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# Microbial-Based Inoculants Impact Nitrous Oxide Emissions from an Incubated Soil Medium Containing Urea Fertilizers

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There is currently much interest in developing crop management practices that will decrease $N_2O$ emissions from agricultural soils. Many different approaches are being investigated, but to date, no studies have been published on how microbial inoculants affect $N_2O$ emissions. This study was conducted to test the hypothesis that microbial-based inoculants known to promote root growth and nutrient uptake can reduce $N_2O$ emissions in the presence of N fertilizers under controlled conditions. Carbon dioxide and $CH_4$ fluxes were also measured to evaluate microbial respiration and determine the aerobic and anaerobic conditions of the incubated soil. The microbial-based treatments investigated were SoilBuilder (SB), a metabolite extract of SoilBuilder (SBF), and a mixture of four strains of plant growth-promoting *Bacillus* spp. Experiments included two different N fertilizer treatments, urea and urea–$NH_4NO_3$ 32% N (UAN), and an unfertilized control. Emissions of $N_2O$ and $CO_2$ were determined from soil incubations and analyzed with gas chromatography. After 29 d of incubation, cumulative $N_2O$ emissions were reduced 80% by SB and 44% by SBF in soils fertilized with UAN. Treatment with *Bacillus* spp. significantly reduced $N_2O$ production on Days 1 and 2 of the incubation in soils fertilized with UAN. In the unfertilized treatment, cumulative emissions of $N_2O$ were significantly reduced 92% by SBF. Microbial-based treatments did not reduce $N_2O$ emissions associated with urea application. Microbial-based treatments increased $CO_2$ emissions from soils fertilized with UAN, suggesting a possible increase in microbial activity. Overall, the results demonstrated that microbial-based inoculants can reduce $N_2O$ emissions associated with N fertilizer application, and this response varies with the type of microbial-based inoculant and fertilizer.

Recent concerns about increased accumulations of greenhouse gases in the atmosphere have stimulated interest in developing better crop management practices to decrease $N_2O$ emissions from agricultural soils. Agriculture is the single largest source of anthropogenic $N_2O$ emissions (Bouwman et al., 2005). Currently, agricultural $N_2O$ emissions are more than twice that of pre-1940 management practices and about six times more than from native vegetation (Del Grosso et al., 2005). Nitrogen fertilization is considered the major source of agricultural $N_2O$ emissions, contributing 60 to 80% of total emissions on a global scale (Dalal et al., 2003; FAO, 2008). To meet growing demands for food, however, N fertilization is needed to optimize crop yields. Thus, considerable effort is being spent extensively studying fertilization practices to reduce $N_2O$ emissions.

Estimations of $N_2O$ emissions from N fertilizers applied to agricultural crops vary widely because $N_2O$ fluxes depend on many factors, such as the type of N fertilizer and the amount of N applied (Eichner, 1990). For instance, losses of $N_2O$ are greater with $NH_4NO_3$ than with urea (Harrison and Webb, 2001). Also, $N_2O$ emission rates are 0.04% for $NO_3$, 0.15 to 0.19% for $NH_4$ and urea, and 5% for anhydrous $NH_3$ (Breitenbeck et al., 1980; Slemr and Seiler, 1984). The concentrations of $NH_4$ and $NO_3$ in the soil, however, have a greater effect on $N_2O$ emissions than the specific fertilizer type applied (Harrison and Webb, 2001).

Microbial interactions in the soil are a very important aspect of $N_2O$ emissions from agricultural soils. Native soil microorganisms are responsible for the degradation and conversion of different forms of N in the soil. The most important chemical reactions that take place in the N cycle are mineralization, immobilization, nitrification, denitrification, $N_2$ fixation, and volatilization. These chemical reactions are largely affected by environmental conditions such as temperature and soil moisture. Because environmental conditions are constantly changing, the interactions among all the chemical reactions are very dynamic. Harrison and Webb (2001) suggested that denitrification is the main process responsible for $N_2O$ emissions



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**Abbreviations**: BM, *Bacillus* mixture; DAI, days of incubation; PGPR, plant growth promoting rhizobacteria; SB, SoilBuilder; SBF, SoilBuilder filtered; UAN, urea–ammonium nitrate.

under anaerobic soil conditions, while nitrification accounts for emissions under aerobic soil conditions.

Due to the great importance of the soil microbial community in N cycling in the soil, alterations in community composition and abundance can change the rate of N cycle processes (Cavigelli and Robertson, 2000). Hence, manipulating native soil microbial communities by chemical treatments or by inoculation with selected microorganisms can potentially alter N cycling in the soil. For example, adding nitrification inhibitors is a widely used method to reduce the rate of nitrification by inhibiting autotrophic $NH_3$–oxidizing bacteria (Singh and Verma, 2007).

During the past few decades, there has been increased interest in the use of beneficial microbial inoculations to improve plant and soil functions. Several microorganisms, such as plant growth-promoting rhizobacteria (PGPR), have been widely studied (Figueiredo et al., 2010). The PGPR stimulate plant growth through either a "biofertilizing" effect or a biocontrol effect. There is currently much interest in PGPR and other microbial-based inoculants specifically as alternatives to or supplements with fertilizers to improve the uptake of nutrients (Adesemoye et al., 2009, 2010; Canbolat et al., 2006; Idriss et al., 2002). Among the PGPR microorganisms, *Bacillus* spp. are widely used, mainly because they can survive as spores and can potentially alter the soil microbial composition. *Bacillus* spp. have a wide metabolic capability that allows them to play important roles in soil ecosystem functions and processes. Due to their heterotrophic nature, *Bacillus* spp. play an important role in the soil C cycle, soil N cycle, soil S cycle, and transformation of other soil nutrients (Mandic-Mulec and Prosser, 2011). Furthermore, they ~~work~~ as biocontrol agents due to the wide range of antiviral, antibacterial, and antifungal compounds they produce, which can control pathogens and have an effect on other soil microorganisms (Chaabouni et al., 2012). Antibiotics are important metabolites that are produced by *Bacillus* spp. They not only can control pathogens but also confer a competitive advantage over other soil microorganisms (Stein, 2005).

Microbial-based inoculants are already on the market and, in recent years, their popularity has increased substantially due to extensive and systematic research to enhance their effectiveness and consistency. SoilBuilder (Advanced Microbial Solutions) is an example of a microbial-based inoculant that is widely marketed.

The treatment of soils and plants with SoilBuilder has been shown to increase root growth and nutrient uptake (Yildirim et al., 2006). In addition, a version of SoilBuilder (AgBlend) induced suppressibility to root-knot nematodes and increased populations of aerobic spore-forming bacteria in the rhizosphere (Burkett-Cadena et al., 2008). Given this demonstrated increase in bacterial populations, we were interested in determining if SoilBuilder can affect bacterial functions related to soil N transformations. Hence, SoilBuilder was selected as a model microbial-based inoculant in the current study and was compared with a mixture of *Bacillus* spp. PGPR as described below. In addition, we were interested in testing the effects that the portion of SoilBuilder that contains metabolites may have directly or indirectly on soil N dynamics that could lead to changes in $N_2O$ emissions from soils.

Although the use of microbial-based inoculants is increasing, currently there is a lack of information about how these products affect $N_2O$ emissions from soils when N fertilizers are present. Thus, the objective of this study was to test the hypothesis that microbial-based inoculants, known to improve nutrient uptake, can reduce emissions of $N_2O$ in the presence of N fertilizers (UAN and urea) under controlled conditions. This study was one of the first to evaluate the use of microbial-based inoculants (*Bacillus* PGPR mix, SoilBuilder, and SoilBuilder filtered) for the purpose of reducing $N_2O$ emissions from soil combined with common agricultural N fertilizers. Carbon dioxide and $CH_4$ were also evaluated in this study to determine the impact of the microbial-based inoculants on microbial respiration ($CO_2$) and whether $N_2O$ production was mainly an effect of aerobic and anaerobic conditions occurring under laboratory incubations.

## Materials and Methods

### Soil Characterization

An initial soil analysis was performed by the Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total C and N were analyzed using an Elementar Vario Macro C-N analyzer (Elementar Americas). The soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of P, K, Mg, and Ca were determined using Mehlich I (double acid extracting solution) (Olsen and Sommers, 1982) and measured using an ICAP 9000 spectrometer (Thermo Jarrell Ash). The cation exchange capacity (CEC) was determined by base summation (Ca, Mg, K, and Na) according to the procedures of Hue and Evans (1986).

### Soil Microcosms

A soil–sand mixture was used as the medium for this study. Sand was mixed with the soil to improve water infiltration and minimize anaerobic conditions during the study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (v/v soil/sand) with white brick or mason sand (particle size: 1/8–1/4 mm). The mixture resulted in a soil medium with the texture of a loamy sand (78.8% sand, 4.4% clay, and 16.8% silt). The soil–sand mixture had a pH of 6.14, CEC of 1.13 cmol $kg^{-1}$, total N concentration of 0.7 g $kg^{-1}$, organic matter concentration of 17 g $kg^{-1}$, total C concentration of 2.6 g $kg^{-1}$, $NO_3$ concentration of 10.53 mg $kg^{-1}$, $NH_4$ concentration of 0.73 mg $kg^{-1}$, Mg concentration of 236 mg $kg^{-1}$, Ca concentration of 305 mg $kg^{-1}$, P concentration of 4 mg $kg^{-1}$, and K concentration of 51 mg $kg^{-1}$.

Soils were incubated for flux measurements in 2-L glass jars containing 400 g of the dry soil–sand mixture with a soil bulk density of 1.15 g $cm^{-3}$. The soil–sand mixture was then adjusted to 20% moisture (gravimetric water content) with the addition of the treatments, which were organized in a complete randomized design with a 4 × 3 factorial arrangement with three microbial-based treatments and a water control (no microbial-based treatment) and two N fertilizer sources and an unfertilized control, each replicated four times. The N fertilizer treatments included (i) UAN-32% and (ii) urea.

Nitrogen fertilization was calculated based on 168 kg $ha^{-1}$. The amount of N applied was calculated based on 1 ha furrow slice (15-cm topsoil), which is equal to 1.98 Gg of soil. Based on this calculation, each jar (400 g of dry soil) received 0.03 g of N in the fertilizer treatments. The specific amount of fertilizer

added was 0.0937 mL of UAN solution and 0.065 g of urea. The experiment was designed to provide the same amount of N regardless of the fertilizer source. Therefore, the quantity of N fertilizer added in each treatment was adjusted for each fertilizer type so that all treatments received the same amount of N.

## Microbial Source Preparation

SoilBuilder, a commercially available microbial soil amendment, is prepared from a bioreactor system consisting of a continuously maintained microbial community. The final product contains bacteria and bacterial metabolites derived from the bioreactor. Based on plate counts using tryptic soy agar (TSA) (incubation for 24 h at 25°C), the most commonly occurring bacteria within the final stabilized product are *Acidovorax facilis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus oleronius*, *Bacillus marinus*, *Bacillus megaterium*, and *Rhodococcus rhodochrous*, each at 10 colony-forming units (cfu) $m^{-3}$.

SoilBuilder filtered (SBF) consisted of SoilBuilder (SB) without microbial cells and was prepared by filtering SB through a 0.45-μm filter and then through a 0.22-μm filter. The SBF contained microbial metabolites derived from the bioreactor production system that, in addition to other components, included organic acids, peptides, and enzymes.

The PGPR *Bacillus* mixture (BM) included four *Bacillus* strains: *Bacillus safensis* T4 (previously called *B. pumilus* T4), *Bacillus pumilus* INR7, *Bacillus subtilis* ssp. *subtilis* IN937a (previously called *B. amyloliquefaciens* IN937a), and *Lysinibacillus xylanilyticus* SE56 (previously called *Bacillus sphaericus* SE56). These strains were obtained from culture collections at the Department of Entomology and Plant Pathology, Auburn University. These strains have been shown to have an important plant growth-promoting effect (Enebak et al., 1998; Jetiyanon et al., 2003; Kokalis-Burelle et al., 2002, 2003).

The microbial-based treatments were applied at a rate of 25 mL $jar^{-1}$. For the BM treatment, the bacterial mix was prepared by mixing each strain's spore suspension, which was previously quantified by plating the spore mix suspension on TSA and incubating for 48 h at 25°C. The spore mix was then adjusted to a concentration of l00 cfu $L^{-1}$. The final concentration in each jar was of $6.2 \times 10^3$ cfu $g^{-1}$ of dry soil. The SB solution was prepared according to the label instructions by mixing 16 mL of SB in 1.0 L of distilled water immediately before setting up the experiment. The SB contained $10^3$ cfu $L^{-1}$, so the final concentration in each jar was $10^3$ cfu $g^{-1}$ of dry soil. The SBF treatment was prepared in the same way as the SB treatment but before applying the 25 mL to the incubated sample, the solution was filtered. Sterility of the filtrate was confirmed by plating onto TSA (48 h at 25°C incubation) and observing no bacterial growth. Unfiltered SB population concentrations were confirmed also by plate count on TSA after incubation for 48 h at 25°C.

## Incubation Methods

The fertilizer source corresponding to each N fertilizer treatment was added, followed by the appropriate microbial-based treatment. Four jars without soil, maintained the same way as the jars with the soil–sand mix, served as a blank. A 118-mL plastic container containing 10 mL of water was placed in each jar to maintain humidity. The soil moisture content of the incubating samples was maintained by weighing the experimental units on each sampling day and adding deionized water as necessary. Shortly following treatment application, the incubation jars were sealed hermetically (jars remained sealed between sampling intervals) with retrofitted lids containing butyl rubber stoppers to allow gas ($CO_2$, $CH_4$, and $N_2O$) sampling. The jars were incubated in the dark at 25°C for 29 d. At the same time, a separate set of jars with the same treatments was incubated simultaneously for destructive sampling to measure soil $NH_4$–N and $NO_3$–N contents. On each sampling day, shortly following gas analysis, the lids of these jars were removed for 5 min to prevent anaerobic conditions from occurring and to allow gases to equilibrate with the ambient atmosphere.

## Gas Flux and Soil Ammonium and Nitrate Sampling

Gas samples were collected at 1, 2, 4, 8, 10, 15, 22, and 29 d after treatment. Soil $NH_4$–N and $NO_3$–N concentrations were determined at 1, 4, 8, 15, 22, and 29 d after treatment (samples were taken from a second set of jars and not from the jars used for gas sampling). Samples for gas analysis, collected by inserting a 23-gauge needle attached to a gastight 10-mL polypropylene syringe through the rubber septum embedded in the lids of the incubation jars, were injected into evacuated 6-mL glass vials fitted with butyl rubber stoppers. The samples were stored at 25°C until analysis, which was done within 2 wk of collection. Gas samples were analyzed using a gas chromatograph (Shimadzu GC-14B) equipped with an electron capture detector for $N_2O$ and a flame ionization detector for $CH_4$ and $CO_2$. The gas chromatograph's detectors were calibrated by comparison with a standard curve using standards obtained from Scott Specialty Gases. Soil flux was determined by dividing the gas concentration ($CO_2$, $CH_4$, or $N_2O$) by the number of days of incubation between samplings. The gas concentrations observed on each sampling day were added together to determine the total flux for the 29-d incubation.

Soil $NH_4$–N and $NO_3$–N concentrations were determined by extracting 5 g of wet soil with 50 mL of 2 mol $L^{-1}$ KCl for determination of the inorganic N content as described by Keeney and Nelson (1982). Soil extracts were measured colorimetrically for $NH_4$ and $NO_2 + NO_3$ using a Bran+Luebbe Auto Analyzer 3.

## Statistical Analysis

Analysis of variance, using a general linear model, was used to analyze each response variable for fertilizer type. Pearson correlations were also used to identify relationships among variables ($CO_2$, $N_2O$, and $CH_4$). All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2004) and a significance level of $\alpha = 0.05$ set a priori. An LSD test was used to identify significant differences among treatments (SB, SBF, BM, and control).

# Results and Discussion

Carbon dioxide and $N_2O$ production rates differed significantly among the fertilizer and microbial treatments (Table 1). In addition, the fertilizer treatment × microbial inoculant interaction was significant (Table 1). These results indicate that emissions of $N_2O$ and $CO_2$ depended on the interaction of

both microbial and fertilizer treatments. This interaction was especially significant during the first 8 d of the incubation.

## Nitrous Oxide Emissions

Urea- and UAN-fertilized soils released 10 times more total $N_2O$ (after 29 d of incubation [DAI]) than the unfertilized treatment (Table 2). These observations confirm reports that as more N cycles through the soil system, a greater quantity of N is converted into $N_2O$ gas (Smith et al., 1997). Previous reports from field studies have indicated that fertilizer-derived $N_2O$ emissions from plots treated with nitrifiable forms of N fertilizer ($NH_3$ or $NH_4$) are greater than those from plots receiving an equivalent application of N as $NO_3$ (Breitenbeck and Bremner, 1986). In this case, UAN had more $NH_4$–N (7.75%) at the beginning of the experiment than the urea (0% $NH_4$–N). When comparing the control treatments (no microbial-based treatment) of UAN and urea (Table 2), total $N_2O$ emissions from UAN were twice the urea treatment.

Nitrous oxide emissions per sampling day peaked on 8 DAI for all microbial-based treatments (SB, SBF, and BM) that received UAN or urea (Fig. 1 and 2). High $N_2O$ emissions measured on the first day after applying N fertilizer were also reported by Pathak et al. (2006), who suggested that higher emissions were due to $N_2O$ formation during nitrification of $NH_4$ produced by hydrolysis of the applied urea. A peak was also observed in all treatments following the addition of N as urea, followed by a decline (Bremner and Blackmer, 1978; Fujinuma et al., 2011; Hou et al., 2000). This peak was not observed in the unfertilized treatment (Fig. 3), suggesting that N fertilizer was responsible for the $N_2O$ peak.

Nitrous oxide production rates between microbial-based treatments varied among different days of the incubation (Fig. 1–3). In the unfertilized treatment (Fig. 3), $N_2O$ production rates were significantly lower for all three microbial-based treatments (SB, SBF, and BM) during the first 8 DAI. After this time, $N_2O$ production from the SB and BM treatments was significantly higher than the control (Fig. 3), while with SBF, emissions stayed lower than the control throughout the experiment. This difference in emissions following treatments with SB or BM compared with SBF could be explained by the fact that both SB and BM contained living microorganisms, while SBF had only microbial metabolites. Accordingly, it is possible that the microorganisms in SB and BM did not survive past 8 DAI due to the low concentration of N in the soil. In support of this interpretation, West et al. (1985) reported that one of the main factors affecting the survival of *Bacillus* species in soil is nutrient availability. In contrast with the SBF treatment, the total $N_2O$ emission (Table 2) was 13 times less than the control (no microbial-based products). It is possible that among the metabolites in SBF are phenolic compounds, which are known to inhibit soil nitrifying bacteria communities (Bending and Lincoln, 2000). In support of this explanation, *Bacillus* spp. have been reported to produce phenolic compounds (Chaabouni et al., 2012).

In UAN-fertilized soils (Fig. 1), the BM treatment resulted in lower rates of $N_2O$ during the first 2 DAI compared with the control (no microbial-based treatment). The SBF treatment

**Table 1. Analysis of variance for the effects of fertilizer and microbial-based treatments on $CO_2$, $N_2O$, and $CH_4$ production (μg trace gas $kg^{-1}$ soil $d^{-1}$, calculated on a dry-soil basis) during 29 d of incubation.**

| Variable | Factor† | ANOVA *P* > *F* LSD (0.05) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 d | 2 d | 4 d | 8 d | 10 d | 15 d | 22 d | 29 d |
| $CO_2$ | fertilizer (F) | <0.0001 | <0.0001 | <0.0001 | 0.0006 | 0.6765 | 0.077 | 0.3013 | 0.0582 |
| | microorganism (M) | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.6289 | 0.0197 | 0.0034 | 0.0012 |
| | M × F | 0.0643 | <0.0001 | 0.0120 | 0.0002 | 0.2450 | 0.0617 | 0.1698 | 0.3262 |
| $N_2O$ | fertilizer (F) | 0.0001 | <0.0001 | <0.0001 | 0.0003 | 0.1084 | 0.0051 | 0.0003 | 0.0685 |
| | microorganism (M) | 0.0274 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| | M × F | 0.2901 | <0.0001 | <0.0001 | <0.0001 | 0.0041 | 0.0026 | <0.0001 | 0.0202 |
| $CH_4$ | fertilizer (F) | 0.2497 | 0.5541 | 0.3897 | 0.2592 | 0.7267 | 0.2870 | 0.5172 | 0.6690 |
| | microorganism (M) | <0.0001 | 0.0073 | <0.0001 | <0.0001 | 0.4617 | <0.0001 | 0.7200 | <0.0001 |
| | M × F | 0.0007 | 0.1784 | 0.0188 | 0.0056 | 0.6101 | 0.0117 | 0.4638 | 0.9120 |

† Fertilizer factors include the treatments urea–$NH_4NO_3$, urea, and unfertilized; microorganism factors include SoilBuilder, SoilBuilder filtered, *Bacillus* mixture, and control (no product applied).

**Table 2. Total $N_2O$ and $CO_2$ production after 29 d of incubation, calculated on a dry-soil basis.**

| Gas | Microbial treatment | Total production | | |
|---|---|---|---|---|
| | | Urea | Urea–$NH_4NO_3$ | Unfertilized |
| | | μg N or C $kg^{-1}$ soil | | |
| $N_2O$ | SoilBuilder | 1,322.2 ab† | 376.7 c | 194.9 a |
| | SoilBuilder filtered | 909.2 bc | 1,029.1 bc | 14.1 b |
| | *Bacillus* mixture | 1,691.8 a | 1,628.8 ab | 155.2 a |
| | control | 808.7 c | 1,639.2 a | 181.8 a |
| $CO_2$ | SoilBuilder | 42,319.6 b | 35,301.2 b | 35,786.5 ab |
| | SoilBuilder filtered | 57,156.8 a | 56,785.6 a | 45,071.7 a |
| | *Bacillus* mixture | 57,814.3 ab | 57,011.6 a | 40,071.4 ab |
| | control | 52,059.1 ab | 48,707.4 a | 31,393.8 b |

† Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.

also showed a similar pattern, but in this case, differences from the control lasted until 4 DAI. The SB treatment produced significantly less $N_2O$ than the control during the first 22 DAI. The SB and SBF treatments significantly reduced the total $N_2O$ production compared with the control treatment (no microbial-based treatment) (Table 2). With these two treatments, $N_2O$ production was almost five times lower than that observed with the control. The SB and SBF treatments also have in common the presence of microbial metabolites, which could be responsible for the $N_2O$ reduction. As mentioned above, it is possible that among the metabolites in SBF are phenolic compounds, which are known to inhibit soil nitrifying bacterial communities (Bending and Lincoln, 2000). The SB treatment, which contained both the living microorganisms and microbial metabolites, resulted in the greatest $N_2O$ reduction. The presence of microorganisms probably increased immobilization of the fertilizer N (Zak et al., 1990), which could potentially reduce nitrification and increased microbial competition for nutrients, which has been attributed before to microorganisms such *Bacillus* spp. (Stein, 2005; West et al., 1985). The SB and SBF could also alter the soil chemistry. Kumar et al. (1988) observed that an increase in soluble salts could inhibit microbial processes such as nitrification. Also, decreases in pH could alter the nitrification and denitrification processes (Bolan et al., 2004; Broos et al., 2007).

For urea-treated soils (Fig. 2), there were no significant differences in $N_2O$ production among the control and SB, SBF, or BM for the first 10 DAI. After 10 DAI, the BM treatment increased $N_2O$ production compared with the control. The total $N_2O$ production after 29 d (Table 2) showed that the highest $N_2O$ emissions were recorded with BM and SB treatments, which were significantly higher than the control treatment. The trends observed with urea fertilizer were different from those observed with UAN, even though both fertilizers contain urea. The amount of urea in UAN is only 16.5%; thus, the mechanisms involved in $N_2O$ production appear to be different.

The treatment effects observed with UAN are probably related more to the presence of $NO_3$–N and $NH_4$–N than to the presence of urea. Chapman and Leibig (1952) and Engel et al. (2010) found that localized application of urea can release significant amounts of $NH_3$, which could be toxic to many microorganisms. Hence, because the experiment was performed in a closed environment, the toxic effect of $NH_3$ could have affected the added microorganisms in the BM and SB treatments. These two treatments also had significantly increased total $N_2O$ emissions (Table 2) compared with the control (no microbial-based treatment). This increase could be explained by the fact that dead bacterial cells become C and N sources that could indirectly increase populations of other microorganisms such as nitrifiers. In contrast, with SBF, the treatment that contained only the metabolites, total $N_2O$ emissions did not differ significantly from the control. There were no reductions in $N_2O$ with SBF in the urea-fertilized soils. This could be explained by the fact that, unlike UAN, urea does not have a significant initial amount of $NH_4$–N. Bending and Lincoln (2000) showed that the inhibition of nitrifiers by phenolic compounds was recorded when an $NH_4$–N based fertilizer was applied to the soil. Hence, in order for SBF to have an inhibitory effect on the population of nitrifiers, a significant initial amount of $NH_4$–N is needed, which was not the case when urea was applied.

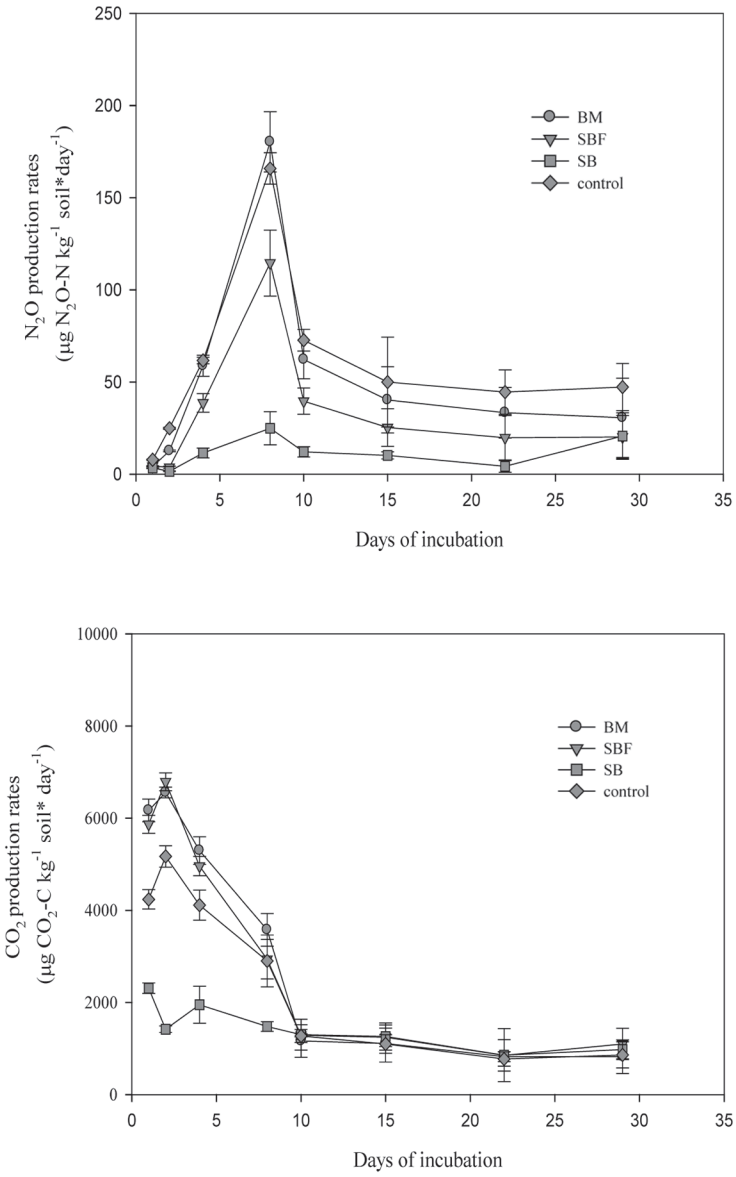


**Fig. 1. Temporal changes in $N_2O$ and $CO_2$ production rates for UAN (urea–$NH_4NO_3$) treatments during 29 d of incubation for SoilBuilder (SB), SoilBuilder filtered (SBF), *Bacillus* plant growth-promoting rhizobacteria mix (BM), and control (no product applied) soil treatments.**

## Carbon Dioxide Emissions

The total $CO_2$ production was less from the unfertilized treatment than from the N fertilizer treatments (Fig. 1–3). This finding differs from observations made by Kowalenko et al. (1978), who found a consistent lowering of microbial activity due to N fertilization. Barabasz et al. (2002), however, suggested that N fertilization would increase microbial activity due to the addition of nutrients. This observation is consistent with our results. The soil used in our experiment was nutrient poor, so when N fertilizer was applied it appears to have enhanced the microbial activity.

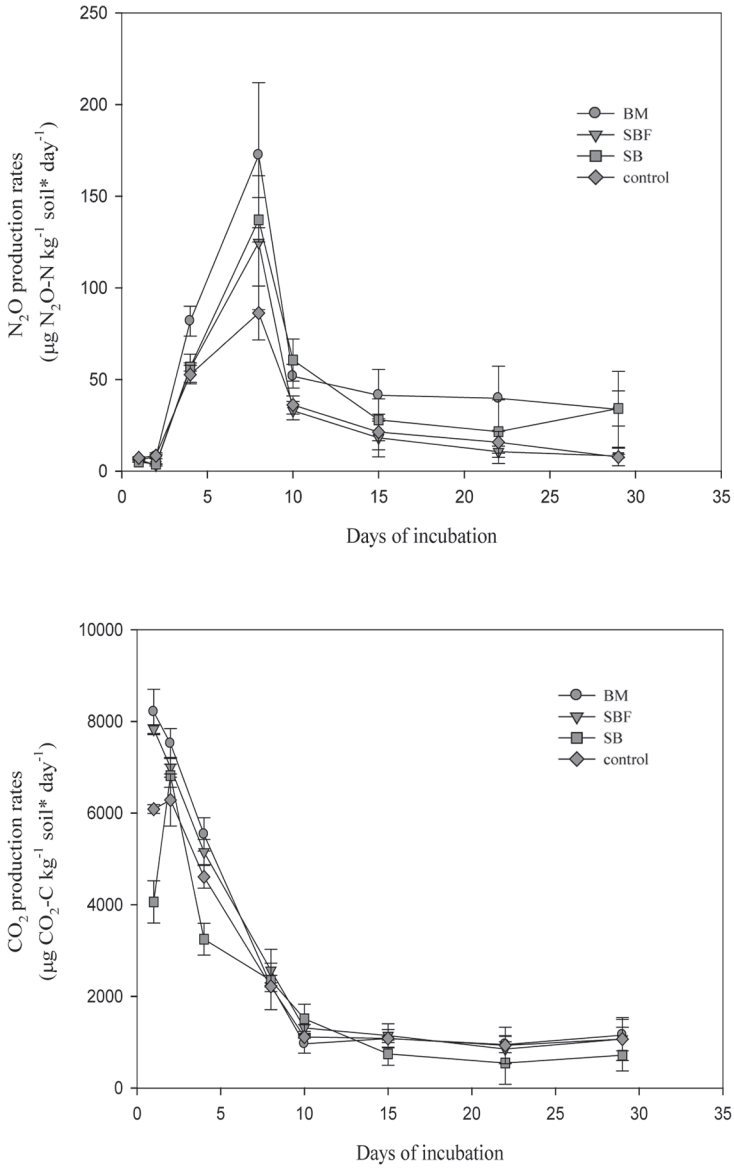


**Fig. 2. Temporal changes in $N_2O$ and $CO_2$ production rates for urea fertilizer treatments during 29 d of incubation for SoilBuilder (SB), SoilBuilder filtered (SBF), *Bacillus* plant growth-promoting rhizobacteria mix (BM), and control (no product applied) soil treatments.**

None of the microbial-based treatments containing microorganisms (SB and BM) differed significantly from the control for $CO_2$ production at 29 DAI (Table 2) in the unfertilized soils. The metabolite-based treatment (SBF) was the only one to significantly increase the total $CO_2$ production. The SBF is the same treatment that had lower total $N_2O$ production, so in this case greater $CO_2$ production was not related to greater $N_2O$ production.

The SB treatment significantly reduced total $CO_2$ production in the UAN- and urea-fertilized soils (Table 2). The SB treatment contained microbes plus metabolites, so the activity of SB could be related more to a decrease in microbial activity in the soil. Furthermore, SB also produced the least $N_2O$ with UAN. In the presence of UAN fertilizer, therefore, the decrease in microbial activity (represented by $CO_2$ production) probably resulted in decreased total $N_2O$ emissions. These results differ from the results of Ullah and Moore (2011), who observed that $N_2O$ fluxes from well-drained soils correlated negatively and significantly with $CO_2$ emission rates.

On the other hand, the SB treatment in the urea-fertilized soil was more related to higher total $N_2O$ production (Table 2). Therefore, these results clearly demonstrate that the dynamics of $N_2O$ and $CO_2$ production are highly affected by fertilizer type. For instance, a treatment that could potentially decrease microbial activity in the presence of one fertilizer could show an opposite effect with another fertilizer. As mentioned above, a possible explanation could be the toxic effect on the added microorganisms of $NH_3$ released from urea. When UAN was present and the toxic effect of $NH_3$ was lower, however, $CO_2$ total production also decreased in the SB treatment. Nannipieri et al. (2003) pointed out an important concept about the link between microbial activity and microbial diversity. Assessing microbial activity by $CO_2$ production in the soil does not take into account the microbial species effectively involved in the measured process. In this case, $CO_2$ production does not reflect the effect of the added microbial treatments (SB and BM). The reduction of $N_2O$ total emissions observed with SB and BM when UAN was present could be related more to a competition with nitrifiers, leading to a change in microbial diversity, than to an increase in total microbial activity.

## Methane Emissions

Methane emissions were low in all incubations, presumably because anaerobic conditions within the jars were negligible during the incubation (data not shown). Methane is formed in soils by the microbial breakdown of organic compounds under strictly anaerobic conditions (Smith et al., 2003; Yu et al., 2001). There were no significant differences in $CH_4$ emissions among the microbial-based treatments, leading to the conclusion that the microbial-based products used for this study do not affect $CH_4$ emissions under aerobic conditions. These results suggest that the soils were well aerated throughout the incubations, which suggests that $N_2O$ fluxes reflect nitrification, not denitrification.

## Nitrate and Ammonium Concentrations in Soil

As expected, $NO_3$–N and $NH_4$–N concentrations in the soil extracts from the unfertilized treatment (Table 3) were much less than the concentrations observed for the N-fertilized treatments (Tables 4 and 5). In the unfertilized treatment, there was significantly less soil $NO_3$–N at 29 DAI with the SB treatment compared with the SBF and BM treatments but not the control. On the other hand, no differences in the $NH_4$–N concentrations in the soil were observed.

Microbial-based treatments increased soil $NO_3$–N levels. Soil $NO_3$–N concentrations among treatments with UAN (Table 4) were significantly different on 8 and 15 DAI. The $NO_3$–N concentration observed from the SB treatment was significantly higher than the control treatment. The SB treatment also produced lower $N_2O$ emissions than the control. Furthermore, the highest $N_2O$ emissions were recorded on 8 and 15 DAI. The reduction in $N_2O$ production

was apparently related to a higher concentration of $NO_3$–N in the soil on 8 and 15 DAI. The microbial-based treatments also increased the soil $NH_4$–N concentration. These differences were significantly higher at the end of the incubation (22 and 29 DAI) for the SB and BM treatments. Thus, higher $NH_4$–N concentrations could be associated with a reduction in the nitrification process that converts $NH_4$–N to $NO_3$–N. Less nitrification is also associated with less production of $N_2O$ (Eichner, 1990).

In the urea treatment, there were no significant differences in the $NO_3$–N or $NH_4$–N concentrations between the microbial-based treatments during the first 8 DAI (Table 5). This indicates that the microbial-based treatments did not delay the release of $NH_4$–N from urea. On the last day of incubation, significantly higher soil $NH_4$–N concentrations were observed with BM than the control. Nevertheless, this higher $NH_4$–N concentration with the BM treatment was not associated with less $N_2O$ production.

Some *Bacillus* spp. were previously reported to have a denitrification capacity that could potentially increase $N_2O$ emissions (Verbaendert and DeVos, 2011). *Bacillus* spp. were present in the SB and BM treatments; however, neither of the results for these treatments showed a consistent pattern regarding an increase of $N_2O$ that could allow us to consider denitrification as an important issue in the present study. Furthermore, the conditions presented in this experiment were not $O_2$ depleted, a condition that is important for denitrification. The PGPR *Bacillus* mix increased $N_2O$ only with urea fertilizer but not with UAN or no fertilizer. Hence, the increase in $N_2O$ emissions could potentially be related more to the presence of urea than to denitrification.

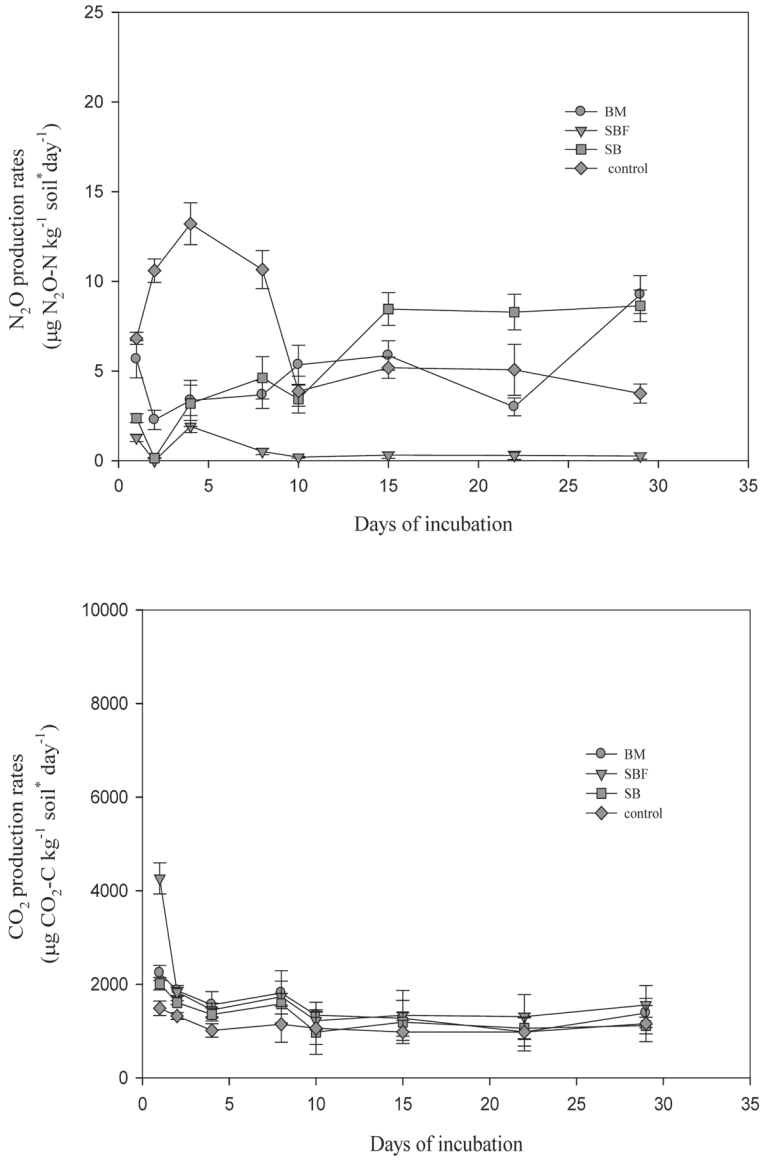


**Fig. 3. Temporal changes in $N_2O$ and $CO_2$ production rates for unfertilized treatments during 29 d of incubation for SoilBuilder (SB), SoilBuilder filtered (SBF), *Bacillus* plant growth-promoting rhizobacteria mix (BM), and control (no product applied) soil treatments.**

## Correlation Analysis

Carbon dioxide and $N_2O$ emissions were correlated in soils fertilized with UAN ($r$ = 0.611, $P$ < 0.0001). Nitrous oxide emissions in the urea treatment were also significant but not highly correlated with $CO_2$ ($r$ = 0.315, $P$ = 0.0003). The relationship between $N_2O$ and $CO_2$ is well documented for cultivated soils (Burford and Bremner, 1975) and also for tropical soils (Garcia-Montiel et al., 2004). To explain why $N_2O$ emissions could be affected by microbial inoculations, however, we must consider that $CO_2$ could only give us information about the total microbial activity, not the microbial diversity in the soil (Nannipieri et al., 2003). No significant correlation was found between $CH_4$ and $CO_2$ or $CH_4$ and $N_2O$.

# Conclusions

The results reported here partially support our hypothesis that microbial-based inoculants can reduce $N_2O$ emissions from soils. The potential reduction of $N_2O$ was affected by the type of fertilizer applied. The use of UAN fertilizer resulted in greater significant reductions in $N_2O$ emissions with SB and SBF. In the unfertilized control, significant reductions with SBF were observed. There was no significant reduction in $N_2O$ emissions, however, when urea was applied to the soil. A possible explanation for this is the toxicity effect that $NH_3$ could have on microbes, which would explain why the microbial treatments did not have an effect when urea was present. The results also showed that the microbes and microbial metabolites have effects on N dynamics and $N_2O$ emissions from soil. Given the lack of an anaerobic environment, some possible mechanisms that could explain the reduction of $N_2O$ emissions include: (i) production or presence of nitrification inhibitors; (ii) inhibition of nitrifying microorganisms; (iii) competition of applied microbial treatments with the native microbial nitrifiers; and (iv) immobilization of N fertilizer by microbes. Emissions of $CO_2$ did not show a clear pattern that could explain the variations in $N_2O$.

The results of this study represent an important starting point for elucidating the effect of microbial and microbial metabolite treatments on the production of $N_2O$ from agricultural soils.

Table 3. The $NO_3$–N and $NH_4$–N concentrations in the soil during 29 d of incubation for the unfertilized treatments, calculated on a dry-soil basis.

| Parameter | Treatment | $NO_3$–N or $NH_4$–N concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 d | 4 d | 8 d | 15 d | 22 d | 29 d |
| | | mg kg$^{-1}$ | | | | | |
| $NO_3$–N | SoilBuilder | 7.91 a† | 8.96 a | 7.76 a | 8.27 a | 7.94 a | 6.42 b |
| | SoilBuilder filtered | 7.65 a | 8.01 a | 8.37 a | 8.01 a | 7.86 a | 8.71 a |
| | *Bacillus* mixture | 7.69 a | 8.63 a | 8.89 a | 8.75 a | 8.87 a | 8.61 a |
| | control | 9.02 a | 8.72 a | 8.23 a | 8.11 a | 7.92 a | 7.44 ab |
| $NH_4$–N | SoilBuilder | 1.09 a | 1.18 a | 1.28 b | 0.98 b | 1.08 bc | 0.63 a |
| | SoilBuilder filtered | 1.08 a | 1.08 a | 1.23 b | 1.28 a | 1.03 c | 0.65 a |
| | *Bacillus* mixture | 0.81 a | 1.14 a | 2.67 a | 0.74 c | 1.29 ab | 0.66 a |
| | control | 0.91 a | 1.11 a | 2.57 a | 0.81bc | 1.41 a | 0.71 a |

† Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.

Table 4. The $NO_3$–N and $NH_4$–N concentrations in the soil during 29 d of incubation for the urea–$NH_4NO_3$ treatments, calculated on a dry-soil basis.

| Parameter | Treatment | $NO_3$–N or $NH_4$–N concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 d | 4 d | 8 d | 15 d | 22 d | 29 d |
| | | mg kg$^{-1}$ | | | | | |
| $NO_3$–N | SoilBuilder | 41.69 a† | 45.14 a | 57.21 a | 110.69 a | 96.52 a | 99.55 a |
| | SoilBuilder filtered | 33.97 a | 43.73 a | 50.93 ab | 71.41 b | 93.34 a | 104.61 a |
| | *Bacillus* mixture | 43.16 a | 44.21 a | 56.45 a | 89.91 b | 102.66 a | 105.42 a |
| | control | 29.82 b | 42.01 a | 41.66 b | 75.76 b | 84.61 a | 86.24 a |
| $NH_4$–N | SoilBuilder | 50.11 ab | 96.29 a | 99.77 ab | 110.78 a | 59.13 a | 42.62 a |
| | SoilBuilder filtered | 38.66 ab | 94.89 a | 98.54 ab | 86.65 a | 53.35 ab | 29.58 ab |
| | *Bacillus* mixture | 57.66 a | 98.63 a | 110.63 a | 96.31 a | 61.25 a | 43.86 a |
| | Control | 32.21 b | 80.26 a | 67.22 b | 55.45 a | 25.41 b | 16.371 b |

† Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.

Table 5. The $NO_3$–N and $NH_4$–N concentrations in the soil during 29 d of incubation for the urea treatments, calculated on a dry-soil basis.

| Parameter | Treatment | $NO_3$–N or $NH_4$–N concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 d | 4 d | 8 d | 15 d | 22 d | 29 d |
| | | mg kg$^{-1}$ | | | | | |
| $NO_3$–N | SoilBuilder | 10.16 a† | 13.94 a | 20.83 a | 56.47 a | 64.99 a | 48.85 a |
| | SoilBuilder filtered | 9.96 a | 14.22 a | 23.21 a | 53.48 a | 55.11 a | 57.67 a |
| | *Bacillus* mixture | 9.51 a | 13.21 a | 21.67 a | 38.55 a | 54.26 a | 49.24 a |
| | control | 9.71 a | 15.91 a | 22.23 a | 52.95 a | 59.38 a | 51.98 a |
| $NH_4$–N | SoilBuilder | 16.83 a | 56.27 a | 52.03 a | 52.91 a | 7.71 a | 1.57 b |
| | SoilBuilder filtered | 17.72 a | 56.03 a | 40.31 a | 22.24 b | 8.47 a | 2.01 ab |
| | *Bacillus* mixture | 16.93 a | 55.51a | 49.08 a | 31.72 ab | 8.15 a | 3.21 a |
| | control | 19.09 a | 53.66 a | 42.78 a | 42.76 ab | 8.41 a | 1.13 b |

† Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.

Microbial-based treatments demonstrated the potential to decrease $N_2O$ emissions from agricultural soils. Further research is needed to better understand the processes involved in the dynamics between microbial-based treatments, the N cycle, and N fertilizers.

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