N_2O production by fungal denitrification in a semiarid soil

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1 Introduction

The increasing nitrous oxide (N_2O) concentration in the atmosphere causes concern due to its contribution to global warming.[1] Soils, especially agricultural soils, contribute approximately half of the world's anthropogenic N_2O emissions and currently this source of N_2O represents 2.4% of the European release of anthropogenic derived greenhouse gasses (GHG).[2,3] Furthermore, N_2O is also directly or indirectly involved in destruction of stratospheric ozone. [4] Modern agriculture must strive to mitigate N_2O emissions by cultivated soils.

The researches have shown that N_2O is released during the microbial nitrification and denitrification process, in principle.[5] Soil heterogeneity permits the coexistence of aerobic and anaerobic zones which allow organisms in the same soil aggregate to function simultaneously, and nitrification and denitrification can take place at the same time.[6,7]

Bacteria and fungi are the two most important microbes for emitting N_2O from agrarian soil. And both of them have the genetic potential to use organic and inorganic sources of N.[8,9] Although the basic mechanism of N_2O formation in soils is well known, there is not a better knowledge of the contribution of these microbes above.

Bacteria has been regarded as the unique microbe to denitrification for a long time, [10,11] but fungi were found to exhibit denitrifying activities in recently.[12-14] Actually, bacteria denitrify rapidly and completely, however it need a strict condition of dissolved oxygen (DO). In contrast, fungi can simultaneously perform denitrification under microanaerobic or aerobic conditions while bacteria.

In this laboratory study, we want to know the influence of different microbes to produce N_2O. Meanwhile we also identify the N_2O production pathways by use of cycloheximide and streptomycin, inhibitors of fungal and bacterial.

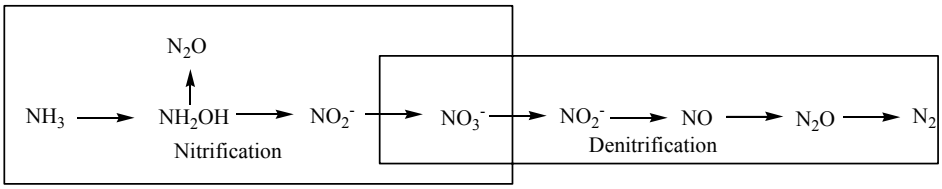

Fig. 1 The mechanism of denitrification and nitrification

2 Materials and methods

2.1 Soil and compost

Soil: surface (0-20 cm) clay loam soil was collected, arable but without plants, in April 2007 from "El Encín" field station, near Alcalá de Henares (Madrid, Spain) (latitude 40º 32'N, longitude 3º 17'W), in the middle of the Henares river basin. The soil was gently broken down by hand after transport to the laboratory, meanwhile it air-dried at room temperature during three days. Then it was sifted through a 2.5 mm sieve, and stored in plastic bag until use. Compost: Urban waste (Madrid, Spain). Inhibitor: streptomycin (Sigma-Aldrich, purity: 95%); cycloheximide (Sigma-Aldrich, purity: 94%).

2.2 Soil sampling and analysis

Soil NO_3^- and NH_4^+ content from each column (at the end of the experiment) and from the additional container (the day following to start the experiment) were determined by extracting 15 g of fresh soil with 50 ml 1M KCl solution; NO_3^- and NH_4^+ were analyzed by colorimetric methods.[15] Soluble organic C was also extracted and analyzed in samples obtained at the end of the experiment as described by Mulvaney et al.[16]

Gravimetric moisture contents for the columns were derived from the relationship between wet weight of the soil column and the dry weight of the soil column. Water filled pore space (WFPS) was calculated by dividing the volumetric water content by total soil porosity. Total soil porosity was calculated according to the relationship: soil porosity = (1- soil bulk density/2.65), assuming a particle density of 2.65 g cm^{-3}. Bulk densities were calculated from the volume of soil in the cores.

3 Laboratory experiments

3.1 Experiment 1

Weighed 2g of soil in vial (20ml). Prepared stock solution: the concentration of streptomycin was 3 mg/ml, solubility of cycloheximide was 1.5 mg/ml so the solution prepared should have dissolved it. Application of the different treatments will be carried out within the vial and these ones will be capped and left for few hours. The headspace will be sample by means of a syringe and this sample transferred to a smaller vial before being analyzed by gas chromatography.

Note: the tables reflect the order of addition of reagents and time of contact, for example: in table 3 soil was weighted and the biocides were added. These were left in contact for 1 hour and then the N solution was added. The headspace was flushed with He (after capping the vial) and the whole system was left in contact overnight. (Each sample repeated 3 times).

The protocol established for the following experiments is described. This was based on the results obtained from the preliminary experiments. Deal with the effect of the application of biocides to soil (bactericide/fungicide) on N_2O production.

3.1.1 The effect of the application of biocides to soil

Table 1. The application of biocides to soil

Sample	Soil (g)	Biocide	Atmosphere	Acetylene
1	2/20ml vial	6.7 ml water	He	No
2	2/20ml vial	C/2ml+4.7ml water	He	No
3	2/20ml vial	S/2ml+4.7ml water	He	No
4	2/20ml vial	C/2ml+S/2ml+2.7ml water	He	No
5	2/20ml vial	6.7ml water	He	1.8ml
6	2/20 ml vial	C/2ml+4.7ml water	He	1.8ml
7	2/20 ml vial	S/2ml+4.7ml water	He	1.8ml
8	2/20 ml vial	C/2ml+S/2ml+2.7ml water	He	1.8ml

C: Cycloheximide, S: streptomycin

Table 2. The resulting concentrations of sample 1-8

Sample	Area (N_2O)	ppm (N_2O)
1	8850.33	2.21
2	11695.33	3.34
3	17602.00	5.71
4	27525.67	9.68
5	103871.33	40.21
6	211922.00	83.43
7	169614.00	66.51

8	258109.00	101.91

The figure 2 shows the effect of both biocides on the fluxes, with and without acetylene. Because of little N and C in the soil and in both cases it is not clear there is inhibition when adding the biocides as the fluxes increased as compared with the blank (no added biocide). The addition of acetylene produced an increase on the fluxes in all the treatments. This could be evidence of the production of N_2 as acetylene blocks the last of denitrification. This could suggest that some of the biocides were being used by the microorganisms as a source of carbon and nitrogen or that materials leaking from the dead cells were used by the microorganisms.[17] The results must be looked at carefully as there is evidence of acetylene being used as a carbon source.[18]

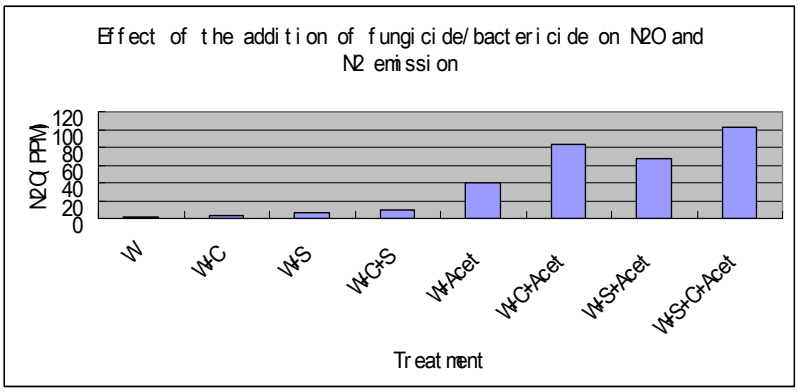

Fig. 2 The effect of biocides on the N_2O emission

3.1.2 The effect of the application of biocides to soil with glucose

Table 3. The application of biocides to soil with glucose

Sample	Soil (g)	Biocide	Atmosphere	Acetylene

9	2/20 ml vial	6ml water+10mg G	He	No
10	2/20 ml vial	C/2ml+4ml water+10mg G	He	No
11	2/20 ml vial	S/2ml+4ml water+10mg G	He	No
12	2/20 ml vial	C/2ml+S/2ml+2ml water+10mg G	He	No
13	2/20 ml vial	3ml water+10mg G	He	1.8ml
14	2/20 ml vial	C/1ml +2ml water+10mg G	He	1.8ml
15	2/20 ml vial	S/1ml +2ml water+10mg G	He	1.8ml
16	2/20 ml vial	C/1ml+S/1ml+1ml water+10mg G	He	1.8ml

G: Glucose

Table 4. The resulting concentrations of sample 9-16

Sample	Area (N_2O)	ppm (N_2O)
9	9794	4.45
10	5857.333	2.09
11	23462	12.65
12	7215.333	2.90
13	62591.33	36.13
14	76447.33	44.44
15	104198	80.54
16	105328	81.56

Figure 3 shows inhibition of the fluxes when using cycloheximide in the glucose treatment. The bottle with streptomycin produced an increase in the fluxes whereas the mixture of both biocides did not produce a significant effect. It showed that the streptomycin didn't work in this condition and it was used as nitrogen source by some fungal.

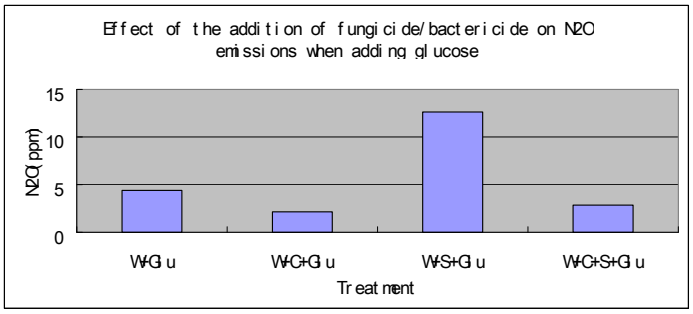

Fig. 3 The effect of biocides on the N_2O emission with glucose

Figure 4 shows mush larger fluxes when adding acetylene compared to no acetylene addition,

and an increase in the fluxes was observed with the addition of streptomycin. Because streptomycin have abundant nitrogen and it can be easily translated to N_2O by microorganisms. And this result also confirms that fungal and bacteria out-of-run mixing with acetylene and He.

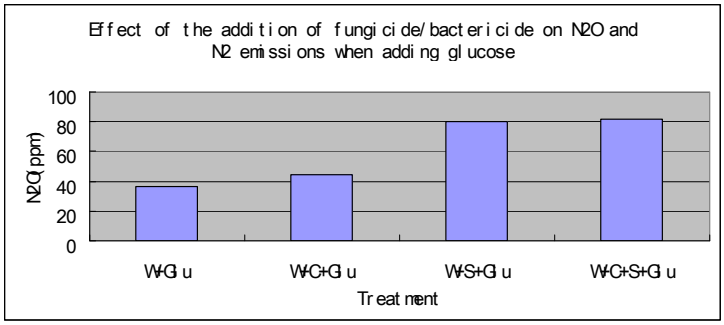

Fig. 4 The effect of biocides on the N_2O emission with acetylene and glucose

3.2 Experiment 2

Each experiment was conducted using 12 vitric jars (6.3 cm height, 5.4 cm diameter) with gas-tight lids fitted with a gas sampling port. And all of the sample bottles must be exhausted air by pump before collected gas simples.

70 g dry soil was weighed into each jar and additional distilled water with $(NH_4)_2SO_4$ 0.43g (200 kg N /ha) to achieve WFPS 60%. The concentrations of streptomycin (3.0mg/g soil) or cycloheximide (1.5mg/g soil) were tested in both soils to determine their optimal concentrations for inhibition. And all of bottles were divide into four groups:

(1) 210 mg (3.0mg/g soil) streptomycin perflask

(2) 105 mg (1.5mg/g soil) cycloheximide perflask

(3) 210mg streptomycin (3.0mg/g soil) and 105 mg streptomycin (1.5 mg/g soil) cycloheximide perflask

(4) N: Control

(Each treatment was replicated 3 times for gas analyses)

All treatments were incubated in the air uncovered for several hours (3h) after solution application, After that they were tightly sealed at room temperature. Gas samples were removed from the flasks on 0 min and 60 min. And soil WFPS has been maintained on a weight basis for 4 days.

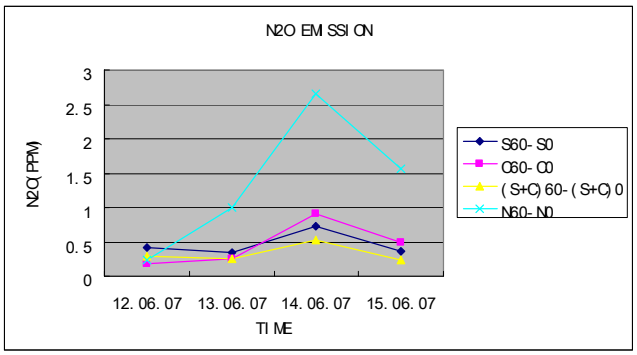

Fig. 5 The effect of time to N_2O emission

Figure 5 indicates that both of bacteria and fungal are work in air atmosphere, furthermore the max emission of N_2O in the 3rd day.

4 Conclusion

Results of this study suggest that the occurrence of fungal denitrification is of ecological significance as N_2O is the dominant gaseous product in this semiarid soil. As fungi have the ability to perform denitrification and O_2 respiration simultaneously in a range of O_2-stress conditions, the potential exists for fungi to produce N_2O in a wider range of soil aeration conditions than bacteria. Fungi are widely distributed in soils and water, hence the potential exists for fungi to make a significant contribution to the global N_2O budget.

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