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## Effect of 3,4-dimethylpyrazole Phosphate on Some Microbial Processes in Soil

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**Abstract:** Laboratory incubation experiments were conducted to study the effect of 3,4-dimethylpyrazole phosphate (DMPP) on i) nitrification, ii) N mineralization potential of the soil, iii) immobilization-remineralization of N and iv) respiratory activity of the soil as well as N<sub>2</sub>O emissions. The inhibitor was applied at 0.35 to 17.5 mg kg<sup>-1</sup> soil in different experiments and the incubation carried out at 25°C for variable lengths of time depending upon the nature of experiment. As expected, the process of nitrification slowed down in the presence of DMPP, rate of nitrification being slower at higher levels of the inhibitor. Measurable quantities of NO<sub>2</sub><sup>-</sup> were found only during the first 3 days of incubation with DMPP having a negligible effect. Potentially mineralizable N showed a small but significant increase in the presence of DMPP i.e., an average of 11 and 9 mg kg<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N accumulating in treated and untreated soil samples, respectively, during the first week of incubation. As expected, only 10% of the NO<sub>3</sub><sup>-</sup>-N present initially was recovered after one week and ca 5% at the end of 2nd week of incubation suggesting significant losses, DMPP had a negligible effect on disappearance of NO<sub>3</sub><sup>-</sup>. Immobilization of NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N in glucose amended soil was significantly retarded by DMPP, the effect being more at higher than recommended concentrations. Immobilization of NO<sub>3</sub><sup>-</sup>-N was retarded more than that of NH<sub>4</sub><sup>+</sup>-N suggesting that the immobilization of two forms of N was affected differently by DMPP. Net accumulation of mineral N (remineralization) was slower in DMPP-treated soil. The flux of N<sub>2</sub>O and respiratory activity (loss of CO<sub>2</sub> and consumption of O<sub>2</sub>) decreased in the presence of DMPP.

**Key words:** Denitrification, DMPP, immobilization-remineralization of N, nitrification, potentially mineralizable N, respiratory quotient

### INTRODUCTION

In general practice, NH<sub>4</sub><sup>+</sup> or NH<sub>4</sub><sup>+</sup> forming fertilisers (e.g., urea) are the predominant source of N applied to agroecosystems. According to FAO statistics for the year 2000, urea comprised 52% of the total nitrogenous fertilisers in 125 countries i.e., 41 million tons out of 178 million tons. In soils, NH<sub>4</sub><sup>+</sup> resulting from the applied fertiliser N is rapidly nitrified with a potential of contributing to environmental pollution through N<sub>2</sub>O emissions into the atmosphere and contamination of subsurface waters with nitrate (NO<sub>3</sub>)<sup>[1,2]</sup>. In addition, N use efficiency of crop plants remains low because of the losses that may range from 30 to 70% of the applied N<sup>[3]</sup>. An increase of 1% in use efficiency of fertiliser N is estimated to save more than 234 million US\$ annually on global basis<sup>[4]</sup>. Controlled release of N from fertilisers and a reduction in the rate of nitrification offer possible solutions to problems of low N use efficiency and environmental pollution<sup>[5,6]</sup>. Attempts have been made to

achieve these targets through the use of nitrification inhibitors (NIs) some of which have shown good promise under practical conditions<sup>[6,7]</sup>. A major consideration during the selection of NIs is their minimum side effects and high effectiveness at the lowest possible application rate.

Of the different NIs that have found practical application, DCD (dicyandiamide) and nitrapyrin [2-chloro-6(trichloromethyl)pyridine] have been the most well known. Recently, 3,4-Dimethyl Pyrazole Phosphate (DMPP) has been introduced in a granular formulation (ENTEC) that also contains NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N<sup>[8,9]</sup>. Under field conditions, DMPP has been shown to reduce nitrification as well as N<sub>2</sub>O emissions and improve crop yields<sup>[8,10]</sup>. This is achieved at least partially by an effective concentration of DMPP at the sites of nitrification i.e., where NH<sub>4</sub><sup>+</sup>-N is available<sup>[11]</sup>. The inhibitor is reported to have no ecotoxicological effects and is hence not hazardous<sup>[9]</sup>. While considerable work has been reported on the side effects of some known

inhibitors like DCD and nitrapyrin<sup>[12-17]</sup>, hardly any published information is available for DMPP, especially in terms of its effects on C and N transformation processes. Present objective was to study the effect of DMPP on i) nitrification, ii) N mineralization potential of the soil, iii) immobilization-remineralization of N and iv) respiratory activity of the soil as well as N<sub>2</sub>O emissions.

## MATERIALS AND METHODS

**Soil:** The experiments were conducted in 2000 on a soil obtained from the top 0-15 cm of experimental fields of the Institute of Agronomy, Weilburger-Grenze, Justus-Liebig-University, Giessen, Germany. The air-dried and sieved (< 2 mm) silty clay soil had the following characteristics: organic C, 1.35%; total N, 0.15%; NH<sub>4</sub><sup>+</sup>-N, 4.7 mg kg<sup>-1</sup>; NO<sub>3</sub><sup>-</sup>-N, 49.3 mg kg<sup>-1</sup>; NO<sub>2</sub><sup>-</sup>-N, 0.6 mg kg<sup>-1</sup>; pH (CaCl<sub>2</sub>), 6.95; maximum water-holding capacity, 45%; clay, 33%; silt, 62 and sand, 5%.

**Effect of DMPP on nitrification:** Fifty grams portions of the soil taken in 250 mL serum bottles were moistened to 20% with a solution of ammonium sulphate and DMPP to obtain NH<sub>4</sub><sup>+</sup>-N concentration in soil of 150 mg kg<sup>-1</sup>, while DMPP was added at 0, 0.35 or 3.5 mg kg<sup>-1</sup> soil. Sufficient replicates were incubated at 25°C to sacrifice triplicate after 0, 1, 3, 7, 11, 15 and 21 days of incubation. The soil samples thus withdrawn were analyzed for N in NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> forms using KCl extraction (1:10, soil:extractant ratio) and a colorimetric method<sup>[18]</sup>.

**Immobilization-remineralization of N as influenced by DMPP:** Portions of soil (50 g) placed in 250 mL serum bottles were moistened to 20% with a solution of glucose, ammonium sulphate and DMPP. The solution delivered 4500 mg C, 150 mg N kg<sup>-1</sup> soil (C/N ratio of the amendment 30) and 0, 0.35, 3.5, or 17.5 µg DMPP g<sup>-1</sup> soil. Bottles covered with perforated parafilm were incubated at 25°C in enough number to sacrifice triplicate from each treatment at 0, 4, 8, 12, 24, 36, 72, 120 and 240 h of incubations. Moisture content of the soil was maintained during incubation by making up the weight loss with distilled water. The soil samples withdrawn at different incubation intervals were analyzed for N content in NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>.

**Gaseous flux as affected by DMPP in soil receiving NH<sub>4</sub><sup>+</sup>+NO<sub>3</sub><sup>-</sup>-N and glucose:** Fifty gram portions of soil placed in 250 ml serum bottles were moistened to 20% with a solution of glucose and NH<sub>4</sub><sup>+</sup> + NO<sub>3</sub><sup>-</sup>-N containing DMPP. Final concentration of glucose and NH<sub>4</sub><sup>+</sup> + NO<sub>3</sub><sup>-</sup>-N in soil was 1% and 100 µg g<sup>-1</sup>, respectively, while that of

DMPP was 0, 0.35, 3.5, 7.0 and 17.5 µg g<sup>-1</sup>. The soil samples were incubated at 25°C. Headspace samples were analyzed for N<sub>2</sub>O, CO<sub>2</sub> and O<sub>2</sub> at 14, 18, 23, 42 and 66 h after incubation. An aliquot of the soil samples were removed for the determination of NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N and moisture content of the remaining soil raised to 30% with glucose solution (soil concentration of glucose, 0.5%) and headspace analyzed for the three gases at different time intervals for 20 h. The final measurement was made after the bottles were kept closed for 12 h.

In another experiment, 50 g portions of the soil were moistened with a solution of glucose, ammonium sulphate and DMPP to obtain uniform level of added C and N (150 and 4500 mg kg<sup>-1</sup> soil, respectively) and DMPP concentrations of 0, 0.35, 3.5 and 17.5 mg kg<sup>-1</sup>. The samples were incubated at 25°C and headspace samples analyzed for N<sub>2</sub>O, CO<sub>2</sub> and O<sub>2</sub> at 4, 8, 12, 24, 36, 72, 120 and 240 h.

**Potentially mineralizable N:** Fifty-g portions of soil were incubated with 50 mL of 1N KCl solution (submerged/anaerobic conditions) without or with DMPP to achieve final concentrations of the latter of 0.35, 3.5 and 17.5 mg kg<sup>-1</sup> soil. One and two weeks after incubation the samples were filtered and the filtrate analyzed for NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>-N. Amount of NH<sub>4</sub><sup>+</sup>-N accumulated after one and two weeks of incubation was taken as potentially mineralizable N.

**Quantification of N<sub>2</sub>O, CO<sub>2</sub> and O<sub>2</sub>:** The incubations were carried out in 250 mL serum bottles, which were sealed with a perforated laboratory foil to allow gas exchange but avoid water loss. At the times of gas flux measurements, the bottles were sealed with a lid that contained a silicon septum to allow headspace sampling. The lid was closed for 1 to 2 h and headspace samples taken with 60 mL disposable syringes equipped with a 3-way valve. Previous experience with the syringes indicated that they were gas tight and kept the gas concentrations for at least 48 h. Gas samples were analyzed immediately after sampling for N<sub>2</sub>O, CO<sub>2</sub> and O<sub>2</sub> on a gas chromatogram (Perkin Elmer, Germany) equipped with Flame Ionization (FID) and Electron Capture Detectors (ECD)<sup>[19]</sup>.

## RESULTS

The process of nitrification slowed down in the presence of DMPP, rate of nitrification being slower at higher levels of the inhibitor (Fig. 1). Measurable quantities of NO<sub>2</sub><sup>-</sup> were found only during the first 3 days

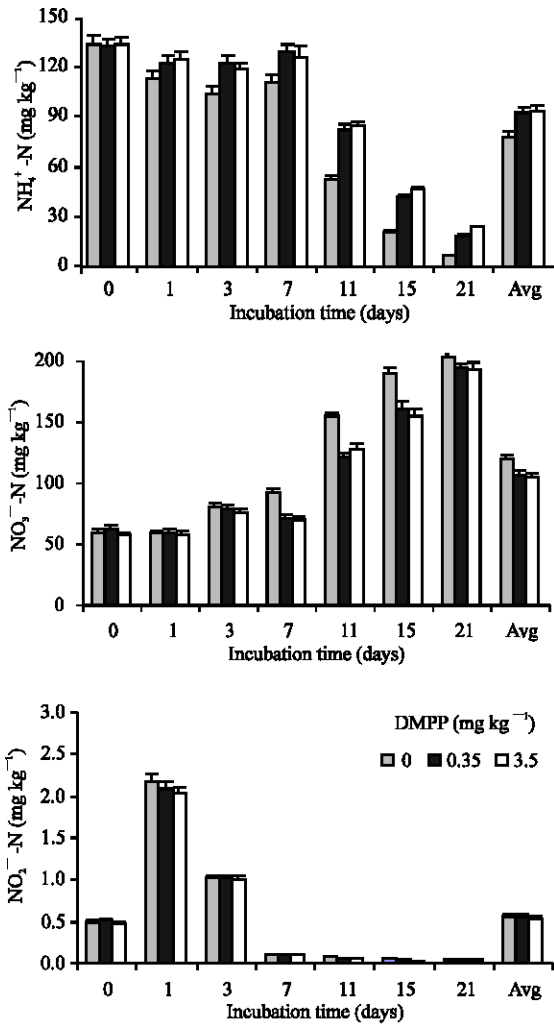


Fig. 1: Changes in the content of ammonium-N (a), nitrate-N (b) and nitrite-N (c) content of soil during incubation with different levels of DMPP; vertical bars represent standard error of means

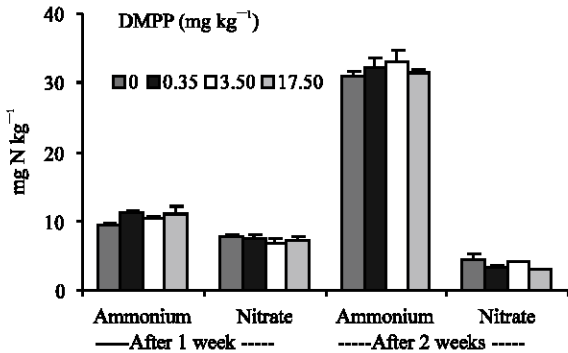


Fig. 2: Content of potentially mineralizable N (ammonium-N) and nitrate-N in soil incubated with different levels of DMPP; vertical bars represent standard error of means

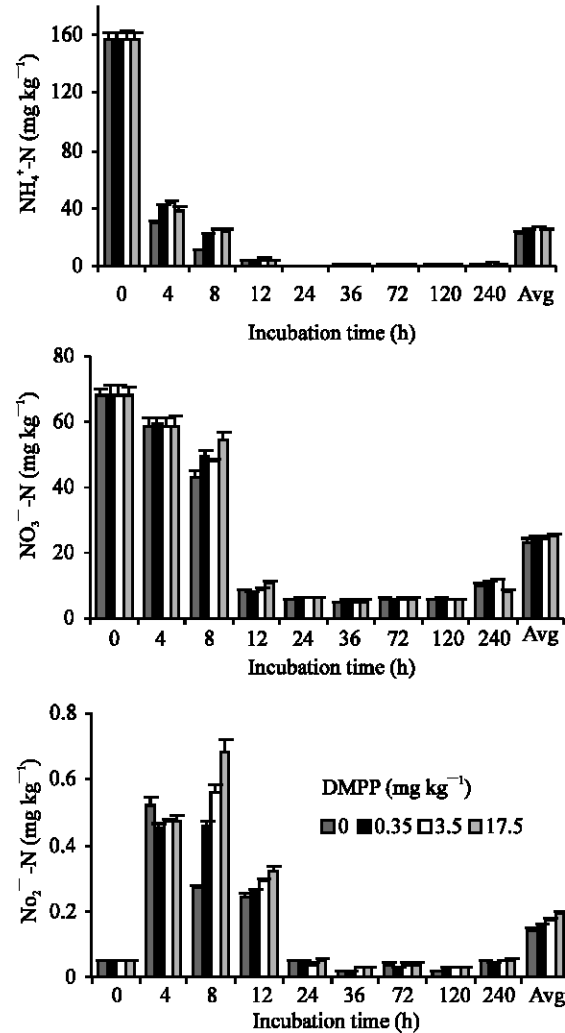


Fig. 3: Immobilization-remobilization of N during incubation of soil [as measured by the amounts of ammonium-N (a), nitrate-N (b) and nitrite-N (c)] content of soil with different levels of DMPP; vertical bars represent standard error of means

of incubation with DMPP having a negligible effect. Average of different samplings showed a small but non-significant effect of DMPP on  $\text{NO}_2^-$ -N. Active nitrification started after 7 days of incubation and the process continued to increase till 21st day. Effect of DMPP on  $\text{NO}_3^-$  formation was more pronounced from 7 to 15 days. At different incubation intervals, application of DMPP had a negligible effect on total mineral N and the average of different samplings was almost similar.

Potentially mineralizable N showed a small but significant increase in the presence of DMPP i.e., an average of 11 and 9  $\text{mg kg}^{-1}$   $\text{NH}_4^+$ -N accumulating in treated and untreated soil samples, respectively, during the first week of incubation (Fig. 2). During the second

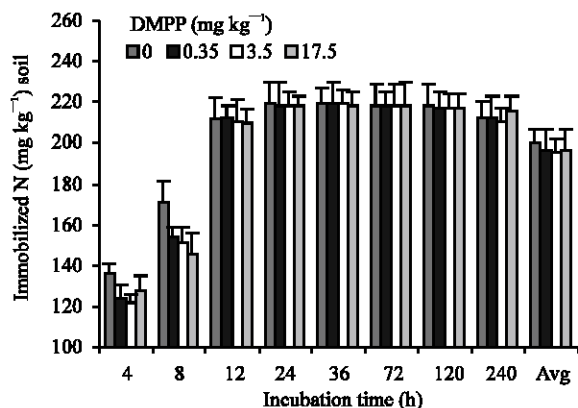


Fig. 4: Calculated values of microbial biomass N (measured as immobilized N) in soil incubated with different levels of DMPP; vertical bars represent standard error of means

week,  $\text{NH}_4^+\text{-N}$  increased by 200% in different treatments with DMPP having a small positive effect. As expected, only 10% of the  $\text{NO}_3^-\text{-N}$  present initially was recovered after one week and Ca 5% at the end of 2nd week of incubation suggesting significant losses, DMPP had a negligible effect on disappearance of  $\text{NO}_3^-$ .

Immobilization of N was significantly retarded by DMPP, the effect being more at higher than recommended concentrations (Fig. 3). Immobilization of N appeared to be complete after 24 h of incubation, with all the treatments showing Ca 6 mg mineral N  $\text{kg}^{-1}$  soil. Immobilization of both  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  was observed in all the treatments. The extent of immobilization was higher for  $\text{NH}_4^+\text{-N}$  than  $\text{NO}_3^-\text{-N}$  since a decrease from 38.3 to 20.7  $\text{mg kg}^{-1}$  in the case of former and from 58.9 to 48.8  $\text{mg kg}^{-1}$  occurred during the incubation period of 4-8 h. In addition, DMPP caused a higher decrease in the immobilization of  $\text{NO}_3^-\text{-N}$  than  $\text{NH}_4^+\text{-N}$ . Apparently, the population immobilization the two forms of N was affected differently by DMPP. Net accumulation of mineral N (reminalization) was found to occur 240 h after incubation in all the treatments, the process being rapid at lower (0.35 and 3.5  $\text{mg kg}^{-1}$  soil, respectively) and slower than control at higher (17.5  $\text{mg kg}^{-1}$ ) concentrations of DMPP. An accumulation of  $\text{NO}_2^-\text{-N}$  was also observed during the first 12 h of incubation followed by a rapid decrease to initial levels; DMPP had a positive effect suggesting a significant inhibition of nitrification. Inhibitory effect on nitrification was also apparent as lowest amounts of  $\text{NO}_3^-\text{-N}$  were found at the highest level of DMPP 240 h after incubation when the experiment was terminated. At this point of time, effective nitrification was found to be taking place as ca 80% of the mineral N was in the form of  $\text{NO}_3^-$  in different treatments; lowest proportion of  $\text{NO}_3^-$  being at the highest level of DMPP.

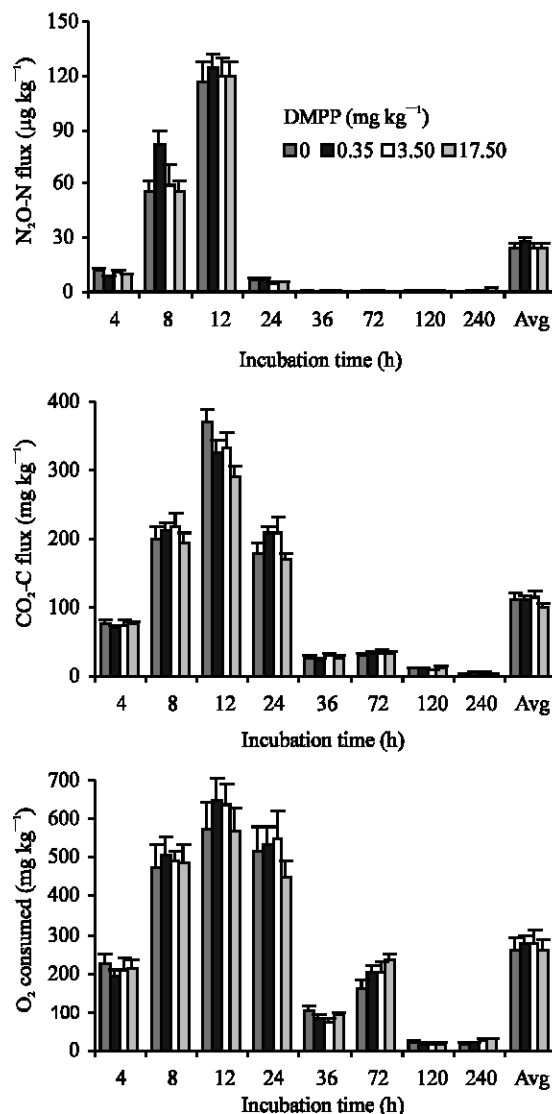


Fig. 5: Flux of  $\text{N}_2\text{O}$  (a) and respiratory activity (loss of  $\text{CO}_2$  and consumption of  $\text{O}_2$ ; b and c, respectively) of soil incubated at 20% moisture with different levels of DMPP; vertical bars represent standard error of means

The results presented in Fig. 3 were used to determine newly developed microbial biomass. Considering a C/N ratio of 8<sup>[20]</sup> and assuming that entire mineral N immobilized was present in the biomass<sup>[21]</sup>, carbon content of biomass varied from 976-1755  $\text{mg kg}^{-1}$  soil (Fig. 4). Maximum build-up of biomass was observed at 24 h after incubation accounting for 39% of the applied glucose C (4500  $\text{mg kg}^{-1}$ ). Negative effect of DMPP on biomass C was noticed only during the initial 8-12 h of incubation.

The data on the flux of  $\text{CO}_2$ ,  $\text{O}_2$  (respiratory activity) and  $\text{N}_2\text{O}$  from soil moistened to 20% with a solution of glucose, ammonium sulphate and different levels of

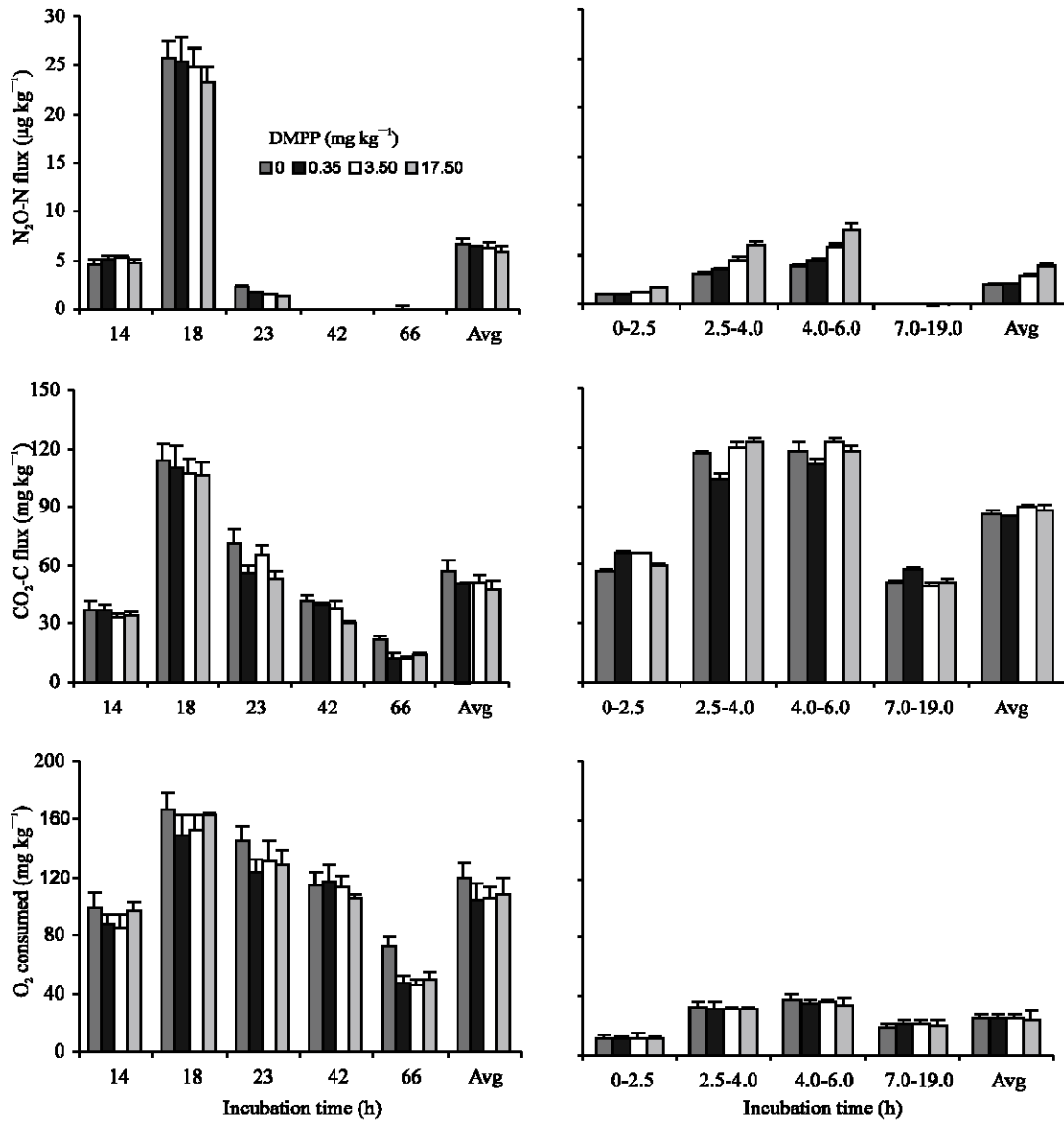


Fig. 6: Flux of N<sub>2</sub>O (a) and respiratory activity (loss of CO<sub>2</sub> and consumption of O<sub>2</sub>; b and c, respectively) of soil incubated at elevated moisture content (22% followed by 30% of soil; left and right parts of the Figure, respectively) with different levels of DMPP; vertical bars represent standard error of means

DMPP are shown in Fig. 5. Maximum microbial activity as suggested by CO<sub>2</sub> evolution and O<sub>2</sub> consumption was recorded after 12 h of incubation; emission of N<sub>2</sub>O was maximum at this time. Within next 24 h, gaseous flux decreased to significantly low levels and showed small changes during the subsequent period of incubation. Computation of data from all treatments and sampling times showed a significant correlation ( $r = 0.87$  to  $0.98$ ; maximum value of correlation being observed between CO<sub>2</sub> and O<sub>2</sub> and minimum between N<sub>2</sub>O and O<sub>2</sub>;  $p=0.05$ ) between the amounts of the three gases that were analyzed. Presence of DMPP in the soil did not have a significant bearing on the emission of N<sub>2</sub>O or CO<sub>2</sub> and

consumption of O<sub>2</sub> although a small decrease was observed in each case. Respiratory Quotient (RQ) varied from 0.14 to 0.57 and generally decreased with the time of incubation. Presence of DMPP had a negligible effect and the average of all incubation intervals was almost similar in different treatments.

In another experiment both NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>-N were used along with glucose and incubation was carried out first at 22% for 66 h followed by that at 30% moisture for 20 h (Fig. 6). Flux of CO<sub>2</sub> and consumption of O<sub>2</sub> decreased in the presence of DMPP. As observed for the first experiment, flux of different gases was significantly correlated amongst themselves ( $r = 0.82$  to  $0.95$ ;  $p=0.05$ ).

Respiratory quotient varied between 0.26 and 0.74 and decreased with time except for an initial increase 18 h after incubation. Except at 14 h after incubation, DMPP caused a substantial decrease in  $N_2O$  emission that was more at higher rates. Increase in the moisture level of soil to 30% and addition of glucose after 66 h of incubation (when respiratory activity was fairly stabilized and  $N_2O$  emission was found to be negligible at 20% moisture), led to an increase in the evolution of  $CO_2$  and consumption of  $CO_2$  till 6th h followed by a decline in all the treatments, DMPP had no effect. Respiratory quotient remained low compared to that at 20% moisture (average of all observations being 0.44 and 0.29, respectively), but showed an increase with incubation time. Emissions of  $N_2O$  significantly increased with the amount of DMPP initially applied and the increase continued until 6th h of incubation. Analysis of soil before raising the moisture to 30% showed substantial amounts of  $NO_3^-$ -N (15-18 mg  $kg^{-1}$  in different treatments; highest being in soil without DMPP) that would have helped sustain emission of  $N_2O$  during subsequent incubation. However, a net consumption of  $N_2O$  that increased with the amount of DMPP was observed when the soil-containing incubation bottles were left overnight with caps tightly closed.

## DISCUSSION

An ideal nitrification inhibitor for use in agriculture specifically blocks oxidation of ammonium but not that of nitrate, does not affect other beneficial organisms and higher plants, remains effective for several weeks after fertiliser application and is economical. Hundreds of chemicals have been evaluated considering these specifications<sup>[22-25]</sup> and the search continues. During the past few years, introduction of DMPP has shown a renewed promise as a potent nitrification inhibitor with positive implications to N economy, environmental cleanliness and crop yields<sup>[8-10]</sup>. There is obvious interest, however, to study the side effects of DMPP on microbial processes other than nitrification.

An important implication of nitrification inhibition is the accumulation of  $NH_4^+$  with a consequent increase in the immobilization of applied N. More  $^{15}N$  in biomass (immobilization) in inhibitor treated soil has been reported<sup>[14]</sup>. Increase in immobilization of N in inhibitor treated soil will be especially obvious when sufficient quantities of easily oxidizable C are available. This is expected in view of the so-called preferential immobilization by soil microorganisms of  $NH_4^+$  as compared to  $NO_3^-$ <sup>[26]</sup>. However, immobilization of  $NH_4^+$ -N will be enhanced only if the inhibitor does not have an inhibitory effect on microorganisms other than nitrifiers.

This was not true for DMPP as it inhibited N immobilization as well as respiratory activities. Apparently, DMPP-mediated prolonged availability of  $NH_4^+$ -N did not cause an increase in the respiratory activity of the soil to an extent that would have led to the development of anaerobic microsites and consequently the denitrification. Ottow and Fabig<sup>[27]</sup> suggested that in bulk soil or sediment, denitrification starts as soon as the trapped  $O_2$  is utilized and the subsequent  $O_2$  diffusion is limited. According to these authors, respiration and denitrification may occur simultaneously even at a relatively high  $O_2$  supply if a sufficient amount of easily decomposable organic matter is made available through organic amendments (as practiced in the present study) or physical treatment of the soil (wetting/drying, freeze/thawing or disturbance etc.).

In the present study, immobilization of N was significantly retarded by DMPP, especially, at concentrations higher than recommended. The inhibitory effect was particularly more for the immobilization of  $NO_3^-$  compared to  $NH_4^+$ -N. In general, immobilization of  $NH_4^+$  and  $NO_3^-$  may not differ significantly in the presence of an easily oxidizable C source like glucose, while immobilization of  $NH_4^+$  may be higher in unamended soil<sup>[28]</sup>. Hence, the microbial population responsible for immobilization of the two forms of N appeared to be affected differently by DMPP in the present study. This may be of significance so far as the availability of  $NO_3^-$ -N in soil and its subsequent fate is concerned. For example, presence of easily oxidizable organic C may facilitate microbial immobilization of  $NH_4^+$  but not  $NO_3^-$ , hence leaving the later available for losses. Such a situation may arise for ENTEC granules that contain both  $NH_4^+$  and  $NO_3^-$  although DMPP is reported to inhibit  $N_2O$  losses<sup>[10]</sup>. In the present study, a small or no reduction in the flux of  $N_2O$  was observed from soil treated with DMPP at recommended or several times higher rates. However, when the moisture level of DMPP-treated soil samples was raised from 20 to 30% with fresh application of oxidizable C (glucose), a significant increase in  $N_2O$  emission was observed over a short period of time 4-6 h, followed by a net scavenging.

Inhibition of N immobilization due to DMPP was also supported by relatively lower level of microbial biomass during the first 4-8 h of incubation. In this period active metabolism of glucose is expected to occur<sup>[29]</sup> leading to a rapid turnover of microbial biomass. Maximum build-up of biomass was observed at 24 h after incubation accounting for 39% of the applied glucose C (4500 mg  $kg^{-1}$ ). Negative effect of DMPP on biomass C was noticed only during the initial 8-12 h of incubation. Not only biomass but the loss of  $CO_2$  was also reduced to some extent by DMPP; maximum losses being observed

after 12 and 18 h. Under field conditions, DMPP is reported to cause a reduction in the loss of CO<sub>2</sub><sup>[10]</sup>, although these results have not been corroborated in model laboratory experiments<sup>[9]</sup>. Irrespective of the changes due to DMPP in respiratory activities, a strong positive correlation was obtained between CO<sub>2</sub>-C evolved and O<sub>2</sub> consumed in the present study. Similar results have been reported earlier<sup>[30]</sup>. The RQ that varied between 0.14 and 0.74 at different sampling occasions in the two experiments was similar in treated and untreated soil suggesting that respiratory activities as a whole were not significantly affected by DMPP. However, when microbial activities were intense, RQ was higher in control compared to DMPP-treated soil suggesting an early onset of anaerobic metabolism in the former as a result of O<sub>2</sub> limitation at the higher respiration rates<sup>[31]</sup>.

In the present study, DMPP had no significant effect on potentially mineralizable N obtained by incubating soil samples under anaerobic conditions. In an incubation study, DCD (dicyandiamide) had no effect on N mineralization from soil organic matter, while N immobilization increased<sup>[12]</sup>. Lodhi *et al.*<sup>[32,33]</sup> reported a strong inhibition of nitrification and increased N mineralization by cyfluthrin (an insecticide) and attributed increased N uptake by plants vis-à-vis improved crop growth to these changes in N transformation process. DCD is reported to be bacteriostatic that blocks conversion of NH<sub>4</sub> to NO<sub>3</sub> for 1-3 months, but has no effect on other soil organisms<sup>[13]</sup>. Chalk *et al.*<sup>[15]</sup> found 90% inhibition of nitrification by N-Serve after 12 days and no effect on net N mineralization, while gross rates of N immobilization and mineralization estimated by isotopic dilution were slightly affected. Nitrification inhibition is reported to cause an increased incorporation of N in soil organic matter<sup>[17]</sup>, whereas in other studies no such effects have been observed<sup>[16]</sup>.

In conclusion, the results of the studies presented above show that in addition to nitrification inhibition, DMPP causes a reduction in activities including respiration and N immobilization. In view of a close relationship between respiratory activities and emission of N<sub>2</sub>O, it may be logical to infer that DMPP will also cause a reduction in the loss of N through denitrification.

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