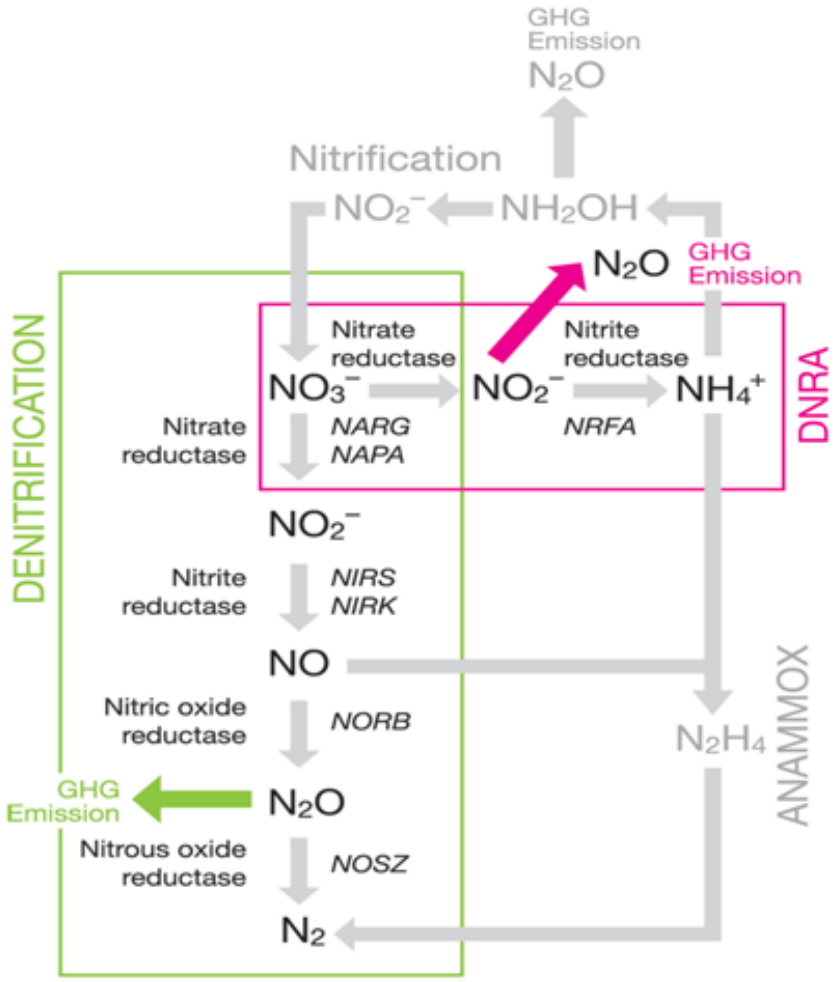

Figure 1: Soil N_2O emitting microbial processes with emphasis on denitrification, reduction enzymes involved, and marker genes. (From Giles and Morley et al. 2012).

Increase in bacterial activity has been associated with NO_3^- abundant conditions, which havebeen recorded to inhibit N_2O reduction, thus leading to increased N_2O production (Blackmer and Bremner 1978; Weier and Doran et al. 1993; Davidson 2009). Denitrifying bacteria have also been seen to prefer NO_3^- to N_2O as an electron acceptor (Schlegel, 1992 as in Wrage and Velthof et al. 2001). Carbon, in different forms and quantities significantly affects N_2O: N_2 molar ratio by improving the denitrifying ability of soils (Dodla and Wang et al. 2013). Carbon sources also account for differences in denitrification rates (Shi and Richardson et al. 2011) and N_2O production; for example, butyrate and glutamic acid addition as energy sources to agricultural soils, produced more N_2O in comparison to the addition of carbohydrates (Morley and Baggs 2010). Optimum denitrification conditions (anaerobic conditions) have further been seen to be created by the amendment of carbon to soil, resulting in contrasting N_2O: N_2 ratios (Morley and Baggs 2010).

Reduction of N_2O to N_2 is catalysed by the copper dependent nitrous oxide reductase; the major biological pathway to N_2 production from N_2O (Pomowski and Zumft et al. 2011; Pauleta and Dell'Acqua et al. 2013). Copper amendment for nitrogen reduction has been employed in various studies (Magalhaes and Machado et al. 2011; Zhu and Chen et al. 2013) with the influence of copper limitation on NO_3^- reduction to N_2O and N_2 gas in NO_3^- rich and depleted conditions, determined on denitrification phenotypes of bacterial denitrifiers in culture, producing varying molar ratios (Felgate and Giannopoulos et al. 2012).

The reduction of N_2O to N_2 during soil denitrification has been identified as a strategy to reduce N_2O emissions (Thomson and Giannopoulos et al. 2012) by enhancing the efficiency of the final step in denitrification. In this current study, the effect of carbon and denitrification dependent elemental copper, on N_2O production and reduction in non-toxic agricultural soils is reported. The presence of copper in soil, is expected to favour the nitrous oxide reductase and thus hypothesized to reflect in a final reduction of N_2O:N_2 molar ratio of carbon amended soils in favour of N_2.

II. Materials and Methods

2.1 Soil

Soils were sampled from Insch, Aberdeenshire (57°20’28’’N;2°36’47’W/57.34102°N; 2.61302°W/ 57.34102; -2.61302) andBrechin, Angus (56° 43’48’’N 2°39’19’’W / 56.72994°N 2.65533°W / 56.72994; -2.65533), both in North East, Scotland. Description of the study areas are shown in **Figure(s) 2and 3**below. Analysed soils were agricultural soils, with varying soil characteristics (**Table 1**). Estimated organic carbon [%] was derived as described by (Ball, 1964) and extractable copper concentration, comparable to Edwards et al. 2012. Soils were air dried to determine gravimetric moisture content of 8 and 5% respectively and were both sandy loam with pH 6.07 (H_2O), 5.18 ($CaCl_2$); pH 5.90 (H_2O), ($CaCl_2$) for Insch and Brechin respectively.

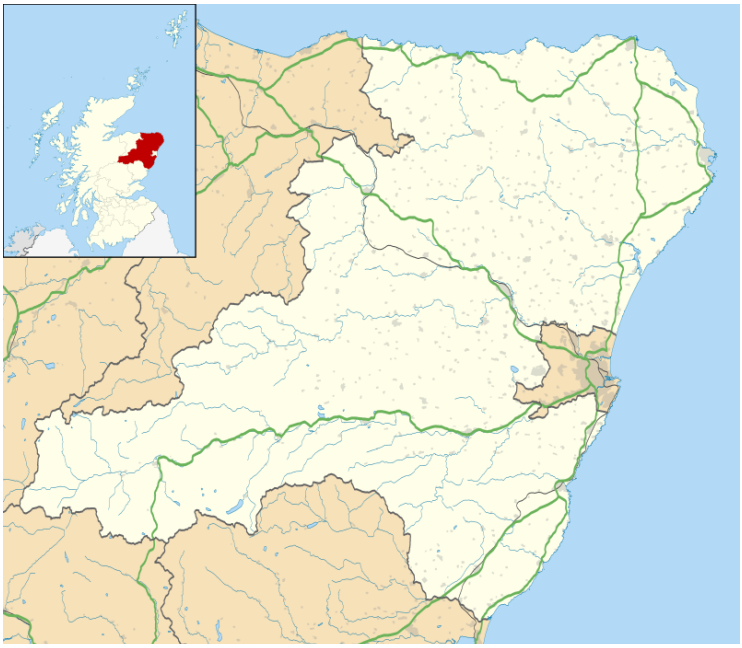

Figure 2: Map of Scotland showing the study area (Insch). (https://en.m.wikipedia.org18/10/2016, 13:47).

Figure 3: Map of Scotland showing the study area (Brechin). (https://en.m.wikipedia.org, 18/10/2016, 12:22).

Table 1: Soil texture properties and gravimetric analysis of soil characteristics

	Clay	**Sand**	**Silt**	**Extractable Cu**	**Moisture**	**Organic Carbon**	
	[%]	[%]	[%]	[Mg/kg]	**Content** %	[%]	
Insch	11.5	57.7	30.8	0.82 (± 0.08)	24.3	12.8	
Brechin	15	70.2	14.8	1.83 (± 0.08)	21.8	10.4	

2.2 Experimental design

Two soils (Insch and Brechin) were amended with 40 kg/H NO_3^- -N as ^{15}N labelled KNO_3^- (30 atom % ^{15}N) in solution, to four replicates each, for four treatments (carbon (ca), copper (cu), carbon plus copper (c/c) and control (co)). 50µg/g glucose ($C_6H_{12}O_6$) in solution was amended to all replicates from the carbon amended treatments, and copper, as 40mg/kg Cu SO_4 added to the copper amended treatments. Sampling was carried out on day (s) 1, 3 and 7 from soil amendments, with the exception of N_2O measurements, which were sampled daily throughout the experiment. Soil pH was maintained by the addition of a KH_2 PO_4 buffer, adjusted with 2M Na OH, to the natural pH of the soil and measurements taken in both H_2O and $CaCl_2$ solutions on each sampling day, with a calibrated pH electrode (Orion Star A211 pH meter).

Sampling was carried out in sets, owing to the destructive nature of some extraction methods.Samples from pH (H_2O) analysis were further used indestructibly, for the dissolved organic carbon analysis, and pH ($CaCl_2$) samples, for the copper analysis, while samples from the gas sampling were used for the destructive extraction of inorganic nitrogen.

2.3.1 Exchangeable Cu determination

Exchangeable copper concentrations present in soil samples, were analysed from 10ml of soil water extracted with rhizons from 40g soil in 1 M $CaCl_2$ solution and collected into evacuated exertainer tubes. Samples were stored between 3 and 5^oC prior to analysis and analysed using the flame atomic absorption spectroscopy (FAAS) at a wavelength of 324.8nm.

2.3.2 Dissolved Organic Carbon analysis

Dissolved organic carbon in soil samples, was obtained by filtering an aqueous solution of 40g soil in 100ml water, through Whattmans glass microfiber 150mm diameter (GF/A), and extract filtrate, further filtered into 15ml plastic tubes using the Pall Acrodisc 32mm syringe filter with 0.45µm supor membrane. Samples were kept cool at temperatures between 3 and 5^oC prior to analysis on a LabTOC aqueous carbon analyser (Pollution and Process Monitoring, Kent, UK).

2.3.3 Extraction and analysis of Mineral N (NO_3^-, NO_2^-)

Inorganic nitrogen was extracted from a solution of 40g of soil in 1M Potassium chloride (KCl), and was shaken on a rotary shaker for an hour before filtering. Samples were filtered through Whattmans glass microfiber 150 mm diameter (GF/A) filters, which were pre-soaked in 50 ml of 1M KCl solution and the filtrate stored in plastic tubes and kept frozen at ˜ -18^oC until analysis. Nitrate and nitrite concentrations in samples were determined using the Fiastar 500 flow injection analyser. Concentrations were determined from an absorbance curve derived from standards of known concentrations.

2.3.4 Gas sampling

2.3.4.1 NO analysis

To analyse the amount of NO produced from all treatments, air samples from soils in air tight Kilner jars were collected in triplicates at 1 minute interval between samples, and measured at 16, 32 and 48 minutes. Samples were analysed using a Sievers Nitric Oxide Analyser (NOA 280i) with the bag program, recording peak NO concentrations.

2.3.4.2 Total N_2O and CO_2 determination

Samples for both analyses were measured by gas chromatography (GC) of headspace samples. Analysed samples were collected from air tight Kilner jars containing soil samples from the different treatments as well as air samples to determine background N_2O and CO_2 levels on each sampling day. Soil headspace gas samples (5ml) were injected into 3ml evacuated exertainer vials, and analysed using an Agilent 6890 series GC system, measuring for both nitrous oxide and carbon dioxide with electron capture detector (ECD) and flame ionization detector (FID) respectively. N_2O and CO_2 concentrations (ppm) were derived from peak areas and a calibration curve of standard gas of known concentrations.

2.3.4.3 ^{15}N-N_2O and ^{15}N-N_2 production

Final denitrification end product, N2 was determined in the laboratory using a trace gas preparation unit (ANCA-TGII, SerCon, UK) coupled to an isotope ratio mass spectrometer (20- 20, SerCon, UK), measuring also, N_2O (denitrification N_2O). Results were calculated using the instrument's signal ratios of different mass to charge ratios of N_2 ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$), with air used as the known standard. Sampling was carried out by extracting air samples from soil samples in air tight Kilner jars, injected into 12ml exertainer bottles (Labco). Exertainer bottles were evacuated and flushed with helium three times before sampling.

2.3.5 Data analysis

Minitab version 16 statistical software was used to determine statistical differences between treatments. To determine significant differences between amended treatments, the general linear model statistical test was carried out for days(s) 1, 4 and 7. A one way analysis of variance was performed to determine significant differences in N_2O:N_2 molar ratio of treatments, and pairwise comparisons were performed by the Tukey method.

III. Results

3.1 Total and denitrification N_2O production

N_2O headspace concentrations from all analysed treatments, varied throughout the experiment. Production was generally observed to be higher in the Brechin soil, with the carbon amended treatments, recording highest production on day 4 (Insch) and day 5 (Brechin) (Fig.2, 3). A general linear model statistical test, performed to determine significant difference in N_2O production between different treatments, showed a significant difference ($p< 0.005$) between the control and copper amended treatments as well as between the carbon and copper plus carbon treatments on day 4 in the Insch soil. Furthermore, a significant difference ($p< 0.005$) was also seen between the control and copper amended treatments on day(s) 1 and 3 of the Brechin soil.

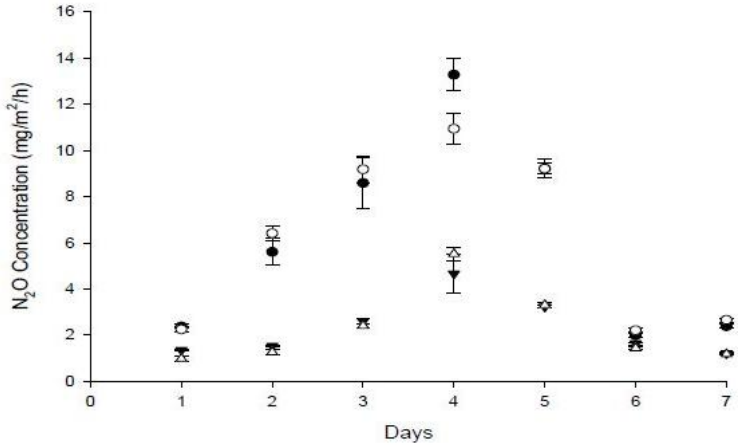

Fig. 2. Total N_2O production Insch (mg/m2/h) over time (days 1-7) following addition of copper and glucoseamendments: (●) carbon, (○) copper plus carbon, (▼) control, (Δ) copper. Values are means (n=4).

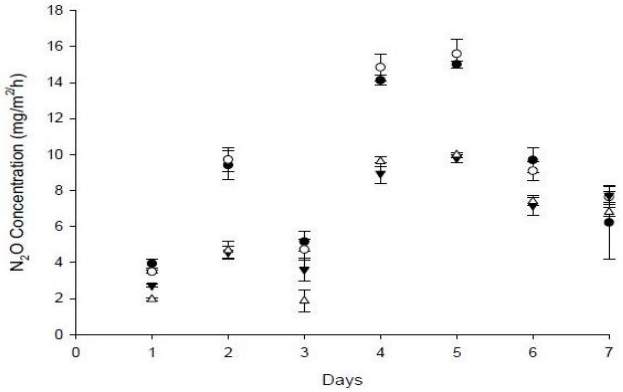

Fig. 3. Total N2O production Brechin (mg/m2/h) over time (days 1-7) following addition of copper and glucoseamendments: (●) carbon, (○) copper plus carbon, (▼) control, (Δ) copper. Values are means (n=4).

3.5.2 Nitric oxide (NO) production

Patterns in NO production (Fig 4 a, b) were similar in both analysed soils, with no significant difference observed between the control and copper amended treatments. However higher NO production was recorded from the carbon plus copper treatments on (day (s) 3 and 1 in Insch and Brechin respectively) in comparison to the carbon amended treatment. A correlation was however observed on day 1 (Insch) between NO production, and dissolved organic carbon concentration (P=0.001, r = 0.805). NO production was generally higher in the Insch soil on days (s) 1, 3; however, there was no significant difference in production from both soils on day 7.

3.5.2.1 Soil treatment N_2O:N_2 molar ratios

Final N_2O:N_2 reduction ratios of the various soil amendments, showed the carbon treatment to have a significantly higher molar ratio when compared to the carbon plus copper treatment in both soils on day 1, (Fig(s) 5, 6). In comparison to the copper amended treatment in Insch on day 1 (Fig (5), the lower molar ratios observed in the control treatment was seen to be at variance with the higher control ratios recorded in the Brechin soil (Fig 6).

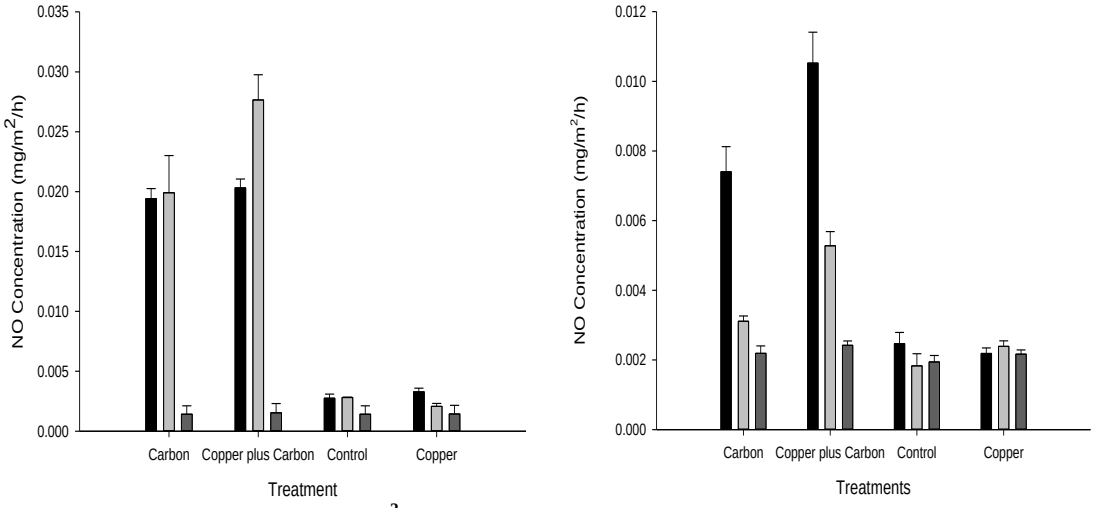

Fig 4. **Nitric oxide production (mg/m^2/h) from soil amended treatments (a) Insch (b) Brechin**: (black) day 1, (light grey) day 3, (dark grey) day 7. Values are means ± 0.003(a), 0.005(b) sem. (n=4).

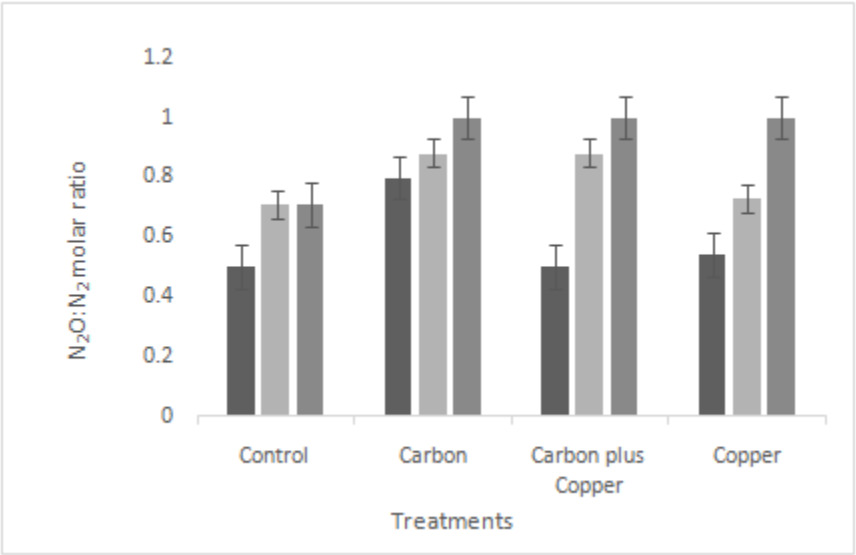

Fig. 5.N_2O:N_2 molar ratio over time (days 1-7) from different soil treatments (Insch): (black) day 1, (light grey) day 3, (dark grey) day 7. Values are means (n=4).

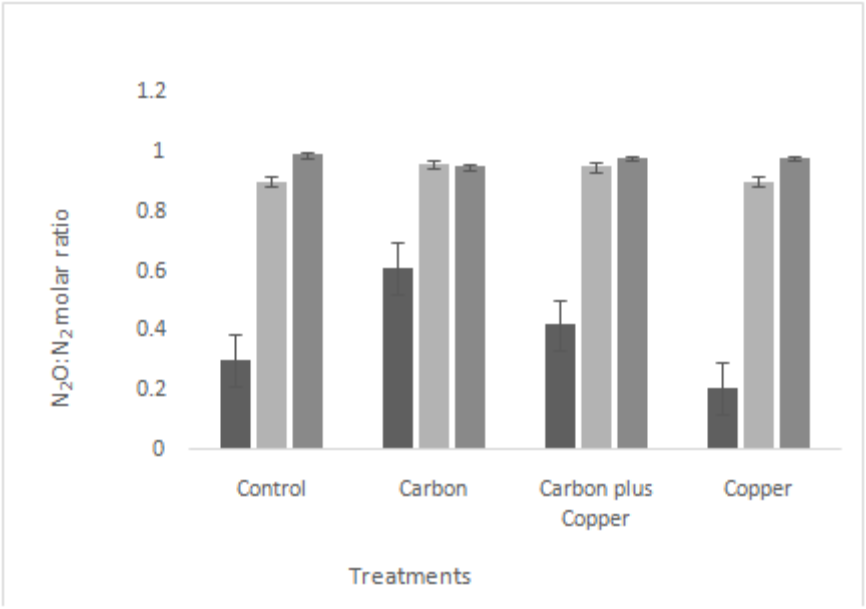

Fig. 6.N_2O:N_2 molar ratio over time (days 1-7) from different soil treatments (Brechin): (black) day 1, (light grey) day 3, (dark grey) day 7. Values are means (n=4).

IV. Discussion

4.1 Copper effects on denitrification gas production

Being copper dependent, it was hypothesized that microbial activity in the final stage of the denitrification process would enhance N_2O reduction as a result of copper availability. In line with our hypothesis, the copper amended treatments, when compared with the control and carbon amended soils, recorded significantly reduced N_2O emissions. Also, final N_2O:N_2 molar ratios were seen to be significantly positively affected by the addition of copper to the carbon amended treatment, as it had in both analysed soils, a significant lower ratio in comparison to the carbon amended treatment.

Although both trends were not observed across all sampling days, this can be attributed to certain factors such as copper concentration (Berti and Jacobs (1996). Heavy metals, though required, can also be detrimental to organisms when in excess and in turn affect denitrifying populations; According to (Magalhaes and Machado et al. 2011), copper amendments up to 60 µg Cu was recorded to decrease nitrous oxide reductase abundance; hence inhibiting the denitrification process. In determining copper toxicity using the denitrification process as a biological indicator, Probanza and Gutiérrez Manero et al. (1996), recorded a significantly reduced N_2O:N_2 molar ratio in soils treated with 100µg/ml Cu solution. Devaney and Hodson et al. (2008) in their study, related large N_2O production to low copper containing soils (46.3mg cu/kg); hence a likely correlation between copper levels and enzyme activity, as large N_2O production recorded, can be attributed to N_2O to N_2 conversion, being catalysed by a copper dependant enzyme . The concentration amended to soil, (40mg/kg Cu SO_4) in this current study, in comparison to a 50 mg/kg^{-1} amendment by (Maderova and Watson et al. (2011), in an earlier study on soil copper toxicity and bioavailability, indicates the non-toxicity status of soil amendment, with concentrations comparable to (Edwards et al. (2012), which according to their method of classification, suggest that copper concentration in both soils were low (Insch) and moderate (Brechin). N_2O reduction to N_2 gas, is not the only copper dependent denitrification reduction process, NO_2^- reduction to NO could either be copper or iron dependent (Felgate and Giannopoulos et al. 2012). Observed increase in NO production in the carbon plus copper treatment in both soils may be attributed to the possible dependence on copper in the earlier reduction process (NO_2^- reduction to NO) and thus probably also implicated in the soil's inability to effectively reduce final N_2O concentrations in the last phase of the denitrification process (N_2O reduction to N_2); as a result of utilization and depletion of the heavy metal (copper) in the earlier reduction process.

In addition to copper concentration, another possible explanation for the inability of a copper amended soil to reduce final denitrification molar ratios can be the absence of specialized genes in microbial community. Some denitrifying bacteria, as described by (Bergaust and Shapleigh et al. (2008) do not possess the genetic information to make the copper dependent nitrous oxide reductase, required to complete the denitrification process, and as such terminate the process in N_2O production. The reduced N_2O:N_2 molar ratio, observed on day 1 in the carbon plus copper treatment, might be indicative of the presence of nitrous oxide reductase in the denitrifying community at the beginning of the experiment. However, according to (Giles and Morley et al. (2011), microbial community present in a particular soil, affect not just the rate of production, but gaseous

products yielded as well, hence might be a reason for the desired reduction not observed on all other sampling days. It is therefore important to determine microbial community in denitrification studies.

4.2 Glucose effects on gas emissions

The effects of carbon as an energy source in denitrification, has been studied extensively with Varying results. According to (Weier and Doran et al. 1993), in their study to determine the effects of abiotic factors such as carbon availability on the denitrification process, they found largest N_2O: N_2 ratios to occur at highest carbon (glucose) amendment (360 kg ha -1). Other studies by (Azam and Muller et al. 2002) have also recorded similar findings of carbon sources added to improve soils denitrifying ability. On the contrary, being dependent on a combination of soil abiotic factors, the denitrification process is largely context specific, as findings from studies for example relating NosZ gene abundance to glucose addition, result in a range of findings, from a positive increase to no effects (Henderson and Dandie et al. 2010; Miller and Zebarth et al. 2008). Also, in their study on interrelationship between denitrification factors, (Morley and Baggs (2010), report on differences in amounts of N_2O and N_2production as a result of different carbon source amendments. Observed effects of glucose amendment to soil in this current study, reveal higher NO production in the carbon plus copper treatment in comparison to the carbon treatment, also reduced N_2O production as well as N_2O:N_2 molar ratio were equally attributed to the carbon plus copper treatment. Lowest recorded NO concentrations were observed on day 7 in both soils and this trend can be attributed to the declining stimulation of denitrifying communities by the carbon source, as a result biological effects.

V. Conclusion

Although not recorded as a trend across all sampling days, our results on N_2O production and N_2O:N_2 molar ratios, in line with our hypothesis indicate that the amendment of carbon and copper to agricultural soils might have a potential reduction effect on denitrification gas production and reduction. It is however important to note that due to varying environmental and soil conditions, effects of amendments such as carbon and copper on the denitrification process cannot be determined based on a few studies; hence the observed potential effect of soil amendments in this current study, is advised to be fully explored in further research such as relating to different carbon sources and concentrations, as well as varying duration of experiment .In addition, total microbial population as well as their denitrification phenotypes is however an important aspect in determining N_2O production from soils.

Acknowledgement

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