

**Evaluation of antibacterial and antifungal compounds for selective inhibition of
denitrification in soils**

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Abstract

Nitrous oxide (N₂O) is an atmospheric constituent implicated in climate warming and stratospheric ozone depletion. Both bacteria and fungi participate in N₂O production, but information is lacking with regard to the relative contribution of bacterial and fungal denitrifiers to the denitrification process in agricultural soils. The selective inhibition technique (SI) is widely used to assess the contribution of different groups of microbes to soil processes, but success of the technique depends on the effectiveness of the inhibitors. In this study, laboratory experiments were conducted to assess the contribution of bacteria and fungi to denitrification using soils from a woodlot, agricultural fields under conventional plowing (PT), and no-till for either 50 years (long-term) or 11 years (medium-term). A selective inhibition (SI) technique was developed using two bactericides (streptomycin, bronopol) and two fungicides (cycloheximide, captan) applied at different rates (0-32 mg g⁻¹ soil). Regardless of application rate, streptomycin and cycloheximide were not effective inhibitors of denitrification, with degree of inhibition only between 2 and 20% relative to controls. These results are significant given the wide use of these products in SI studies. However, the bactericide bronopol and the fungicide captan effectively inhibited denitrification, with the strongest inhibition observed at an application rate of 16 mg g⁻¹ soil. The ratio of fungal to bacterial denitrification activity (F:B) was generally less than 1, indicating a dominance of bacteria in denitrification activity in the soils investigated. However, an increase in F:B ratio from 0.24 in medium-term NT to 0.87 in long-term NT soils was noted, suggesting perhaps a progressive increase in the role of fungal denitrifiers with longer duration of NT farming.

Introduction

Nitrous oxide (N_2O) is a by-product of nitrogen (N) cycling processes in soil ecosystems, and an important atmospheric constituent implicated in the accelerated greenhouse effect and stratospheric ozone depletion.¹ Although N_2O can also originate from nitrification, denitrification remains the dominant N_2O production process in soils.¹ Denitrification is the dissimilatory reduction of nitrate (NO_3^-) to $\text{N}_2\text{O}/\text{N}_2$ by mostly facultative anaerobes as a substitute for oxygen during respiration under oxygen-deficient conditions.^{1,2} As denitrifying microbes are largely heterotrophs, N_2O production is often limited by the availability of metabolizable organic carbon.³

Both fungi and bacteria participate in N_2O production in soils¹⁸, and therefore their relative contribution to the denitrification process can be affected by land-use and management. Among anthropogenic activities, agriculture has been identified as the largest contributor to global N_2O emission, largely due to tillage operations and application of N fertilizers.^{4, 5, 6, 7,8, 9, 10} No-till (NT) is a farming practice that has gained wide acceptance in recent decades, and has been proposed as an alternative to the conventional plow-till (PT) practice. In contrast to PT, the land surface remains relatively undisturbed under NT, and the current year's crop seeds are sown directly into the residue left by the previous crop. The effects of no-till farming on soil moisture, temperature, organic carbon (SOC) availability, size and composition of the soil microbial community are well documented.¹¹ Several past studies have reported an increase in the fungi:bacteria ratio of the soil microbiota with NT adoption.^{2,11,12, 13, 14} It has been suggested that litter mixing with soil (and thus direct contact between the soil bacteria and substrate) caused by plowing creates conditions favorable to bacterial

growth under PT.¹⁴ However, in NT systems in which there is a spatial separation between microorganisms and decomposable litter on the soil surface, the presence of hyphae confers an ecological advantage to fungi.¹⁵ Kladvko¹⁶ suggested that higher soil moisture content under NT management may contribute to fungal dominance in the soil microflora. This shift in soil microbial community composition has been linked to SOC accretion, with land management practices favorable to fungal dominance generally resulting in enhanced SOC storage^{11,17}, but it remains unclear whether a similar connection can be made with regard to denitrification in agricultural soils. Thus, an objective of this study was to assess the relative contribution of fungi and bacteria to denitrification in PT and NT soils (both medium- and long-term). It was hypothesized that, as the soil microbial community becomes fungal-dominated with longer duration of NT, there will be a parallel increase in the relative contribution of fungi to denitrification in agricultural soils.

To examine the role of fungi and bacteria in soil processes, different approaches have been adopted, but the selective inhibition (SI) has been the most widely-used technique.^{13,19} The technique was first introduced by Anderson and Domsch²⁰ and was then modified for application to agricultural and forest soils.²¹ Different bactericides (e.g. streptomycin sulphate, bronopol and oxytetracycline) and fungicides (e.g. captan, cycloheximide, ketoconazole, benomyl and nystatin) have been employed in past studies^{13,17,22,23,24}, including studies investigating denitrification in soils and sediments.^{13, 25,26, 27,28} In these studies, different types of biocides were used and at different application rates. Inhibition efficiency has been variable, and after analysis of published results, it has not been possible to identify the type and the optimum concentration of biocide that is

most inhibitory to denitrification in agricultural soils. Therefore, in this study, a standardized method was developed and applied to several US Midwest soils to determine the relative contribution of fungi and bacteria to denitrification.

Materials and methods

Soil samples collection

This study was conducted with soil samples (0-10 cm) collected from a farmer's fields in Indiana (39°51' 49"N, 86°21'31"W) and from experimental plots in Ohio (39°51' 48"N, 83°40'20"W) (USA). Management practices included conventional tillage (PT₁), long-term no-till (NT₁, 50 years) at the Ohio plots, and included conventional tillage (PT₂) and medium-term no-till (NT₂, 11 years) at the farmer's fields in Indiana. Soil samples were also collected from a nearby deciduous forest (woodlot, WL), serving as a local relatively undisturbed site for comparison. The Ohio plots are under continuous corn (*Zea mays*, L.), and typically receive 16 kg N ha⁻¹ at planting and 184 kg N ha⁻¹ as anhydrous NH₃ (side-dress). The farmer's fields in Indiana are under corn-soybean (*Glycine max*, L.) rotation. During the corn year, the fields receive 5-10 kg N ha⁻¹ at planting plus 150-180 kg N ha⁻¹ as anhydrous NH₃. No N fertilizer is applied during the soybean crop. At the sampling sites, soils are classified as Crosby (aeric Epiaqualfs) and Brookston (typic Argiaquolls) developed from glacial till. Soil samples were transported to the laboratory in plastic bags, sieved (2 mm) and kept in a refrigerator (4 °C) until used in the experiments described below. A portion of each soil sample was air-dried and used for determination of chemical properties (Table 1).

Selection of fungal and bacterial denitrification inhibitors

The selective inhibition (SI) technique²¹ was used with adaptation. First, a series of assays was conducted to identify the biocides (bactericide and fungicide), and application rates that yield maximum inhibition of denitrification. The tested biocides included some of the compounds most commonly used in past studies as well as some novel products. This evaluation was conducted using soil samples collected from the NT1 site (Table 1).

Field-moist (0.16 ± 0.02 g water g⁻¹ soil) soil samples were left overnight at room temperature (22 °C) in the laboratory for acclimation. Then, 10 g of soil subsamples were placed in serum bottles (250 mL) and amended with 1.44 ml of denitrification enzyme activity (DEA) media (100 mg NO₃-N kg⁻¹, and 40 mg dextrose-C kg⁻¹). Bottles were divided into three groups with one group receiving no treatment (control) and the other two groups treated either with a bactericide or a fungicide. The bactericides evaluated in this study were streptomycin sulfate (C₄₂H₇₈N₁₄O₂₄·3H₂SO₄, CAS#3810-74-0) and bronopol (C₃H₆BrNO₄, CAS# 52-51-7) obtained from Fisher Scientific. The fungicides used in this study included cycloheximide (C₁₅H₂₃NO₄, CAS#66-81-9) and captan (C₉H₈Cl₃NO₂S, CAS#000133-06-2) also from Fisher Scientific. Biocides, received in dry powder formulation, were used to prepare biocide solutions. Biocides were applied at different concentrations (1, 2, 4, 8 and 16 mg g⁻¹ soil), and the final volume of suspension (DEA media and dissolved biocide) in each serum bottle was adjusted with deionized water as needed to reach a final volume of 20 mL. Each treatment was applied in triplicate.

Serum bottles were stoppered, shaken vigorously to make a slurry, then successively evacuated and flushed with ultra-high purity (UHP) N₂ at least 3 times, and

finally injected with acetylene (C_2H_2) for a partial pressure of 10 kPa C_2H_2 to stop the conversion of N_2O to N_2 .¹³ Serum bottles were incubated at 25 °C. Gas samples were taken from bottles headspace after 3, 8, 24 and 48 h of incubation, and stored in evacuated glass vials to determine N_2O concentration. Based on the results of this first SI test, additional assays were conducted using only the two most effective inhibitors, but increasing their application rate to 32 mg g^{-1} soil to determine if more pronounced inhibition can be achieved at higher application rates.

Assessing fungal and bacterial denitrification in plowed and no-till soils

Based on the previous results, the SI technique was applied to different soils (PT1, NT1, and WL from Ohio; PT2 and NT2 from Indiana; Table 1) to determine the relative contribution of fungal and bacterial microflora to denitrification. Field moist (10 g) soil subsamples were placed in serum bottles and amended with DEA media as described before. The following biocide treatments were applied: control (no biocide), bactericide (bronopol, 16 mg g^{-1} soil), fungicide (captan, 16 mg g^{-1} soil) and BroCap (mixture of bronopol and captan, each at 16 mg g^{-1} soil). Each treatment was applied in triplicate. The final volume (DEA media and dissolved biocide) of solution in each serum bottle was 20 mL. Serum bottles were evacuated, flushed with UHP N_2 , injected with C_2H_2 (10 kPa) as previously described. Bottles were incubated at 25 °C, and gas samples were taken from the headspace after 3, 8, 24, 48, 72, 96, 120, 144 and 168 h of incubation for determination of N_2O and CO_2 concentration.

Analytical methods

Soil pH was measured using a soil suspension (1:2 soil to water) and an Accumet-25 pH/ion meter. Particle size analysis was conducted using the hydrometer method, with sodium hexametaphosphate ($\text{Na}_6\text{P}_6\text{O}_{18}$, 5%) as a dispersing agent. Finely-ground (150 μm) soil samples were analyzed for total carbon and nitrogen using a Vario-Cube analyzer (Elementar Americas, Mt Laurel, NJ). Concentration of N_2O and CO_2 in gas was measured using a Varian CP-3800 gas chromatograph (Palo Alto, CA) interfaced with a Combipal headspace auto-sampler (CTC Analytics) and equipped with a thermal conductivity detector (CO_2 detection) and an electron capture detector (N_2O detection) in parallel. The stationary phase consisted of a pre-column (length: 0.3 m; i.d.: 2 mm) and an analytical column (length: 1.8 m; i.d.: 2 mm) filled with Porapak Q (80-100 mesh). Operating conditions of the gas chromatograph were as follows: carrier gas (UHP He at 20 mL min^{-1} for CO_2 , and UHP N_2 at 60 ml min^{-1} for N_2O), oven temperature (90 $^\circ\text{C}$), detector temperature (TCD at 150 $^\circ\text{C}$, and ECD at 300 $^\circ\text{C}$). The gas chromatograph was calibrated using certified CO_2 and N_2O standards obtained from Matheson Tri-Gas.

Computations

The percentage (%) inhibition caused by a biocide was computed through comparison of gas production in biocide-treated bottles with the corresponding control (same soil) using the equation:

$$\text{Inhibition \%} = (X_i - X_{\text{Control}}/X_{\text{Control}}) \times 100$$

Where, X_i and X_{Control} represent the amount of N_2O (or CO_2) produced during the incubation in biocide-treated bottles and control, respectively. Similar to the

computational procedure adopted in several past studies^{22,29,30} the fungi to bacteria (F:B) ratio was calculated based on CO₂ and N₂O production in the control relative to gaseous production in soils treated with biocide. The ratio was calculated as:

$$F:B = (A - B)/(A - C)$$

Where, A= respiration measured (as cumulative CO₂ concentration evolved) in the absence of inhibitors; B= respiration in the presence of the fungicide; and C= respiration in the presence of the bactericide. Since some biocides can affect non-target microorganisms, an inhibitor additivity ratio (IAR) was calculated to account for synergistic and antagonistic effects:

$$IAR = [(A - B) + (A - C)/(A - D)]$$

Where, A, B and C are cumulative CO₂ concentrations as described above, and D=respiration in the presence of both biocides (fungicide and bactericide).^{13,31} An IAR of 1 indicates no overlap in the antibiotic action on non-target organisms, and no antagonistic effect of one antibiotic on the other. An overlap is identified by an IAR>1 and an antagonistic effect by an IAR<1.¹³

Statistical analyses

Data were first tested for normal distribution using the normality test available in the Sigma Plot software (Systat, San Jose, CA). Since most of the data were not normally distributed, Kruskal-Wallis test was used to determine the significance of the experimental factors (soil type, biocide type and application rate) on N₂O and CO₂ production. The Kruskal-Wallis test was followed by Mann-Whitney pairwise test when a significant difference was detected. Unless otherwise noted, statistical significance in this

study was determined at $\alpha=0.05$. Statistical tests were conducted using PAST software (ver. 2.17c) downloaded from <http://nhm2.uio.no/norlex/past/download.html> (University of Oslo).

Results

Selection of optimum biocides and inhibitory concentrations

The production of N₂O was observed in all treatments, but the rate of production varied significantly depending on the treatment (Figs. 1 and 2). Regardless of application rate, no inhibition of N₂O production was observed with the bactericide streptomycin. Instead, streptomycin addition resulted in a slight stimulation of N₂O production (Fig. 1a). In contrast, bronopol, the other bactericide used in this study, decreased N₂O production at all application levels, with the most inhibitory effect observed at an application rate of 16 mg g⁻¹ soil (Fig. 1b). With regard to the fungicides, cycloheximide (Fig. 2a) was a less effective inhibitor of denitrification compared to captan (Fig. 2b). With both fungicides, the strongest inhibition was observed at the 16 mg g⁻¹ soil application rate. Overall, the cumulative amount of N₂O produced during the incubation was significantly ($P < 0.05$) lower with the bactericide bronopol and the fungicide captan (both at 16 mg g⁻¹) compared to the control (Figs. 1b, 2b).

Since the most inhibitory effect of bronopol and captan was observed at the highest biocide application rate (16 mg g⁻¹ soil) used in the initial assays, additional tests were conducted by extending biocide application to 32 mg g⁻¹ soil to determine whether a higher degree of inhibition can be achieved. Incubation was conducted with the same NT₁ soil amended with bronopol or captan (32 mg g⁻¹ soil). Gas production was monitored

during a 72-h period. Results showed that both biocides decreased N₂O production compared to controls, but the cumulative amount of N₂O produced during the incubation was statistically similar ($P > 0.05$) in bottles treated with 16 and 32 mg g⁻¹ soil (Fig. 3). Since the two highest application rates (16 mg g⁻¹ and 32 mg g⁻¹ soil) of bronopol and captan produced the same degree of inhibition in N₂O production (Fig. 3), the lower level (16 mg g⁻¹ soil) was used in subsequent tests to determine the relative contribution of bacterial vs fungal denitrifiers to N₂O production in agricultural soils.

Degree of inhibition of denitrification in different soils

Although some minor deviations were noted in the NT₁ soil, N₂O evolution was generally linear during the incubation period. As expected, N₂O concentration was highest in the control followed by the captan-treated soils (Fig. 4). The addition of captan marginally affected N₂O production in the PT₂ and NT₂ soils (Fig. 4d-e), but resulted in noticeable N₂O production reduction in the PT₁, NT₁ and WL soils (Fig. 4a-c). At all sampling times, both bronopol and BroCap treatments resulted in the highest inhibition in N₂O production and, in all the soils investigated, cumulative N₂O concentrations in these treatments were significantly ($P < 0.05$) lower than in controls.

Across treatments, addition of the bactericide bronopol resulted in 85±7 % inhibition of N₂O production. A similar level of inhibition (84.7±4.7 %) was measured when bronopol was applied concurrently with the fungicide captan. In contrast, a smaller and more variable (36±21 %) degree of inhibition was measured with the fungicide captan, applied alone (Fig. 5). The highest N₂O inhibition with captan was recorded in the NT₁ soil (NT for 50 y).

Respiratory response of soils to biocide treatments

A steady accumulation of CO₂ was observed during the 7-day incubation period in almost all treatments (Fig. 6). As expected, CO₂ production was higher in the controls than in the biocide-treated soils, although in the PT₂ soil difference was only marginal (Fig. 6d). Among the control, the highest rate of CO₂ accumulation was recorded in the NT₁ soil (NT for 50 years) and the lowest in both PT soils (Fig. 6). The effect of biocides application on CO₂ production was more variable and less pronounced than observed with N₂O production. Like with N₂O production, addition of the bactericide bronopol resulted in greater respiration inhibition than the other treatments (51.4±13.3 % in bronopol treatments vs 31±11.6 % in BroCap and captan treatments; Fig.7). A positive relationship (r^2 : 0.41, $P < 0.01$) was found between % inhibition of N₂O production and % inhibition of CO₂ production.

Fungi:bacteria ratio

Fungi:bacteria ratios (F:B) were calculated using both the reduction in CO₂ and N₂O production in biocide-treated soils relative to the controls. For all the soil and biocide treatment combinations, F:B values were < 1, suggesting that bacteria were the dominant group of microorganisms responsible for CO₂ and N₂O production in the soils tested (Table 2). The highest and lowest F:B values were observed in the NT₁ and PT₂ soils, respectively.

Inhibitor additivity ratio

Data from the treatments involving the combination of bronopol and captan (both at 16 mg g⁻¹ soil; BroCap) was used to calculate IAR. Results showed that the IAR was >1 in all the soils tested, indicating a synergistic effect of the applied biocides (Table 3). In general, IAR values tended to be the highest in the NT and lowest in the PT soils.

Discussion

Biocides efficiency in inhibiting bacterial and fungal activity

The concentration of biocides used in past selective inhibition assays varies greatly, with optimum concentration reported in the literature ranging between 1 and 16 mg biocide g⁻¹ soil.^{2,13,17,22,23,32} The optimum concentration (16 mg biocide g⁻¹ soil) found in the present study was in the upper end of that range. It has been suggested that high soil clay content can reduce the efficiency of biocides, and that higher concentrations are needed to obtain significant reduction in respiratory activity.²² Given the fine texture of the soils used in this investigation (Table 1), this reasoning would be consistent with the results.

Therefore, as done in the present study, preliminary tests must be first conducted to determine optimum concentration of inhibitors for each new set of soils under investigation.

In this study, different types and levels of biocides were used to find the most effective products against denitrification and respiratory activity mediated by bacteria and fungi in agricultural soils. These products include inhibitors that were tested in some of the pioneering work to develop the selective inhibition procedure²⁰ as well as some inhibitors introduced more recently in the literature to distinguish fungal and bacterial

activity.^{13,17,23,24, 33} Streptomycin, the bactericide traditionally used in SI procedure, surprisingly showed almost no inhibitory effect on the activity of bacterial denitrifiers (Fig. 1a). This result was somewhat unexpected given the large number of past investigations in which streptomycin was used as a bactericide.^{18,22,31,34} However, this result is in agreement with several past studies that have documented instances of inefficient inhibition of bacterial growth by streptomycin.^{2,35} Boyle *et al.*³³ also reported that streptomycin was a much less effective bactericide than bronopol and oxytetracycline-HCl in controlling bacterial activity. Identifying the factors contributing to streptomycin inefficiency remains a challenge, but soil redox condition is likely not a contributing factor since streptomycin inefficiency has been reported in studies using both water-saturated and unsaturated soils.²⁶

Cycloheximide was another product that exhibited surprisingly low biocidal effect. In fact, cycloheximide was the least effective of the biocides examined, and resulted in slightly higher N₂O production compared to control (Fig. 2a). These results contrast with those of other studies in which cycloheximide was found to inhibit fungal activity even at low concentrations (e.g. 1-2 mg g⁻¹ soil).^{2,18} It has not been possible to find information in the literature to satisfactorily explain the inefficiency of cycloheximide observed in the present study. Overall, these mixed results with popular products such as streptomycin and cycloheximide underscore the need for experimentalists to first evaluate the biocides they plan to use in SI studies.

Bronopol (bactericide) and captan (fungicides) were found to be effective inhibitors of N₂O production in this study. Several investigators have also successfully applied these products in past studies examining bacterial and fungal contribution to N₂O

production and respiration^{13,17,24}. Although bronopol and captan were effective inhibitors of N₂O production, some level of microbial activity was still maintained, as manifested by the slow accumulation over time of N₂O and CO₂ in the incubation bottles (Figs. 4 and 6). Even in the presence of both bactericide and fungicide (BroCap treatments) some gaseous production was observed. Similar observations were reported in selective inhibition studies involving desert, prairie, forest and agricultural soils^{17,18}. This residual N₂O and CO₂ production in biocide-treated soils can be ascribed to the activity of surviving decomposers at the expense of metabolizable C and N released from dead microbes in the biocide-treated soils. Moreover, it needs to be noted that denitrifiers are only a small fraction (~ 5%) of the total soil microbial community and, therefore other microorganisms remain active even when N₂O producers are inhibited.³⁶ This line of reasoning is supported by the generally lower (1.8 times) rate of CO₂ inhibition compared to that of N₂O (Figs. 5 and 7).

Bacteria and fungi contribution to N₂O production in agricultural soils

With all the soils tested in this study, the inhibition of N₂O production was consistently stronger with addition of the bactericide bronopol than with the fungicide captan (Fig. 4). These results suggest that bacteria were the main group of microorganisms contributing to denitrification in the soils investigated. The F:B ratios (Table 2) further supports this statement. A study by Herold *et al.*² using arable soils also reported similar results, with fungi and bacteria contributing 18% and 54% respectively of the total N₂O production. In contrast, data from Laughlin *et al.*²⁶ showed a fungal dominance in N₂O production in well-aerated grassland soils. The results of Seo and DeLaune³¹ suggested that bacteria

were the primary drivers of denitrification under strongly-reducing conditions, whereas fungi play a greater role under aerobic and moderately-reducing conditions. Results of the present study also contrasted with those of Chen *et al.*²⁸ that documented higher fungal contribution than bacteria to N₂O production in soils from various ecosystems. It is unclear that the pH (5.3-6.5 vs 5.5-7.4; Table 1) and texture (sandy vs clay-loam) of the soils evaluated by Chen *et al.*²⁸, the antibiotics used (streptomycin/cycloheximide vs bronopol/captan in the present study) and their incubation method (< 90% water-filled pore space vs fully anoxic in the present study) may contribute to these contrasting results. Therefore, in light of these considerations and the variety of methodologies adopted in past studies, it is prudent to caution against generalization at this point. Therefore, future studies should examine these factors and, most importantly, the effect of redox status on the partitioning of N₂O production between fungi and bacteria in agricultural soils.

Tillage practices and N₂O production partitioning between soil bacteria and fungi

Under NT management, soils are less disturbed in comparison to PT and are generally covered with crop residue cover. This contributes to higher moisture content in NT, a soil environment that is likely favorable to the proliferation of denitrifying microbes.^{37,38} In addition, fungi are more likely to succeed in soil systems that are left undisturbed, allowing for the development of fungal hyphae which are in contact with crop residue on the land surface.¹¹ In contrast, because of soil mixing and the direct contact between decomposers and substrates in PT soil systems, the microbial community is generally dominated by bacteria.¹⁷

Despite its wide use in soil biochemistry research, the SI method has inherent shortcomings. The IAR ratios provide a way to assess the validity of F:B ratios derived from the method. IAR values close to 1 are usually taken as an indication of the accuracy of F:B estimations.³¹ Values for IAR found in this study are in the same range as those reported in several past investigations.^{13,35} These deviations have generally been ascribed to inhibition of non-target microorganisms by the biocides applied.¹⁷

The F:B ratios for N₂O production were less than 1, indicating the dominance of bacteria as N₂O producers in the soils tested. This conclusion is at variance with the hypothesis of the study regarding fungal dominance of NT soils. The N₂O inhibition data (Fig. 5) showed that, among the soils tested, the fungicide captan induced its highest level (66%) of denitrification inhibition in the NT₁ soil (50 y under NT). In comparison, the inhibition measured in the mid-term (11 y) NT soil was only 18 % (Fig. 5). Similarly, the highest F:B ratios (computed using either CO₂ or N₂O production data) were measured in the NT₁ soil (Table 2), although difference was not significant. This trend could indicate the evolution of a larger population of fungal denitrifiers in agricultural soils with longer duration of no-till management (e.g. NT₁). Chronosequence studies, using soils under NT for varying length of time, are needed to test the merit of that suggestion.

Conclusions

One of the objectives of this study was to identify the types and levels of biocide leading to optimum inhibition of denitrification in agricultural soils. Streptomycin and cycloheximide, the biocides most commonly used in selective inhibition assays, hardly resulted in any inhibition of N₂O production. In fact, in some of the soils, streptomycin

stimulated the process. The reason for this lack of inhibition remains to be elucidated. Captan and bronopol, however, inhibited N₂O and CO₂ production, with an optimum concentration of 16 mg g⁻¹ for both biocides. Fungi:bacteria ratios smaller than 1 were measured in all the soils tested, suggesting that bacteria were the dominant N₂O producers in the soils investigated. Although the difference was not significant, this ratio was highest in the long-term NT soil (NT₁), suggesting a progressively greater role for fungal denitrifiers in the denitrification process with longer NT duration.

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Table 1 Chemical properties of soils (0-10 cm) used in the study

Location of sampling sites	Tillage practice	pH	Total C (g C kg ⁻¹ soil)	Total N (g N kg ⁻¹ soil)	Soil texture
S. Charleston (Ohio)	PT ₁	7.16	12.6±1.00	1.5±0.23	Silt clay loam
	NT ₁	6.14	21.2±0.20	2.0±0.50	Silt clay loam
	WL	5.54	31.4±1.20	2.5±0.31	Silt loam
Starkey farms (Indiana)	PT ₂	6.41	13.3±2.3	1.9±0.60	Silt loam
	NT ₂	7.42	18.5±1.6	1.7±0.20	Silt loam

Table 2 Fungi:bacteria ratio (F:B) based on the cumulative CO₂ and N₂O concentration during a 168-h incubation of soils treated with either bronopol (bactericide) or captan (fungicide) applied at a rate of 16 mg g⁻¹ soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison

Soil type	F:B	F:B
	(based on CO ₂ concentration)	(based on N ₂ O concentration)
PT ₁	0.50±0.21	0.50±0.13
NT ₁	0.67±0.31	0.87±0.18
WL	0.45±0.18	0.46±0.24
PT ₂	0.43±0.22	0.24±0.09
NT ₂	0.49±0.15	0.22±0.11

Table 3 Inhibitor additivity ratio (IAR) based on the cumulative CO₂ concentration during a 168-h incubation of soils treated with either bronopol (bactericide) or captan (fungicide) applied at a rate of 16 mg g⁻¹ soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison

Soil type	IAR
PT ₁	1.02±0.18
NT ₁	1.80±0.51
WL	1.46±0.11
PT ₂	1.22±0.35
NT ₂	1.71±0.24

Fig. 1 Nitrous oxide production in control and soils treated with different concentrations of bactericide, (a) control vs. streptomycin, (b) control vs. bronopol. Error bars represent SD from a mean of three replicates.

Fig. 2 Nitrous oxide production in control and soils treated with different concentrations of fungicide, (a) control vs. cycloheximide, (b) control vs. captan. Error bars represent SD from a mean of three replicates.

Fig. 3 Nitrous oxide production in control and soils treated with different concentrations of biocides, (a) control vs. bronopol, (b) control vs. captan. Error bars represent SD from a mean of three replicates.

Fig. 4 Nitrous oxide production in control and soils treated with either bronopol (bactericide), captan (fungicide) or their mixture (BroCap). Biocide was applied at a rate of 16 mg g^{-1} soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison. Data are presented in the following graph panels: (a) PT₁, (b) NT₁ (50 years), (c) WL, (d) PT₂, and (e) NT₂ (11 years). Error bars represent SD from a mean of three replicates.

Fig. 5 Percent inhibition (%) of nitrous oxide production in soils treated with either bronopol (bactericide), captan (fungicide) or their mixture (BroCap). Biocide was applied at a rate of 16 mg g^{-1} soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison. Data are presented in the following graph panels: (a) PT₁, (b) NT₁ (50 years), (c) WL, (d) PT₂, and (e) NT₂ (11 years). Within a biocide treatment, bars are labelled with different letters to indicate a significant difference between tillage practices. Error bars represent SD from a mean of three replicates.

Fig. 6 Carbon dioxide production in control and soils treated with either bronopol (bactericide), captan (fungicide) or their mixture (BroCap). Biocide was applied at a rate of 16 mg g^{-1} soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison. Data are presented in the following graph panels: (a) PT₁, (b) NT₁ (50 years), (c) WL, (d) PT₂, and (e) NT₂ (11 years). Error bars represent SD from a mean of three replicates.

Fig. 7 Percent inhibition (%) of carbon dioxide production in soils treated with either bronopol (bactericide), captan (fungicide) or their mixture (BroCap). Biocide was applied at a rate of 16 mg g^{-1} soil. Soils used in these assays were from sites under plow-till (PT₁ and PT₂), long-term (50 years, NT₁), and medium-term no-till (11 years, NT₂). Soils from a woodlot (WL) were also incubated for comparison.

Data are presented in the following graph panels: (a) PT₁, (b) NT₁ (50 years), (c) WL, (d) PT₂, and (e) NT₂ (11 years). Within a biocide treatment, bars are labelled with different letters to indicate a significant difference between tillage practices. Error bars represent SD from a mean of three replicates.