Effects of sterilization techniques on chemodenitrification and $\text{N}_2\text{O}$ production in tropical peat soil microcosms

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Abstract. Chemodenitrification – the non-enzymatic process of nitrite reduction – may be an important sink for fixed nitrogen in tropical peatlands. Rates and products of chemodenitrification are dependent on $\text{O}_2$, pH, $\text{Fe}^{2+}$ concentration, and organic matter composition, which are variable across peat soils. Assessing abiotic reaction pathways is difficult because sterilization and inhibition agents can alter the availability of reactants by changing iron speciation and organic matter composition. We compared six commonly used soil sterilization techniques – $\gamma$ irradiation, chloroform, autoclaving, and the use of three different chemical inhibitors (mercury, zinc, and azide) – for their compatibility with chemodenitrification assays for tropical peatland soils (organic-rich, low-pH soil from the eastern Amazon). Out of the six techniques, $\gamma$ irradiation resulted in soil treatments with the lowest cell viability and denitrification activity and the least effect on pH, iron speciation, and organic matter composition. Nitrite depletion rates in $\gamma$-irradiated soils were highly similar to untreated (live) soils, whereas other sterilization techniques showed deviations. Chemodenitrification was a dominant process of nitrite consumption in tropical peatland soils assayed in this study. Nitrous oxide ($\text{N}_2\text{O}$) is one possible product of chemodenitrification reactions. Abiotic $\text{N}_2\text{O}$ production was low to moderate (3 %–16 % of converted nitrite), and different sterilization techniques lead to significant variations on production rates due to inherent processes or potential artifacts. Our work represents the first methodological basis for testing the abiotic denitrification and $\text{N}_2\text{O}$ production potential in tropical peatland soil.

1 Introduction

Across ecosystems, physical and chemical factors, such as solar radiation or redox gradients, can drive abiotic chemical transformations. The nitrogen (N) cycle, in particular, includes abiotic reactions that can affect the retention of nutrients or substrates (Clark, 1962; McCalley and Sparks, 2009; Parton et al., 2007). Abiotic formation of N-containing gases has long been known (Jun et al., 1970; Wullstein and Gilmour, 1966). A major abiotic process in the N cycle is chemodenitrification, the stepwise reduction of nitrite ($\text{NO}_2^-$) to gaseous products, namely nitric oxide (NO), nitrous oxide ($\text{N}_2\text{O}$), or dinitrogen ($\text{N}_2$), often coupled to iron ($\text{Fe}^{2+}$) oxidation, as described in Eqs. (1) and (2) (Davidson et al., 2003; Kampschreur et al., 2011; Zhu et al., 2013; Zhu-Barker et al., 2013; Zhu-Barker et al., 2013).
NO$_2^-$ + Fe$^{2+}$ + 2H$^+$ → NO + Fe$^{3+}$ + H$_2$O (1)
2NO + 2Fe$^{2+}$ + 2H$^+$ → N$_2$O + 2Fe$^{3+}$ + H$_2$O (2)

Equations (1) and (2) are plausible in soils and sediments (Jones et al., 2015). The abiotic reduction of N$_2$O to N$_2$ is not well known. It has been associated with the presence of copper (Moraghan and Buresh, 1977), but this species is unlikely to be present at sufficient levels in peat soils to promote this reaction. Anoxic tropical peat soils are expected to have the ideal conditions for chemodenitrification: low O$_2$, low pH, high organic matter (OM), and high Fe$^{2+}$ (Kappelmeyer et al., 2003; Nelson and Bremner, 1969; Porter, 1969; Van Cleemput et al., 1976). In these ecosystems, N$_2$O$^-$ is supplied by nitrification fueled by organic N mineralization or from external sources (fertilization, wet or dry deposition). Besides metals, reduction of NO$_2^-$ compounds can also be mediated by organic functional groups found in soils. Abiotic phenol oxidation occurs at oxic–anoxic interfaces in tropical soils and may be linked to the N cycle (Hall and Silver, 2013). In such reactions, NO$_2^-$ can be reduced by phenolic groups to form the nitrosonium cation NO$_2^+$, which can either (1) remain fixed within the organic compound as nitrosophenol (Thorn and Mikita, 2000; Thorn et al., 2010) or (2) be emitted in gaseous form. After tautomerization to an oxime (Raczyńska et al., 2005) and reaction with NO$^+$ derived from a second NO$_2^-$ ion, hydroxylammonium (H$_2$N$_2$O$_2$) can be produced, which further decomposes to N$_2$O (e.g., Scheme 1; Porter, 1969; Stevenson et al., 1970).

Other OM-dependent NO$_2^-$ reduction pathways can produce NO and N$_2$ (McKenney et al., 1990; Thorn et al., 2010) instead of N$_2$O.

The importance of abiotic N transformations in environmental samples has been notoriously difficult to quantify due to the artifacts emerging from physical or chemical “killing” methods intended to eliminate biological activity but affecting metals, organic matter, or other pools. In order to distinguish denitrification from chemodenitrification, enzymes contributing to gaseous N production must be inactivated, most commonly by addition of sterilants or inhibitors. An efficient sterilization treatment ideally (1) contains a negligible number of live cells, (2) eliminates biological activity, and (3) has little or no effect, directly or indirectly, on abiotic reactions (e.g., it should neither alter mineral structure nor lyse cells because release of cellular contents could influence abiotic reactions). Because rates and products of chemodenitrification are dependent on O$_2$, pH, Fe$^{2+}$ concentration, and OM composition, it is important to assess whether a sterilant or inhibitor elicits a physicochemical change that can affect the availability or interaction of these reactants.

Soil sterilization techniques include γ irradiation, chloroform (CHCl$_3$) fumigation, autoclaving, and addition of chemical inhibitors such as mercury (Hg), zinc (Zn), or azide (N$_3$). Highly energetic γ irradiation damages enzymes and cell components, rendering cells nonviable and inactive, generally with minimal effect on soil chemistry (Trevors, 1996). Autoclaving with high-pressure steam disrupts cell membranes, denatures proteins, and decreases aromaticity and polycondensation of soil OM (Berns et al., 2008; Jenkinson and Powlson, 1976b; Trevors, 1996). Fumigation with CHCl$_3$ induces cell lysis and has minimal effect on enzymes (Blankinship et al., 2014). Chemicals like Hg, Zn, and N$_3$ do the opposite: they inhibit enzymes (Bowler et al., 2006; McDevitt et al., 2011) but do not lyse cells (Wolf et al., 1989).

We evaluated the appropriateness of six sterilants (γ irradiation, autoclaving, CHCl$_3$, Hg, Zn, and N$_3$) for chemodenitrification measurements in low-O$_2$, low-pH, high-OM soils from a tropical peatland. First, we tested the effects of sterilants on cell membrane viability and biological denitrification activity. Next, we evaluated the effects of sterilants on soil chemistry (pH, OM composition, and extractable Fe). Finally, we assessed the effects of the six sterilants on chemodenitrification measured by NO$_2^-$ depletion and N$_2$O production.

2 Materials and methods

2.1 Sample characteristics

Soil samples were collected in October 2015 from a tropical peatland, locally known as Quistococha (03°50.024′S 73°19.235′W), near Iquitos (Loreto, Peru). The soil geochemistry of this site has been described previously (Lawson et al., 2014; Lähteenoja et al., 2009). The samples were obtained from depths of 15–30 cm below the water table and kept strictly anoxic during transport and storage at 4°C in the dark. Water saturation and organic carbon content were determined by oven drying and loss on ignition, respectively. Dissolved organic carbon (DOC) was determined by high-temperature combustion using a Shimadzu TOC-V Total Organic Carbon Analyzer (Shimadzu Scientific Instruments, Columbia, MD). Inorganic N species were quantified photometrically using an AQ2 Discrete Analyzer (Seal Analytical, Southampton, UK) and method EPA-103-A Rev.10 for ammonium (NH$_4^+$; LoD 0.004 mg N L$^{-1}$, range 0.02–2.0 mg N L$^{-1}$) and method EPA-127-A for nitrate (NO$_3^-$)/nitrite (NO$_2^-$; LoD 0.003 mg N L$^{-1}$, range 0.012–2.0 mg N L$^{-1}$). Hydroxylamine was measured photometrically using the iodate method (Afkhami et al., 2006).

2.2 Soil sterilization and slurry incubations

Experiments were started within 6 weeks of soil collection. For each sterilization procedure, anoxic wet soil was exposed to the chemical sterilant 48 h prior to start of the NO$_2^-$ incubation or sterilized by physical treatment and allowed to equilibrate for at least 12 h. The untreated (live) control was incubated as a slurry without any additions or treatments for 48 h prior to start of the NO$_2^-$ incubation. Anoxic vials filled with...
wet soil were irradiated with a $^{60}$Co source for 7 d, yielding a final radiation dose of 4 Mrad (40 kGy). The irradiated soil was then prepared for incubation in an anoxic glove box (0.5% $\text{H}_2$ in $\text{N}_2$) with disinfected surfaces and sterilized materials to prevent contamination. For autoclaved samples, soil was prepared for incubation in closed vials and autoclaved at 121°C and 1.1 atm for 90 min. The CHCl$_3$-treated samples were fumigated for 48 h under a 100% $\text{N}_2$ atmosphere. Because volatilized CHCl$_3$ corrodes electron capture detectors used for $\text{N}_2\text{O}$ detection (see below), CHCl$_3$ was removed by flushing the vials with $\text{N}_2$ for 5–7 min immediately before the start of incubations.

In contrast to the physical sterilization treatments, soil samples were continuously exposed to the chemical inhibitors throughout their incubation. Sodium azide (NaN$_3$, Eastman Organic Chemicals), zinc chloride (ZnCl$_2$, Fisher Scientific), and mercuric chloride (HgCl$_2$, 99.5%, Acros Organics) were added from anoxic stock solutions to final concentrations of 150, 87.5, and 3.7 mM, respectively. The Hg concentration was the minimum needed to eliminate microbial heterotrophic growth based on visual inspection of soil extract on agar plates exposed to 0.5 to 92.1 ng L$^{-1}$, which includes concentrations demonstrated to be effective previously (Tuominen et al., 1994).

After the initial physical or chemical treatment, triplicate incubations were diluted 1:10 in 20 mL of autoclaved 18.2 MΩ cm water in 60 mL glass serum vials. All microcosms were prepared in an anaerobic glove box (0.5% $\text{H}_2$ in $\text{N}_2$) prior to incubation. Triplicate soil slurries were amended from an anoxic, sterile stock solution to a final concentration of 300 µM NO$_3^-$ (6 µmol in 20 mL) and sealed with thick butyl rubber stoppers. A parallel set of samples was amended with 300 µM NO$_3^-$ to evaluate denitrification potential with CO$_2$ measurements. Control incubations received an equivalent volume of autoclaved 18.2 MΩ cm water without NO$_3^-$. Soil microcosms were incubated in the dark at a constant temperature of 25°C. NO$_3^-$ was quantified in all soil treatments using the Griess assay (Promega, Kit G2930), pH measurements were taken with an Orion 3 Star meter (Thermo Scientific) before and after sterilization, and at the end of the experiment after 70–76 h of incubation.

2.3 Gas chromatography

To quantify $\text{N}_2\text{O}$ and CO$_2$ production, 200 µL of headspace gas was sampled with a gas-tight syringe (VICI Precision Sampling) and injected onto a gas chromatograph (GC, SRI Instruments) equipped with both an electron-capture detector (ECD) and a flame-ionization detector (FID). Two continuous HayeSep-D columns were kept at 90°C (oven temperature); $\text{N}_2$ (UHP grade 99.999%, Praxair Inc.) was used as carrier gas, and for FID combustion $\text{H}_2$ was supplied by a $\text{H}_2$ generator (GC8970, Parker Balston). For CO$_2$ measurements, a methanizer (which reduces CO$_2$ to the detectable CH$_4$ via a Ni catalyst at 355°C) was run in line before the FID. The ECD current was 250 mV and the ECD cell was kept at 350°C. The $\text{N}_2$O and CO$_2$ measurements were calibrated using customized standard mixtures (Scott Specialty Gases, accuracy ±5%) over a range of 1–400 and 5–5000 ppmv, respectively. Gas accumulation in the incubation vials was monitored over time. Gas concentrations were corrected using Henry’s law and the dimensionless concentration constants $k^C_{\text{H}}(\text{N}_2\text{O}) = 0.6112$ and $k^C_{\text{H}}(\text{CO}_2) = 0.8313$ (Stumm and Morgan, 2012) to account for gas partitioning into the aqueous phase at 25°C.

2.4 Live or dead cell staining

To assess the efficacy of sterilants or inhibitors visually, the bacterial viability kit LIVE/DEAD BacLight L7012 (Molecular Probes, Invitrogen) containing SYTO9 and propidium iodide dyes was used to stain and distinguish dead and living cells on the basis of intact cell walls. The green (live) and red (dead) signals were counted at 60× magnification from 10 squares of 0.01 mm$^2$ randomly distributed in the center of a 5 µL Neubauer chamber, using an Olympus BX-61 microscope with the FITC/Cy5 filter set. Photographs were taken with an Olympus DP-70 camera attached to the microscope. Particles were counted with ImageJ software version 1.50i (Abramoff et al., 2004).

2.5 Fe extraction and quantification

Dissolved Fe species were extracted from peat soil incubations following the protocol of Veverica et al. (2016). The method is based on an ionic liquid extraction using bis-2-ethylhexyl phosphoric acid (Pepper et al., 2010), which was

Scheme 1. Putative nitrous oxide formation pathway formed by NO$_2^-$ reduction by phenolic groups.
shown to be more suitable for extraction of Fe from humic-rich matrices than the traditional ferrozine or phenanthroline methods. Briefly, 2.5 mL of soil slurry was filtered (0.2 μm nylon filter; Celltreat Scientific Products) and mixed with 7.5 mL of HCl (0.67 N) in an extraction vial in a 0.5% H₂ in N₂ glove box. The O₂ concentration in the glove box was continuously monitored and remained < 10 ppm. To separate Fe³⁺ from Fe²⁺, 10 mL of 0.1 M bis-2-ethylhexyl phosphate (95%, Alfa Aesar) in n-heptane (99.5%, Acros Organics) was added to the acidified sample. Next, the organo-aqueous emulsion was shaken at 250 rpm in closed extraction vials for 2 h. The bis-2-ethylhexyl phosphate chelates Fe³⁺ more effectively than it chelates Fe²⁺. The Fe²⁺-containing aqueous phase was sampled into a 3-fold HCl-washed HDPE vial (Nalgene) in the glove box. The Fe³⁺ fraction chelated in the organic phase was then back-extracted into an aqueous phase by the addition of 10 mL 4 N HCl and shaking at 250 rpm in closed extraction vials for 20 min. Fe³⁺ and Fe²⁺ fractions were quantified separately in acidified aqueous solution by inductively coupled plasma–optical emission spectrometry (ICP-OES; Thermo iCAP6300 at the Goldwater Environmental Laboratory at Arizona State University). The ICP-OES pump rate for the Ar carrier was set to 50 rpm, and Fe2395 and Fe2599 lines were used for Fe quantification.

The tropical peat soil used for the incubation experiments had 5.5–5.8 pH, 92.2% water content, 307 ± 9 g total Fe kg⁻¹ soil. The native soil pore water had 13.2 ± 1.2 mg L⁻¹ DOC, 436 ± 79 μg NL⁻¹ NH₄⁺, 9.7 ± 1.3 μg NL⁻¹NO₃⁻, and 3.9 ± 0.2 μg NL⁻¹NO₂⁻. Hydroxylamine was below detection in all cases (< 3 μM). Soil pH dropped from 5.5 to 5.8 in untreated soil to 3.6, 4.8, 5.0, 5.2, and 5.4 after treatment with Hg, Zn, γ irradiation, autoclaving, and CHCl₃, respectively. Only N₃ treatment increased soil pH (to 6.4).

### 3 Results

#### 3.1 Composition of high-OM tropical soils

The tropical peat soil used for the incubation experiments had 5.5–5.8 pH, 92.2% water content, 307 ± 5 mg TOC g⁻¹ dry weight, and 3.8 ± 0.9 g total Fe kg⁻¹ soil. The extractable iron fraction partitioned as 54 ± 3 μM extractable Fe³⁺ and 213 ± 16 μM extractable Fe²⁺. The native soil pore water had 13.2 ± 1.2 mg L⁻¹ DOC, 436 ± 79 μg NL⁻¹ NH₄⁺, 9.7 ± 1.3 μg NL⁻¹NO₃⁻, and 3.9 ± 0.2 μg NL⁻¹NO₂⁻. Hydroxylamine was below detection in all cases (< 3 μM). Soil pH dropped from 5.5 to 5.8 in untreated soil to 3.6, 4.8, 5.0, 5.2, and 5.4 after treatment with Hg, Zn, γ irradiation, autoclaving, and CHCl₃, respectively. Only N₃ treatment increased soil pH (to 6.4).

#### 3.2 Effects of sterilants on cell integrity and potential of denitrifying activity

Live and dead dyes were used to assess microbial viability by means of membrane integrity, where a “dead” signal indicates disrupted or broken cell membranes (Stiefel et al., 2015). The majority (74%) of cells in the live incubation displayed the “live” signal (Fig. 1). The CHCl₃ and...
γ-irradiated treatments were most effective at reducing the number of viable cells (∼15 % intact membranes after sterilization). Chemical inhibitors (Hg, Zn, and N3) were less effective at killing cells (∼30 % intact membranes after sterilization). Autoclaved samples did not fluoresce, likely due to cell lysis during steam pressurization.

Biological denitrification activity was measured over 3 d in live and sterilized soils based on the difference in CO2 production with and without added NO3−. An efficient sterilization treatment would show no changes in CO2 beyond those due to equilibration between the gas phase and aqueous phase. Nitrate stimulated CO2 production in live soil (ANOVA, p < 0.05) and not in the γ-irradiated, Zn, Hg, N3, or autoclaved incubations (Fig. 2), indicating that residual cells in the sterilized treatments were not capable of denitrification.

3.3 Effects of sterilants on soil chemistry

In general, sterilization increased extractable Fe2+ and Fe3+ relative to live controls (Fig. 3). This trend was particularly pronounced in Zn treatments, which had 9× higher extractable Fe2+ (1915 ± 26 µM) and 1.6× higher extractable Fe3+ (87 ± 3 µM) than live controls. The Hg treatment showed the second-largest increases. In the presence of NO3−, extractable Fe2+ decreased and extractable Fe3+ increased in live, Zn, and CHCl3-fumigated treatments, as expected if Fe2+ was oxidized by NO3− during chemodenitrification. However, autoclaving, γ irradiation, and N3 lowered Fe3+ concentrations, suggesting the influence of unknown concomitant reactions. For instance, autoclaving (largest drop in Fe3+) already showed lower Fe3+ concentrations after sterilization. Production of Fe3+-reduction artifacts in treatments could lead to Fe3+ depletion and, hence, mask increase in Fe3+ due to chemodenitrification. NO3− addition resulted in near-complete depletion of extractable Fe2+ in live, CHCl3-fumigated, and γ-irradiated soils. Changes in Fe speciation with other sterilants were more moderate. Minimal changes were observed for other metals in soil samples (e.g., Mn, Al, Cu, and Zn; data not shown).

Fluorescence analysis of soil extracts using excitation-emission matrices (EEMs) was used to evaluate changes in DOM-containing aromatic moieties or conjugated double bonds (Stedmon et al., 2003; Fig. 4). The NO3 treatment was excluded from this analysis due to an interference with N3− absorbance that prevented inner-filter corrections from being made. The EEM signals showed the greatest change in the “humic” region (λEx < 240–270 nm, and λEm = 460–500 nm; Fellman et al., 2010), especially in Zn and Hg treatments, which significantly increased the FI from 1.20 (in live soil baseline, prior to NO3− incubation) to 1.49 (Table 1). Zn and Hg may elicit direct fluorescence quenching through the formation of Zn and Hg metal complexes (McKnight et al., 2001) or possibly due to indirect quenching by more highly dissolved Fe2+. Signal strength in the humic region was enhanced by NO3− addition in the live, CHCl3-fumigated, and γ-irradiated treatments. All five sterilization treatments had lower aromaticity (HIX) than live controls (Table 1). Autoclaved samples had 10-fold higher total fluorescence compared to live soils, suggesting that autoclaving degraded insoluble humics into more soluble and less condensed OM.
3.4 Effects of sterilants on chemodenitrification and 
abiotic N₂O production

In the first 48 h, NO₃⁻ consumption rates were the highest in live soil (5.2 µM h⁻¹), closely followed by irradiated 
samples (4.5 µM h⁻¹, Fig. 5). The major chemodenitrification 
pathway for N₂O formation was likely NO₃⁻ reduction by Fe²⁺, 
resulting in consumption of ~1.5 µmol Fe²⁺ and accumulation of ~1.1 µmol Fe³⁺ in the live control 
(Fig. 3). After 48 h, NO₃⁻ depletion continued to completion in the live control but slowed in all treatments other 
than the metal additions. After 72 h of incubation, 3%–
16% of NO₃⁻-N was converted to N₂O-N across treatments. N₂O production rates were assessed by linear 
regression of data points over the whole duration of the 
experiment. Higher rates were observed in live, Zn²⁺,
and N₃ treatments (0.5–0.7 nmol N₂O g⁻¹ h⁻¹, $r^2 > 0.95$) 
than in γ-irradiated, CHCl₃-fumigated, autoclaved, and Hg 
treatments (0.1–0.2 nmol N₂O g⁻¹ h⁻¹, $r^2 > 0.9$). Production 
rates within treatments showing high or low rates were 
not significantly different (Student’s $t$, $p > 0.05$), although 
comparisons across treatments with high or low rates were 
statistically different (Student’s $t$, $p < 0.05$). Thus, we 
identified a higher and lower group of sterilant-dependent N₂O 
production rates from the same soil samples. The live 
control showed logarithmic N₂O accumulation, while the steril-
ized treatments had linear accumulation over time; the latter 
is expected in abiotic accumulation (Fig. 5).

4 Discussion

4.1 Chemodenitrification is a dominant NO₃⁻ 
consumption process in slurry incubations of 
tropical peat soils

Similar NO₃⁻ consumption rates between live and irradiated 
treatments imply that NO₃⁻ depletion was dominated by abiotic 
processes over the first 48 h. In general, abiotic reactions 
tend to be linear processes, whereas microbially mediated 
reactions can be affected by enhanced expression of genes or
Table 1. Characteristics of dissolved organic matter in soil extracts from incubations of peat from Quistococha, Peru. Fl, HIX, and freshness indices were calculated as described in the Methods section. The “tyrosine-like” region is defined at an excitation of 270–275 nm and an emission of 304–312 nm (Fellman et al., 2010). The signal for that region was averaged across replicates and expressed as percent difference between NO$_3^-$ additions and controls $\pm$ SD of replicates. A drop in the signal intensity was consistently apparent but clear differences between the treatments were not, due to high standard deviation of replicates.

| Treatment       | FI** | HIX*** | Freshness | Drop in mean fluorescence of the tyrosine-like region (% over control) |
|-----------------|------|--------|-----------|---------------------------------------------------------------------|
| Live soil       |      |        |           |                                                                     |
| Baseline        | 1.20 | a      | 5.57 a    | 0.44                                                                |
| Control         | 1.21 |        | 4.72 a    | 0.41                                                                |
| Nitrite-added   | 1.16 | *      | 7.11 *    | 0.40                                                                |
| Zn              |      |        |           |                                                                     |
| Baseline        | 1.49 | b      | 2.70 b    | 0.58                                                                |
| Control         | 1.50 |        | 2.27 b    | 0.59                                                                |
| Nitrite-added   | 1.55 | *      | 2.05 *    | 0.62                                                                |
| Autoclaving     |      |        |           |                                                                     |
| Baseline        | 1.20 | a      | 2.54 a    | 0.47                                                                |
| Control         | 1.20 |        | 2.83 a    | 0.46                                                                |
| Nitrite-added   | 1.20 |        | 2.97 a    | 0.43                                                                |
| Chloroform      |      |        |           |                                                                     |
| Baseline        | 1.23 | c      | 2.79 b    | 0.43                                                                |
| Control         | 1.27 |        | 2.70 b    | 0.44                                                                |
| Nitrite-added   | 1.14 | *      | 4.12 *    | 0.40                                                                |
| $\gamma$ irradiation | | | | |
| Baseline        | 1.30 | d      | 1.90 b    | 0.57                                                                |
| Control         | 1.27 |        | 2.35 b    | 0.56                                                                |
| Nitrite added   | 1.21 | *      | 2.95 *    | 0.52                                                                |
| Hg              |      |        |           |                                                                     |
| Baseline        | 1.49 | b      | 2.20 b    | 0.57                                                                |
| Control         | 1.50 |        | 1.60 b    | 0.56                                                                |
| Nitrite added   | 1.44 | *      | 2.12 *    | 0.51                                                                |

* indicates significant difference to control.

** Fluorescence index.
** Humification index.
Mean values marked with the same letter are insignificantly different from each other.

cell reproduction in a nonlinear fashion (Duggleby, 1995). Linearity is more reflected in the N$_2$O curve than in the NO$_3^-$ curve. The difference in linearity of N$_2$O production in sterilized vs. live treatments (Fig. 5) suggests that biological denitrification did not occur in sterilized soils.

Compared to our study, incubations of artificial media with 200 µM NO$_3^-$, 0.5–8.1 mM Fe$^{2+}$, and pH 7–8 had similar rates of Fe$^{2+}$ depletion but 10× higher rates of NO$_3^-$ reduction, and higher (~10%–50%) N$_2$O yields (Buchwald et al., 2016; Jones et al., 2015). In our peat incubations, reactive OM likely trapped NO$_3^-$ in the soil matrix via OM-bound nitrosation reactions (Thorn and Mikita, 2000; Thorn et al., 2010) and the lower pH likely promoted conversion of NO$_2^-$ to NO (Kappelmeyer et al., 2003; Porter, 1969) or N$_2$ (Stevenson et al., 1970). Studies in low-pH northern temperate peat soils have shown the primary product of abiotic NO$_2^-$ reduction was NO and not N$_2$O (McKenney et al., 1990).

4.2 Artifacts due to sterilization methods for chemodenitrification assays

Azide and Zn exhibited enhanced NO$_3^-$ conversion to N$_2$O, at rates at least 2 to 5 times as high as those measured for the other sterilants (Fig. 5), likely due to higher pH and Fe availability, respectively. In the N$_3$ treatments, elevated N$_2$O production could be explained by the reaction of protonated NO$_3^-$ with N$_3$ in a pH-dependent manner (Stedman, 1959) and other changes in soil solution that originated from the increase in pH. Nitrite reaction with N$_3$ has been characterized in marine and freshwater solutions reaching its maximum at pH 4.5 and proceeding slowly yet significantly (20% conversion in 1 h) at pH > 5 (McIlvin and Altabet, 2005), as in our slurries. Moreover, N$_3$’s self-fluorescence impeded OM measurements, making N$_3$ an incompatible sterilizing agent for chemodenitrification studies. Zn increased Fe availability and may have increased NO$_3^-$ affinity for reactive OM groups; both effects would lead to an abiotic increase in N$_2$O production (Clark, 1962; McCalley and Sparks, 2009; Parton...
creased reactivity of humic acid with NO\textsuperscript{-}\textsubscript{3} and can diminish its reducing power, as indicated by decomposition of nitrous acid (HNO\textsubscript{2}) contributed to \textit{O} production. At low pH (3.6), 98\% of Hg was sorbed to humic acids, whereas only 29\% of Zn was sorbed at pH \textasciitilde{}8 (Kerndorff et al., 2007). Chlorination of natural OM may prompt formation of quinones (Criquet et al., 2015), which are intermediates in the OM-based abiotic N\textsubscript{2}O production (Thorn and Mikita, 2000); indeed, regions of the EEMs corresponding to hydroquinones (Cory and McKnight, 2005) appear to be slightly higher in CHCl\textsubscript{3} treatments. The benzene derivative produced during nitrosophenol reaction with NO\textsubscript{2} leads to reduced \textpi{} electron delocalization (Scheme 1). Because excitation of \textpi{} electrons produces fluorescence, reactions with NO\textsubscript{2} might be expected to reduce OM fluorescence. However, the experiment duration is important, and if microbial cells indeed reproduce after the treatment, short experimental periods (e.g., hours or days) or reapplication of CHCl\textsubscript{3} might keep down the numbers of any potential denitrifiers improving the use of this method.

4.3 Gamma irradiation is the preferred sterilization method for chemodenitrification assays

The fewest chemical artifacts were observed in \gamma{}-irradiated samples. Soil that had been exposed to \gamma{} rays showed the lowest N\textsubscript{2}O production rates, approximately one-fifth of those observed in live samples. Irradiation also caused only very small changes in Fe speciation relative to live controls.
and yielded EEMs that were remarkably similar to those obtained from live soil extracts. Our measurements of sterility and respiratory activity indicated the lowest potential for biological activity and hence the least amount of interference for the time period tested. We therefore confirmed γ irradiation to be a preferred method for sterilizing soil (Trevors, 1996) and for assessing abiotic N₂O production potential. In practice, the long preparation time needed to reach a sufficient dose (dependent on radiation source; see Methods) was compensated for by the lack of chemical artifacts during the experiment and the reduced number of hazardous waste products. Limited accessibility to irradiation facilities and the absence of a field portable option remain the main challenges to wide distribution of this approach.

5 Conclusion

High N₂O emissions occur in tropical regions with water-saturated soils (Liengaard et al., 2014; Park et al., 2011; Pérez et al., 2001). Whether these tropical N emissions are solely biotic or have abiotic contributions is not well known because rates of chemodenitrification are not commonly evaluated. Abiotic processes in the N cycle remain overlooked, partly due to the lack of reliable means of quantifying abiotic reactions. This study showed that chemodenitrification occurs in a tropical peat soil, leading to a low to moderate fraction of N₂O conversion from nitrite amendment. We also demonstrated that γ irradiation is the “gold standard” for chemodenitrification assays. The application of N₂ to quantify abiotic N₂O production is unsuitable because changes associated with the fraction of the sterilant itself may react to form N₂O and affect increased pH. CHCl₃ and γ rays have slightly reducing effects on the soil Fe pool and might lead to a weak discrimination against pathways involving Fe as a reactant. CHCl₃ fumigation was another approach with limited effects on Fe chemistry that lowered the number of viable cells greatly; however, the potential for microbial regrowth after CHCl₃ removal is its main drawback. Autoclaving seemed to have minor disadvantages for abiotic N₂O production, despite the substantial changes to soil OM.

Unlike other lab-intensive treatments, the application of Zn and Hg are amenable for field experiments; however, we observed distinct chemical artifacts when using both of these options. Care is warranted if using Zn and Hg chemical inhibitors, which can increase Fe availability and may thus overestimate Fe-dependent abiotic N₂O production rate. A potential disadvantage of the application of toxic metals is a decrease in soil pH. We cannot exclude pH-driven effects on N intermediates; however, no major deviation in the final N₂O production rate related to acidification was observed. With the methodological evaluation presented here, we determined that a directed selection of approaches can allow for better constrained and more detailed studies of the role of abiotic pathways and soil components shaping denitrification and N₂O fluxes from soil ecosystems.

Data availability. All data presented in this paper are available in the Figshare repository under the following DOI: https://doi.org/10.6084/m9.figshare.10043177.v1 (Buessecker, 2019).

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Competing interests. The authors declare that they have no conflict of interest.

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