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Mechanisms of N_2O production following chloropicrin fumigation

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Abstract

Soil fumigation has recently been shown to affect the greenhouse gas balance by increasing emissions of nitrous oxide (N_2O) following chloropicrin (CP) application. However, the exact mechanisms of this increase were not investigated. The purpose of this study was to elucidate potential mechanisms of CP-induced N_2O production through laboratory incubations using chemical inhibitors (acetylene, antibacterial, antifungal, and oxygen), isotopically labeled ^{15}N-CP, and pH modifications of a forest nursery soil. Results showed that N_2O production increased by 12.6 times following CP fumigation. Microbial activity contributed 82% to the CP-induced N_2O production, with the remaining 18% from abiotic processes as determined by incubation with sterilized soil. Inhibitor studies suggested that 20% of the N_2O production was from bacteria and 70% from fungi. There were no significant differences in N_2O production following CP fumigation under various levels of acetylene (0, 10, and 10 kPa), suggesting that traditional nitrification and denitification reactions did not significantly contribute to N_2O production following CP fumigation. ^{15}N labeled studies indicated that 12% of fumigant source N was incorporated into the produced N_2O. No enrichment in N_2 was observed, indicating that N_2O was one of the terminal biotic mineralization products of CP. Production of N_2O is aerobic and production rates increased with increasing oxygen concentrations. Our data strongly suggested that fungal mediated denitrification reactions under aerobic conditions were the primary mechanism for CP-induced N_2O production.
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Keywords: Nitrous oxide; Soil fumigation; Fungi; Greenhouse gas

1. Introduction

Soil fumigation is an agricultural practice of using various chemicals to reduce the threat of soil borne plant pathogens, insects, and weeds for production crops. Typically, fumigation is only performed for high-value crops, such as tomatoes, strawberries, and in seedling nurseries. Methyl bromide has been the most widely used soil fumigant since the 1930's (MBGC, 1994). Due to its ozone depleting characteristics (Wofsy et al., 1975; Butler, 1995) and large volatilization losses following fumigation (Majewski

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et al., 1995; Wang et al., 1997; Williams et al., 1999), the use of methyl bromide as a soil fumigant is now prohibited in the US. Chloropicrin (CP) is one potential methyl bromide substitute due to its lack of ozone depleting properties and efficacy against a wide spectrum of soil borne diseases (Wilhelm et al., 1997; UNEP, 1998).

Soil fumigation has been documented to reduce overall microbial populations and diversity (e.g. Lebbink and Kolenbrander, 1974; Ridge, 1976; Ingham and Thies, 1996; Miller et al., 1997; Ibekwe et al., 2001; Dungan et al., 2003; De Cal et al., in press). However, other studies have observed enhanced fumigant degradation after repeated soil application (van Dijk, 1974; Smelt et al., 1989; Verhagen et al., 1996; Dungan and Yates, 2003). These enhanced degradation rates indicate that some soil biota survived the fumigation process, thereby potentially altering the dynamics of soil microbial functionality following fumigation. In addition to fumigant degradation effects, the alteration in soil greenhouse gas processes following fumigation need to be assessed. Recently, it has been reported that the use of CP increased N_2O gas production in fumigated soils (Spokas and Wang, 2003; Spokas et al., 2005). However, the mechanisms for this stimulation effect were not understood. This information is critical due to the importance in ecological function that soil microbial communities have on global greenhouse gas budgets.

The purpose of this study was to identify the soil biotic group responsible for the production of N_2O following CP fumigation as well as to investigate the potential mechanisms of this N_2O formation. These objectives were accomplished through laboratory incubations with chemical inhibitors, labeled ^{15}N-CP, and pH modified soils.

2. Materials and methods

2.1. Soil

Soil from a forest nursery in Byromville, GA (32.169° N; 83.974° W) was used in this study. The soil is a Eustis loamy sand (siliceous, thermic psammentic paleudult) with $1.86 \pm 0.01\%$ organic matter content, 69 mg N kg-1 total N, <1.2 ppm nitrate, 2.8 ppm ammonia, volumetric moisture of $9.0 \pm 0.1\%$, a pH of 5.6, and a microbial biomass of 219 ± 23 μg $C_{Biomass}$ g^{-1} following the method of Jenkinson and Powlson (1976). The GA soil is representative of forest nursery soils that are fumigated. Soil was sieved (2 mm), homogenized, and stored at 4 °C (±2 °C) for 6 weeks until incubations could be preformed.

2.2. Laboratory incubations

Incubation experiments were conducted to determine mechanisms of N_2O formation after CP fumigation, following procedures similar to Spokas et al. (2005). Soil was pre-incubated for 10 days in a humidified environment (100% relative humidity) at 22 °C before starting incubations to allow equilibration of the microbial populations after cold storage (Wu et al., 1996). Triplicate sub-samples (5 g) were placed in sterilized 125 ml serum vials (Wheaton Glass, Milville, NJ) and sealed with Teflon-lined butyl rubber septa (Agilent Technologies, Palo Alto, CA). Chloropicrin (CP; Cl_3CNO_2) (ChemService, West Chester, PA) was injected through the sealed serum vial to prevent volatilization losses. CP was added at a concentration of 65 μg g^{-1}, which was the calculated concentration based on typical field application rates. Samples were incubated for 10 days in the dark (22 ± 2 °C). Headspace O_2 concentrations in the vials were consistently above 185 ml l^{-1} at 10 days, indicating that headspace conditions were aerobic throughout the incubations. N_2O in the headspace was analyzed by a gas chromatography (GC) system described in Spokas and Wang (2003). Rates of N_2O production were calculated from changes in headspace concentration over the 10 days period on a soil dry weight basis. Incubations were repeated with chemical inhibitors (acetylene, oxygen, antibiotic, and antifungal), ^{15}N-CP, and pH modified soils. Procedures unique to each treatment are described below.

2.2.1. Chemical inhibitors

Selective inhibition procedures developed by Webster and Hopkins (1996) were used to assess the potential origins of N_2O. Acetylene was added to reach a partial pressure of 10 and 10 kPa in the serum vial's headspace before injecting CP. Acetylene inhibits ammonia monoxygenase (nitrification) activity at low concentrations (10 Pa) and at higher

concentrations inhibits both N_2O reductase (denitrification) and ammonia monoxygenase (Webster and Hopkins, 1996). Additionally, triplicate fumigant and control incubations with an enriched O_2 concentration (30% O_2, 70% N_2) were conducted, since O_2 is a selective inhibitor for anaerobic denitrification (Tiedje, 1988).

Selective inhibitors of microbial activity were used to assess the fungal and bacterial contribution to the N_2O produced (Anderson and Domsch, 1975). Tetracycline (10 mg g^{-1}; $C_{22}H_{24}N_2O_8$) and streptomycin (10 mg g^{-1}; $C_{21}H_{39}N_7O_{12}$) were used as antibiotic agents and cycloheximide (4-{(2R)-2-[(1S,3S,5S)-3,5-dimethyl-2-oxocyclohexyl]-2-hydroxyethyl}piperidine-2,6-dione; 15 mg g^{-1}) and benomyl (methyl-1-[(butylamino)carbonyl]-H-benzimidazol-2-ylcarbamate; 5 mg g^{-1}) were used as antifungal agents. These inhibitors have been used as selective indicators to verify the active role of bacteria and fungi in other xenobiotic degradation processes (e.g. Dodard et al., 2004). Lin and Brookes (1999) confirmed that selective inhibitor incubations and direct microscopy counts gave similar results for the proportions of bacteria and fungi in soils. Incubations were performed in triplicate with each of these inhibitors and CP. Inhibitors were thoroughly mixed with 100 g of soil before the 5 g sub-samples were taken, improving measurement accuracy.

2.2.2. *^{15}N chloropicrin incubations.*

^{15}N-CP (98% labeled) was used to track the potential pathways of N mineralization in soil. ^{15}N-CP was synthesized by initially dissolving 2.21 g (31.6 mmole) of ^{15}N sodium nitrite ($Na^{15}NO_2$ at 98+ at.% ^{15}N) in 50 ml of water. The solution was chilled to $<5^{\circ}$ in an ice bath. After cooling, 5.80 g of $AgNO_3$ (34.1 mmole) was dissolved in 50 ml of water and added drop-wise to the $Na^{15}NO_2$ solution with continuous stirring over 10 min as described in Rajendran et al. (1987). Stirring was continued for an additional 20 min following which the water layer was drawn off with a filter stick (glass tube with a fritted glass disc on the end). The solid material was rinsed sequentially with 50 ml each of water, ethanol, and methyl tertiary-butyl ether. The solid material was mixed with 50 ml ethanol and 3.9 ml (9.22 g, 65.0 mmole) methyl iodide (CH_3I) (Gensler and Dheer, 1981). The flask was capped and stirred for 4 days, then filtered. For the chlorination, 50 g ice was added to the mixture and then while stirring 45 ml of 5% bleach (NaOCl) solution was added (Sparks et al., 1997). The mixture was stirred for 5 min then extracted five times with 10 ml dichloromethane, with the extracts monitored by GC–MS followed by subsequent distilling in a short path apparatus to yield 220 mg of ^{15}N-CP.

Non-sterile and sterile soil was incubated with and without ^{15}N-CP treatment under aerobic conditions. These incubations were only carried out in duplicate due to the cost of the labeled compound and analyses. Samples were analyzed for ^{15}N-N_2O and ^{15}N-N_2 at the University of California Davis Stable Isotope Facility.

2.2.3. *pH Modification*

To investigate the pH dependency on CP-induced production of N_2O, soil prior to incubation was adjusted to lower and higher pH values by using 0.25 ml additions of 0.10 N HCl or 0.10 N NaOH, which altered the 1:1 distilled water pH from the original 5.6 to 2.8, and 8.5, respectively. Soil pH has been shown to affect biotic fungal/bacterial ratios (Blagodatskaya and Anderson, 1998) as well as abitoic mechanisms (Harter and Naidu, 2001).

2.3. *Statistical analysis*

Results presented are arithmetic means of triplicate analyses, and N_2O production rates are expressed on a soil dry weight basis. Data were analyzed using an analysis of variance (ANOVA) procedure for independent samples to test for statistical significance using GraphPad InStat (version 3.00, GraphPad Software, San Diego, CA). If significant differences existed among the factors, as indicated by the F-ratio, the Tukey's Honestly Significant Difference (HSD) test was performed to determine which pair-wise interactions were significantly different at the $P < 0.05$ level.

3. Results

3.1. *Acetylene block and O_2 inhibition incubations*

For the forest nursery soil, N_2O production increased 12.6 times following CP fumigation

compared to non-CP amended control in the absence of any inhibitor additions (Fig. 1A). The amount of N_2O produced with CP addition in the acetylene block incubations and CP fumigated control was not significantly different ($P > 0.05$, Fig. 1A) regardless of the amount of acetylene.

When the O_2 content in the headspace was increased to 30%, N_2O production increased to 9.35 μg N_2O-N kg^{-1} h^{-1} following CP injection. This increased represented a 134-fold increase over the ambient O_2 control (no CP) and a 235-fold increase in N_2O production following CP treatment compared to the non-fumigated 30% O_2 control. The increased O_2 content also caused a 5.4-fold increase compared to the ambient O_2 CP-fumigated treatment (Fig. 1A).

3.2. Antibiotic and antifungal incubations

Streptomycin significantly reduced CP-induced N_2O production compared to the control (Fig. 1B). Lack of statistical significance of the tetracycline reduction could be a direct result of the prevalence of bacteria resistant to tetracycline in the environment (Trevors, 1987; Agersø et al., 2004). On average, the antibacterial inhibitors reduced N_2O production by 20% as compared to equivalent controls with no antibacterial agents. This indicates that 20% of the N_2O production results from biota sensitive to these antibacterial agents.

The antifungal compound, benomyl, reduced N_2O production by 80% and cycloheximide reduced N_2O production by 70% (Fig. 1B). This suppression was

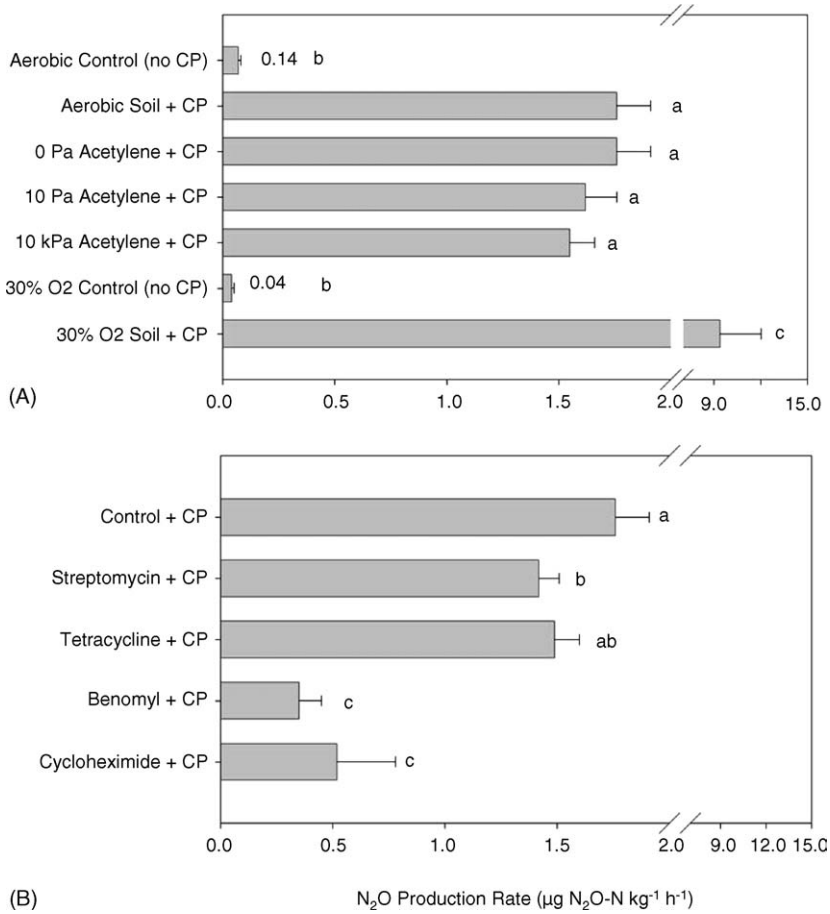

Fig. 1. N_2O production rates from (A) acetylene and O_2 inhibitor incubations and (B) antibacterial and antifungal inhibitor incubations. Data presented are averages of measurements ($n = 3$) with the bars representing one standard deviation. Different letters indicate significant differences in the treatments ($P < 0.05$).

greater than that observed with the antibacterial inhibitors. Jakobsen (1994) has shown that benomyl acts on additional soil organisms besides fungi in laboratory studies, and therefore could artificially bias the results for more than just fungal communities. However, this reduction in soil biota has not been universally established, as shown by Hart and Brookes (1996).

3.3. ^{15}N chloropicrin incubations

Incubations with ^{15}N-CP clearly showed that a significant amount of ^{15}N was present in the produced N_2O (Fig. 2A). There was a significant difference between the percentage of ^{15}N in N_2O at 10 days resulting from both sterile and non-sterile ^{15}N-CP incubations compared to controls. Even though the soil was steam sterilized for three cycles, each 45 min with 24 h intervals, there could be residual biomass that survived. Since conclusive data do not exist, it was assumed that the production observed in the steam sterilized soil was solely attributed to abiotic mechanisms which accounted for 18% of the non-sterile production. Additional N sources were involved, since the percent of ^{15}N excess in N_2O was only 19%. This would indicate that 81% of the N in N_2O produced was from other pools and not directly from the mineralization of the fumigant. From mass balance calculations, there was 27.7 μg N added to the soil incubation from CP treatment. N_2O production accounts for 17.0 μg N (1.2 μmoles N) and at 19% ^{15}N excess corresponds to a total of 3.2 μg ^{15}N. Coupling these calculations with the initial injected ^{15}N indicates that approximately 12% of the N from the CP was incorporated into N_2O at 10 days.

Distribution of the isotopic composition of the N_2 remained remarkably unchanged following fumigation (Fig. 2B). There was no significant enrichment in N_2 from ^{15}N-CP incubations. This indicates that there was no direct formation of N_2 from ^{15}N-CP. Coincidentally, the lack of enriched to N_2 also indicates that there was no significant reduction of the enriched N_2O ($\delta^{15}N \approx 64000$ for the ^{15}N-CP incubations) to N_2 during the incubation.

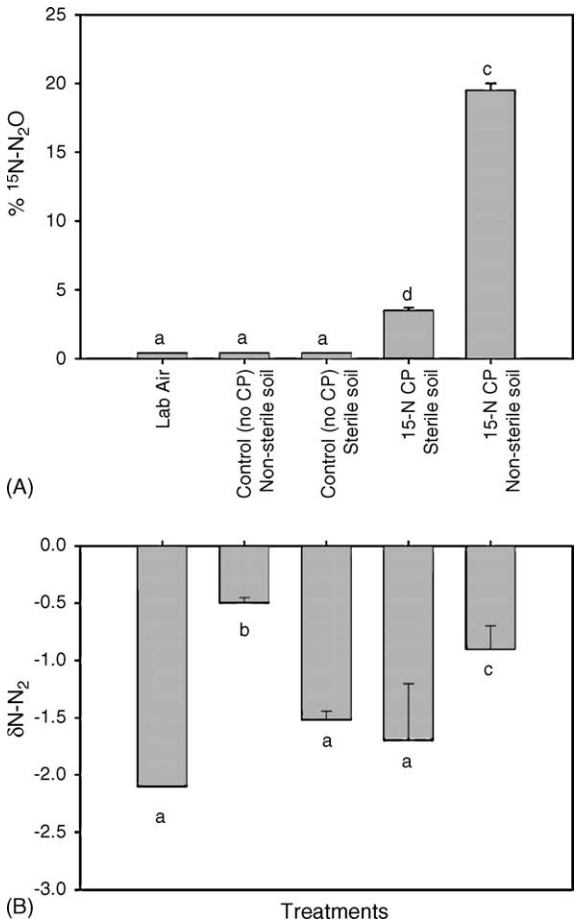

Fig. 2. Isotopic ^{15}N in (A) N_2O and (B) N_2 measured after 10 days incubation after injection of ^{15}N-labeled chloropicrin (CP). Data presented are averages of measurements ($n = 2$) with the bars representing one standard deviation. Different letters indicate significant differences in the treatments ($P < 0.05$).

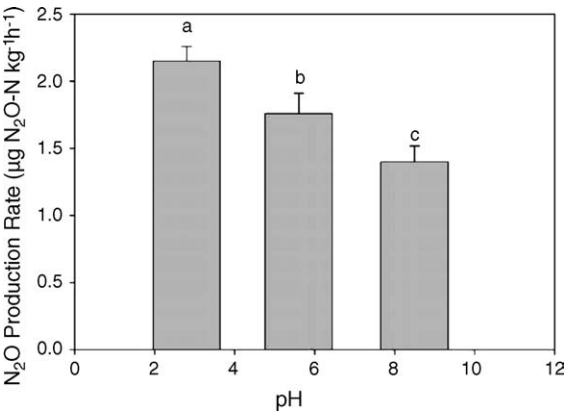

Fig. 3. N_2O production rates as a function of soil pH. Data presented are averages of measurements ($n = 3$) with the bars representing one standard deviation. Different letters indicate significant differences in the treatments ($P < 0.05$).

3.4. pH Adjustment incubations

There was a direct relationship found between pH and observed N_2O production rates ($r^2 = 0.999$; $P = 0.02$). Production rates at pH values of 2.8, 5.6, and 8.5 were 2.15 ± 0.11, 1.76 ± 0.15, and 1.40 ± 0.12, respectively (Fig. 3). Lower pH values were observed to favor N_2O production in these soil incubations.

4. Discussion

Theoretically, acetylene (>10 Pa) inhibits ammonia monoxygenase activity which is an enzyme transforming NH_3 to NH_2OH (Bollmann and Conrad, 1997). Our results suggest that this oxidation step does not play an active role in the production of N_2O from CP. This agrees with field observations that have measured 10-fold increases in soil ammonia levels following CP fumigation (Winfree and Cox, 1958). Denitrification is typically estimated from the difference in N_2O in the headspace between incubations containing 10 kPa and 10 Pa acetylene (Knowles, 1982). However, these differences were not significant in this study. A limitation with the acetylene block is that it can only differentiate between *pure* nitrification and denitrification, and can not distinguish variants like nitrifier denitrification, chemodenitrification, or heterotrophic nitrification (Robertson and Tiedje, 1987; Wrage et al., 2004). The general conclusion from the acetylene block experiments is that traditional anaerobic denitrification does not significantly contribute to N_2O production. We observed increased N_2O production with increasing O_2 concentrations and there was no increased N_2O production at 10 kPa acetylene (Fig. 1A). The 30% O_2 incubation indicates the importance of O_2 in the process, and also further supports the lack of N_2O reductase activity since O_2 would inhibit its functionality and synthesis (Tiedje, 1988). Incidentally, Shoun et al. (1992) reported fungi species to be lacking N_2O reductase and this could attribute to the reason for no significant differences observed in 10 kPa acetylene incubations. However, non-traditional denitrification mechanisms (e.g. aerobic denitirifaction) can not be excluded, since inhibitors for these pathways are not available (Wrage et al., 2004).

Net result of the selective biomass inhibitors suggested that fungi were the predominant biota responsible for N_2O production following CP fumigation in this particular soil. In the investigated soil, the fungal consortium responsible for the majority of N_2O production appears to be CP-tolerant. The pH dependency of the observed N_2O production also agrees with fungi being the dominant biota, since lower pH values also favors their activity (Atlas and Bartha, 1998). These results are surprising in light of the fact that CP is a very effective anti-fungal agent for pathogenic fungi (Maas, 1984), but CP's effect on non-pathogenic species has not been well studied. Fungi can dominate the microbial biomass in soil (Ruzicka et al., 2000; Laughlin and Stevens, 2002) and significantly contribute to the production of N_2O both in grassland (Laughlin and Stevens, 2002) and woodland soils (Laverman et al., 2000).

N_2O sources are not limited to microbial nitrification and denitrification reactions. Abiotic pathways have been identified for the production of N_2O from HNO_2 in sterile soils (Venterea and Rolston, 2000). The increased production with reduced pH observed here (Fig. 3) is consistent with these previously observed abiotic mechanisms which require sufficient acidity to form HNO_2. No conclusive data exist to support these abiotic pathways in this soil. From the sterile soil incubation, a maximum of 18% of N_2O production could be attributed to completely abiotic mechanisms. In the non-sterile soils, there could have been additional contribution from abiotic pathways after an initial biotic degradation step (e.g. dechlorinaton of CP; Fig. 4), which would not have been accounted for in the steam sterilized incubation. However, steam sterilization can result in increased concentrations of reduced forms of ions, particularly Fe and Mn (Lopes and Wollum, 1976), which is important to remember particularly since Fe(II) has been shown to be involved in abiotic transformations of CP (Cervini-Silva et al., 2000). This potentially could have artificially biased the estimated abiotic contribution in this study.

There is sufficient evidence to conclude that a biotic component is involved in the production of NO_2^- (Castro et al., 1983). This NO_2^- could form HNO_2 under acidic conditions leading to abiotic N_2O. We feel that if this NO_2^- was abiotically converted to N_2O the ^{15}N labeled percentage should have been at least 50%,

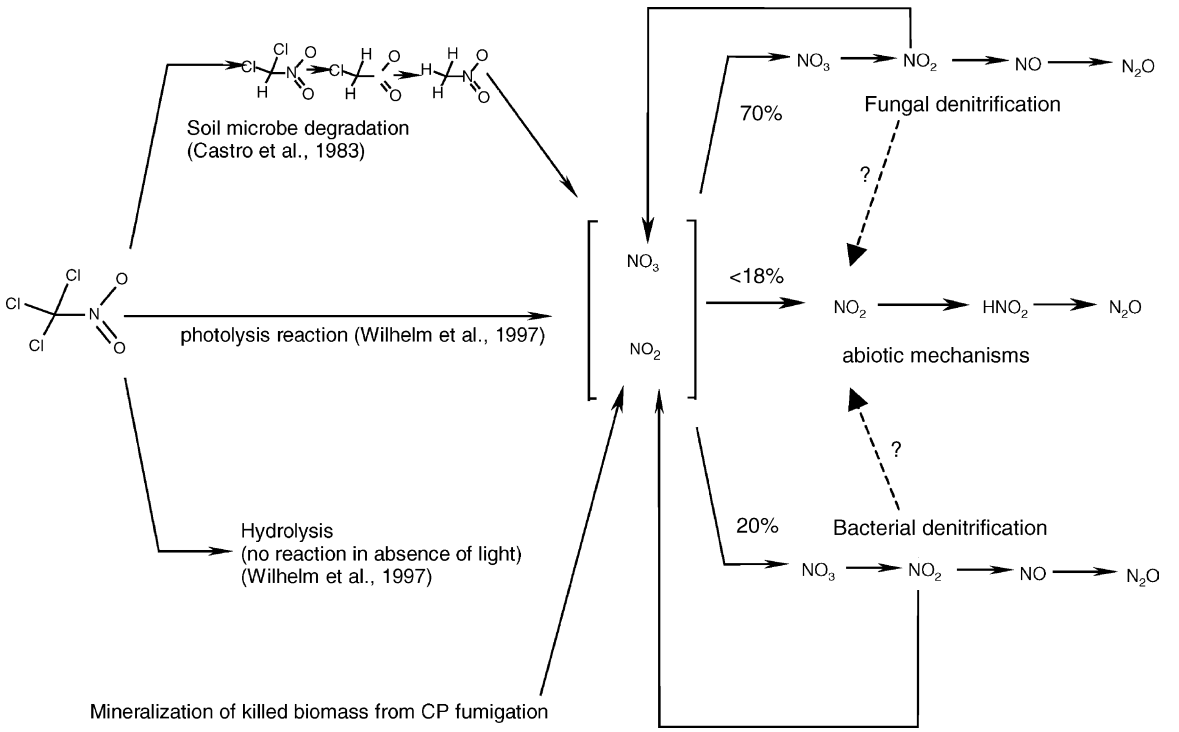

Fig. 4. Summary of potential mechanisms for N_2O formation following chloropicrin fumigation. Contributions of the pathways were elucidated through the use of antibacterial and antifungal inhibitors. Fungal and bacterial pathways may include an undetermined abiotic contribution as indicated by the dashed arrows. Inhibitors of abiotic processes are not available.

suggesting other concurrent mechanisms. The observation that there still was significant N_2O production under basic conditions casts doubt on the idea that abiotic pathways involving acidic intermediates are the sole reason for the N_2O production. However, abiotic reactions cannot be completely excluded as potential sources of N_2O under basic conditions.

5. Conclusions

Based on the results from these incubation studies we hypothesize that the production of N_2O following CP fumigation is predominantly due to microbial activity, chiefly involving fungi (Fig. 4). Observations have revealed that fungi can play a dominant role in the N_2O production in non-fumigated soils (e.g. Laughlin and Stevens, 2002). Our data suggests that the N_2O producing fungi are resistant to CP, but sensitive to other fumigants and fungicides. This conclusion is consistent with previous results indicating that the mixtures of CP and methyl isothiocyanate, which is another soil fumigant, reduced N_2O production by 83% compared to sole application of CP in laboratory incubations (Spokas et al., 2005). The hypothesized primary mechanism is fungal denitrification (nitrate → nitrite → N_2O) occurring under aerobic conditions with a maximal contribution from abiotic mechanisms of 18% (Fig. 4). Fungi posses the ability to perform both denitrification and O_2 respiration simultaneously (Shoun et al., 1992; Laughlin and Stevens, 2002) and this has been observed to occur over a wider range of O_2 concentrations than corresponding bacterial denitrification, which requires anaerobic conditions (Firestone and Davidson, 1989; Granli and Bockman, 1994; Murray and Knowles, 2004).

The low ^{15}N label percentage observed in the N_2O suggests other sources of nitrite and nitrate are also involved (Fig. 4). One potential source is from the mineralization of the organic N from the killed biomass. Bacterial nitrification and denitrification do

not significantly contribute to N_2O production as suggested by the acetylene block and bacterial inhibitor experiments. In addition, the isotopic results here agree with the conclusions of Shoun et al. (1992) that the fungal denitrification sequence terminates with N_2O, since there was no significant enrichment in labeled N_2 produced. However, the universality of this conclusion needs to be examined especially since soil fumigation with CP has long term effects on soil N_2O production (Spokas et al., 2005).

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