Role of Microorganisms in Emission of Nitrous Oxide and Methane in Pulse Cultivated Soil Under Laboratory Incubation Condition

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Abstract Soil from a pulse cultivated farmers land of Odisha, India, have been subjected to incubation studies for 40 consecutive days, to establish the impact of various nitrogenous fertilizers and water filled pore space (WFPS) on green house gas emission (N_2O & CH_4). C_2H_2 inhibition technique was followed to have a comprehensive understanding about the individual contribution of nitrifiers and denitrifiers towards the emission of N_2O . Nevertheless, low concentration of C_2H_2 (5 ml: flow rate 0.1 kg/cm^2) is hypothesized to partially impede the metabolic pathways of denitrifying bacterial population, thus reducing the overall N_2O emission rate. Different soil parameters of the experimental soil such as moisture, total organic carbon, ammonium content and nitrate–nitrogen contents were measured at regular intervals. Application of external N-sources under different WFPS conditions revealed the diverse role played by the indigenous soil microorganism towards green house gas emission. Isolation of heterotrophic microorganisms (*Pseudomonas*) from the soil samples, further supported the fact that denitrification might be prevailing during specific conditions thus contributing to N_2O emission. Statistical analysis showed that WFPS was the most influential parameter affecting N_2O formation in soil in absence of an inhibitor like C_2H_2 .

Keywords Incubation · Nitrification · Denitrification · N_2O · CH_4

Introduction

Green house gases like CO_2 , CH_4 , and N_2O , etc. trap the outgoing solar radiation from earth surface and play a vital role in the warming up process of the atmosphere [1–3]. Accumulation of these gases over time due to lack of sinks is one of the present global concerns [4]. Amongst various sources agricultural activities like raising crops and animal husbandry are important sinks as well as potential source for certain green house gases like CH_4 and N_2O [5, 6]. However, vast uncertainties exist in the estimated values of individual sources and sinks [7, 8] thus signifying the prerequisite of such studies not only on global levels but also at regional basis. Globally agriculture contributes a significant lot of the total green house gas, consisting of CH_4 (52 %) and N_2O (84 %) as the major trace constituents [9]. The global emission of CH_4 and N_2O from Indian agricultural activities is 0.1 and 0.23 % respectively [10, 11]. Some major processes responsible for such emissions can be regarded as a series of successive oxidation and reduction reaction, mediated by various soil microorganisms [11, 12].

Annual emission of N_2O is increasing at a rate of 50 ppb and nearly 70 % of this emission, both direct and indirect, is believed to be from the agricultural sector [13–17]. Though N_2O contributes to 9 % of total green house gas emission, its global warming potential is 300 times higher than CO_2 . N_2O is produced naturally from soil through major microbial metabolic pathways such as nitrification and denitrification [16]. Nitrification occurs whenever soil $\text{NH}_4\text{-N}$ is available and environmental conditions (such as temperature and moisture) are favorable for the nitrifier population. These situations are normally prevalent in many agronomic zones [17]. During oxygen deficient conditions, microorganisms use nitrate as the electron acceptor for the anaerobic oxidation process known as

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denitrification. The later generally occurs in wet soils where diffusion of oxygen is inhibited by saturated condition inside the soil aggregate (compaction) [18].

Study of the overall nitrification and denitrification in the soil, in presence of external nitrogen source coupled with various edaphic parameters, have revealed substantial information regarding the rapid utilization as well as loss of nitrogen in the fertile lands. This has lead to an assessment of the emission rate of green house gases like N_2O and CH_4 , specifically from the agricultural sector [17].

With the availability of a plethora of information regarding the soil microbial association with the green house gas emission from agricultural fields, researchers are able to predict the intricate soil-microbe-gas emission interaction processes. These processes are extensively influenced by soil, atmospheric and other edaphic conditions [19].

Pulse cultivation in India has been an ancient practice and pulses, like horse gram, green gram and black gram are being cultivated in almost all parts of the country [19]. However there is little information on the emission of CH_4 and N_2O from the source and the specific role played by the soil microbial consortium in the same, especially in the pulse cultivated soil [16]. In the current study, an attempt has been made to determine the emission rate of N_2O and CH_4 under incubated conditions from the soil samples collected from a specific farmer's field growing pulses, from Soroda village ($19^{\circ}50'N$ $84^{\circ}19'N$ 140 m above sea level) located in southern part of Odisha, a coastal state in India. This village is regarded as a tribal belt and the inhabitants follow traditional agricultural practices. They use bullock for tilling instead of mechanical tiller. Chemical fertilizer or pesticides are never applied in the pulse cultivated fields instead farm yard manure is used as plant supplement. Both the green house gases have been monitored by varying different soil parameters. Isolation and identification of microbes (nitrifiers and denitrifiers) involved in the emission of the gases, has been aimed towards further clarification of the processes.

Materials and Methods

Soil Sampling

Soil samples were collected from a farmer's field, cultivating black gram (*Vigna mungo*). The field under investigation was not treated with any chemical fertilizer. It is located near Soroda in Ganjam district of Odisha. It is rain fed during monsoons and being upland, has a well drained soil. The area receives 150 mm rain fall annually, mostly during monsoon (July–September). Soil samples were collected from a depth of ~15 cm during fallow period [20].

After collection the samples were immediately transported to laboratory in air-tight containers followed by crushing and sieving through 500 μm sieve. To obtain definite moisture content, the soil samples were sprayed with water and kept at 60 °C for 24 h to stabilize. Soil moisture was measured by subtracting the weight of wet soil (soil with moisture), from the weight of normal moisture free soil, and expressed as the maximum water holding capacity [16]. Finally different water filled pore space (WFPS) conditions were maintained by adding required quantity of water to the sample bottles containing soil samples and were numbered accordingly.

Different Parameters Investigated

Influence of various parameters like nitrogen source (ammonium nitrate, ammonium acetate, and ammonium sulfate), moisture content of the soil (70, 80, 90, and 100 %), and injection of an inhibitor like C_2H_2 were studied. C_2H_2 was added to quantify the denitrification rate in the soil samples [19].

Incubation Experiments

100 g of sieved soil were placed in each serum bottle of 250 ml. 1 ml (1,000 ppm) of NH_4 salt solution (N source) was added to the serum bottles. WFPS was varied from 70 to 100 %. The serum bottles were accordingly leveled and the set of related experiments were carried out in quartet. In the control set only moisture variables were taken. One set of the serum bottles were flushed with 5 ml of C_2H_2 (flow rate 0.1 kg/cm²) for 5 min in order to estimate the amount of denitrification [19]. The serum bottles were sealed with air tight polypropylene rubber stoppers and incubated continuously (Remi incubator) at 37 °C. The gas in the head-space of bottles were drawn (at a regular interval of 24 h) through a syringe and analyzed using two gas chromatographs standardized for the detection of N_2O (Simadzu AA1 equipped with electron capture detector) and CH_4 (Perkin Elmer auto system equipped with flame ionization detector). XL grade© certified nitrogen gas of 99.99 % purity obtained from BOC, India, was used as carrier gas in both the systems.

Measurement of Other Soil Parameters

Different soil parameters like, total organic carbon (TOC), ammonia-N and nitrate-N were estimated from the treated soil on a weekly basis by titrimetric method [21], automated phenate method and cadmium reduction method respectively [22].

Isolation of Autotrophs and Heterotrophs

An attempt was made to isolate the microbes (autotrophs and heterotrophs) associated with the nitrification and denitrification processes taking place in the soil. For the isolation of the microorganisms a specific enrichment media was used with the following composition: $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/l), KH_2PO_4 (0.2 g/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.04 g/l), $\text{Fe}_2(\text{SO}_4)_3$ (0.25 g/l), Phenol red (0.25 g/l). pH of the medium was adjusted between 6.8 and 7.8 by adding sodium carbonate solution (1 M). 1 g of soil (collected from the experimental field in air tight containers) was mixed with sterilized liquid enrichment media. The conical flask containing the media was placed in a shaker incubator (Julabo SW 22) for 48 h at 37 °C followed by centrifugation of the full grown media. The biomass pellet was then inoculated to fresh sterile media and incubated as described above. Similarly, sub-culturing was repeated once more before plating into the agar plates of the enrichment media (2 g of Agar/100 ml of enrichment media) for isolation of pure bacterial colonies. Plating was done by spreading 0.1 ml of the microbe enriched media over the agar plates. The entire process was carried out under sterile conditions. Culture plates were then incubated at 37 °C for 2 days. Pure colonies were isolated from the plates and sent to the Institute of Microbial Technology, Chandigarh, India in sealed vials for identification. The heterotrophic bacteria were isolated in the same way as autotroph but the enrichment media used for heterotrophic bacteria isolation contained an additional source of carbon (sodium acetate). The microbial strains were identified based on biochemical and physiological characteristics of isolates [23].

Results and Discussion

N_2O Emission

Variation of WFPS

WFPS is one of the key factors affecting N_2O formation in agricultural soils [24]. In order to study the effect of different WFPS conditions on the emission of N_2O , two sets of experiments were designed by varying the moisture content at 70, 80, 90 and 100 % in the soil samples without providing external N-source. One of the sets was exposed to C_2H_2 whereas the other set was taken as such.

It can be observed from Fig. 1 that in the absence of external N-source, the indigenous microbial community in the soil was active throughout the 40 days of incubation period with emission peaks obtained over various WFPS conditions. Maximum peaks at 80 and 90 % WFPS conditions may be due

to the metabolic activity of denitrifying population. The soil sample under study was collected from a pulse cultivated land treated with farmyard manure which served as nutrient for the microorganisms even in the absence of external N-source. With gradual increase in soil moisture content, the oxygen diffusion reduces creating favorable conditions for the metabolism of the otherwise dormant denitrifiers. With exposure to C_2H_2 , the initial N_2O emission rate was higher at 70 % WFPS. But gradually there was a sharp decline in the same. This observation supports the adverse effect of C_2H_2 on the aerobic microbial community, i.e., the nitrifiers [25]. Maximum numbers of N_2O peaks were obtained at higher WFPS conditions, i.e., 80, 90 and 100 %. The N_2O emission from the soil is believed to be directly influenced by the denitrifiers under increasing moisture content of the soil due to prevalence of anaerobic conditions, as has been explained earlier [26, 27]. The major speculation is regarding the initial higher emission rate followed by a reduction, even at higher WFPS as seen in Fig. 1. Such observations raise the possibility of C_2H_2 obstructing the metabolic rates of denitrifiers as well.

Variation of Nitrogen Source

The link between N_2O emission and amount of N-fertilizer applied has given rise to the concept of the emission factor (EF), where EF is the amount of N_2O -N emitted expressed as a fraction (or a percentage) of the N applied [28]. Therefore, N_2O and CH_4 emission rates are studied in the incubated soil samples by applying various external N sources.

Ammonium Acetate

Ammonium acetate was added to the soil sample in order to study its effect on N_2O emission. WFPS of the soil samples treated with ammonium acetate were maintained at 70, 80, 90 and 100 %. One set was exposed to C_2H_2 , whereas in the replicate set, C_2H_2 was absent (Fig. 2). In

Fig. 1 N_2O emission at different WFPS conditions, exposed and not exposed to C_2H_2

Fig. 2 N_2O emission in presence of ammonium acetate under different WFPS condition, exposed and not exposed to C_2H_2

presence of low concentration of C_2H_2 , the enzyme ammonium monooxygenase (one of the key enzymes in the nitrification process), is inhibited and thus the growth of the nitrifiers is affected [29, 30]. Based on the availability of a suitable electron acceptor, such circumstances become congenial for denitrifiers to play a major role in the emission of N_2O .

Our study is in accordance with earlier reports, as significant N_2O emission has been observed at 80 % WFPS (Fig. 2) which supports the augmentation of both nitrifier and denitrifier population, in absence of C_2H_2 [31]. Experimental set with C_2H_2 exposure showed a different result. N_2O emission at various WFPS levels did not show much variation (Fig. 2); however, a larger number of peaks were obtained at 100 % WFPS condition. This observation can be considered as an indication that, high WFPS conditions might lead to water logging and oxygen scarcity in the medium, which in turn enhances the metabolic activity of denitrifier population and a subsequent rise in N_2O emission.

However with exposure to low concentrations of C_2H_2 , the nitrification process is affected thus having a negative impact on the denitrification process in general. It has been reported that nitrate formed during nitrification acts an electron acceptor by the denitrifiers [32, 33]. Presence of a low concentration of C_2H_2 , has a direct deleterious impact on the nitrification and an indirect one on denitrification process, with relation to availability of nitrate. But further investigation is necessary to determine the accuracy of low partial pressures of C_2H_2 having inhibitory effects only on nitrification process [31].

Ammonium Sulfate

In presence of ammonium sulfate and absence of C_2H_2 , maximum N_2O peaks were obtained at 70 % WFPS condition, even after 35 days of incubation. This observation

Fig. 3 N_2O emission in presence of ammonium sulfate under different WFPS condition, exposed and not exposed to C_2H_2

supports the vibrant metabolic rate of nitrifiers owing to low moisture conditions in the incubated soil. The overall N_2O emission, as observed in Fig. 3, would have been due to combined metabolic action of both nitrifier and denitrifier population in the soil.

The parallel set of experiments conducted in the presence of C_2H_2 showed maximum N_2O peaks with 90 % WFPS condition maintained in the soil. A consistent fall in the overall N_2O emission rate has been observed (Fig. 2) with subsequent increase in the incubation period. These observations were similar to those obtained in the presence of ammonium acetate. The initial higher N_2O peaks may be due to the simultaneous action of the nitrifiers as well as the denitrifiers, whereas C_2H_2 gradually impedes the nitrifier metabolism rate thus enthralling the denitrifiers towards the emission of N_2O . Such observations have also been reported earlier [18]. On the other hand in presence of C_2H_2 the overall N_2O emission showed a descending trend for all the four WFPS conditions, like the earlier case. Such observation further questions the exact role of C_2H_2 on the growth rate of indigenous microorganisms generating N_2O .

Fig. 4 N_2O emission in presence of ammonium nitrate under different WFPS condition, exposed and not exposed to C_2H_2

Table 1 N₂O emission in presence of ammonium nitrate with C₂H₂ (for 40 consecutive days)

Analysis of variance (ANOVA)					
Source of variation	Degrees of freedom	Sum of squares	Mean squares	<i>F</i> (calculated)	<i>F</i> (tabulated)
Between groups	3	51068778.06	17,022,926		
Within groups (error)	156	517420413.7	3,316,798		2.67 (5 % level of significance)
Total	159	568489191.8		5.13	

Ammonium sulfate as external N-source did not enhance the N₂O emission rate in the incubated soil samples just like ammonium acetate.

Ammonium Nitrate

Ammonium nitrate has been observed to be the paramount nitrogen source for the maximum N₂O emission from the incubated soil. The emission rate of N₂O has been observed (Fig. 4) to be 6 times higher than that emitted in the presence of the other two N-sources. In this case maximum number of N₂O peaks was obtained at 70 % WFPS condition. Figure 4 shows that the emission rate of N₂O, even in presence of C₂H₂ is much higher (around 6 times) in comparison to the other two N-sources. Though a decline in the rate of emission was observed after 25 days of incubation, yet significant numbers of N₂O peaks were obtained in presence of ammonium nitrate.

Use of ammonium nitrate as synthetic fertilizer triggers high N₂O emission [34] from different soil types due to the readily available nitrate. Assimilation of nitrate by the denitrifiers has always resulted in direct emission of N₂O. This is a crucial observation depicting the influence of N source that plays a major role in enhancing the metabolic

rates of the nitrifier and denitrifier community in the soil, thus giving rise to higher N₂O emission.

Supplementation of ammonium nitrate to the soil sample maintained under various WFPS conditions lead to maximum N₂O emission. For further determining the correlation between N₂O emission rate and presence of C₂H₂, statistical interpretation of the overall data has been done by applying *F*-test followed by determination of least significant difference (LSD) values using Microsoft excel program.

It can be observed from Table 1 that the critical value of *F*_{0.05} at (3, 156) d.f. = 2.67. *F*_c value >2.67 indicates that *F* value is significant at 5 % level.

There was no significant difference between the average N₂O emission rate at higher WFPS conditions in presence of C₂H₂ (Table 2). However, when the mean values of the minimum WFPS (70 %) was compared with that obtained at higher WFPS (80, 90, 100 %), a significant difference was observed.

The critical value of *F*_{0.05} at (3, 156) d.f. = 2.67. *F*_c value is >2.67 specifies that *F* value is significant at 5 % level (Table 3). It can be observed from Table 4 that significant difference exists between the means of N₂O emission rate at various WFPS conditions; hence the differences are statistically significant.

Table 2 Least significance difference (for 40 consecutive days)

Different moisture	Difference of mean value	LSD value (5 %)
70:80	1160.15	>924.42
70:90	1360.78	>924.42
70:100	1353.35	>924.42
80:90	200.64	<924.42
80:100	193.21	<924.42
100:90	7.43	<924.42

Table 4 Least significance difference (for 40 consecutive days)

Different moisture	Difference of mean value	LSD value (5 %)
70:80	147.58	>97.86
70:90	703.22	>97.86
70:100	557.60	>97.86
80:90	555.64	>97.86
80:100	410.02	>97.86
100:90	145.62	>97.86

Table 3 N₂O emission in presence of ammonium nitrate without C₂H₂ (for 40 consecutive days)

Analysis of variance (ANOVA)					
Source of variation	Degrees of freedom	Sum of squares	Mean squares	<i>F</i> (calculated)	<i>F</i> (tabulated)
Between groups	3	231,817,858	77,272,619		
Within groups (error)	156	5799216.7	37174.466	13.32	2.67 (5 % level of significance)
Total	159	237,617,075			

Thus it can be concluded that, in presence of C_2H_2 and ammonium nitrate as N-supplement, N_2O emission rate is higher at high WFPS conditions, though there is a gradual decline in the emission rate after a certain period. This observation further supports the fact that the readily available nitrate acts a good source of nutritional supplement for the growth of denitrifying population that ultimately participates in high N_2O emission at higher WFPS conditions.

CH₄ Emission

CH₄ emission was estimated in the soil samples with different moisture contents for which no external N source

Fig. 5 CH₄ emission rate during variation in % WFPS

was provided as explained earlier. Figure 5 shows the highest CH₄ peak when the soil WFPS was 100 % and gradually declined with the decline in the moisture content. Another crucial observation was that CH₄ emission increased significantly after 30 days of incubation. It has been reported [34, 35] that there is a trade-off relationship between the CH₄ and N_2O emissions in the soil. It can be observed from Fig. 1 that, under higher WFPS, the N_2O emission is comparatively lower and there is a gradual decline in the same with time whereas for CH₄ maximum peaks were obtained at higher WFPS and the emission rate increased with time. Thus the factors like WFPS and consequently oxygen availability have a strong impact on formation of N_2O and CH₄ in soil and both are opposite to each other. This observation indicates that at higher WFPS in the soil anaerobic condition is prevailed leading to dominance of methanogenic bacterial activity.

Microbial Isolation

Soil samples were inoculated in liquid selective enrichment media for the isolation of autotrophic and heterotrophic microorganisms. Followed by isolation of the microorganisms in selective enriched solid media, samples were sent to the Institute of Microbial Technology, Chandigarh for further identification. The strains have been identified

Table 5 Analysis of various soil parameters

Week:	Ammonia				TOC				Nitrate			
	1	2	3	4	1	2	3	4	1	2	3	4
Control with different moisture												
70 %	1.16	1.40	0.91	0.13	0.49	1.46	1.14	0.49	1.11	1.02	0.97	0.99
80 %	1.19	1.50	1.46	1.08	0.49	1.95	1.52	0.97	1.25	1.04	1.02	0.01
90 %	1.40	1.72	1.38	0.36	1.46	2.44	1.90	0.97	1.20	0.96	0.25	0.12
100 %	1.45	1.46	1.05	0.79	0.97	1.46	1.14	1.46	1.25	1.15	0.63	0.09
Ammonium acetate with different moisture												
70 %	1.46	1.78	1.31	1.18	1.46	2.92	2.28	2.46	1.02	0.91	0.82	0.14
80 %	1.54	1.82	1.36	1.02	0.97	1.70	1.33	1.97	1.08	1.13	0.95	0.09
90 %	1.65	2.27	1.50	1.09	0.49	1.06	1.14	1.46	1.15	1.24	1.12	0.12
100 %	1.58	1.74	1.64	1.03	0.56	0.97	0.76	1.49	1.14	1.10	0.96	0.44
Ammonium sulfate with different moisture												
70 %	1.25	1.82	1.45	1.08	0.49	1.04	1.14	1.46	1.44	1.01	0.87	0.08
80 %	1.24	2.09	1.47	1.04	0.97	1.06	1.14	1.97	1.35	1.02	0.95	0.09
90 %	1.22	2.05	1.54	1.14	0.49	0.97	1.16	1.46	1.14	1.08	0.85	0.11
100 %	1.47	1.74	1.54	1.11	0.49	0.97	1.14	1.46	1.07	1.03	0.85	0.01
Ammonium nitrate with different moisture												
70 %	4.22	2.10	1.57	1.55	0.95	1.43	1.76	1.95	2.94	1.09	1.08	0.23
80 %	2.13	3.77	1.67	1.33	0.49	0.97	1.52	1.95	2.63	1.16	0.93	0.12
90 %	2.53	3.92	1.74	1.68	0.76	0.97	1.46	1.49	2.73	1.21	0.47	0.24
100 %	2.18	1.40	1.90	1.12	0.83	1.46	1.52	1.62	2.59	1.32	0.78	0.23

as *Pseudomonas aeruginosa* (MTCC No. 10,069, 10,070) and *Pseudomonas cepacia* (MTCC NO. 10,071). Here the MTCC No. indicates the gene-bank accession numbers of the isolated strains. The presence of *Pseudomonas* is concordant with our observations as the heterotrophic microbe is known to play a key role in the soil denitrification process.

Soil Parameters

Soil moisture content remains almost unchanged throughout the experiment. Table 5 shows that the TOC increases gradually over time. This supports the increased rate of CH₄ emission [35]. Ammonia concentration increased initially, followed by a decline after 1 week which may be due to the conversion of ammonia into nitrate. Table 5 also indicates a decreasing value of NO₃[−], which again points out the sequential conversion of nitrate to nitrite and N₂O.

Conclusion

The present study suggests that WFPS had a considerable effect on the emission rate of N₂O and CH₄ in the incubated soil samples. In the presence of various external nitrogen sources (ammonium acetate, ammonium sulfate) as well as different WFPS conditions, the exposure to C₂H₂ showed a gradual decline in the overall N₂O emission. Thus it can be concluded that though the activity of the nitrifiers are vulnerable to the presence of low concentration of C₂H₂, the metabolic pathway of the denitrifiers might also be obstructed. At the same time emission rate of N₂O was maximum at higher percentage of WFPS and ammonium nitrate supplementation. Thus readily available nitrate along with oxygen deficient conditions probably made the environment suitable, for the growth of denitrifiers with simultaneous N₂O emission. The heterotrophic microorganisms isolated from the soil samples were identified as *Pseudomonas aeruginosa* (MTCC No. 10,069, 10,070) and *Pseudomonas cepacia* (MTCC NO. 10,071) at the Institute of Microbial Technology, Chandigarh. Soil analysis showed that there was a gradual increase in the TOC which concur the gradual increase of CH₄ emission. Statistical interpretation of the overall N₂O emission rate in presence of ammonium nitrate as the external nitrogen source depicts significant correlation between the emission rate and prevailing WFPS conditions in the soil.

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