Effects of sterilization techniques on chemodenitrification and N$_2$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 with low oxygen, low pH, high organic matter, and variable ferrous iron concentrations. Assessing abiotic reaction pathways is difficult because sterilization/inhibition agents can alter the availability of reactants by changing iron speciation and organic matter composition. We compared six commonly used soil sterilization techniques – γ-irradiation, chloroform, autoclaving, and 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, γ-irradiation resulted in soil treatments with lowest cell viability and denitrification activity, and least effect on pH, iron speciation, and organic matter composition. Nitrite depletion rates in γ-irradiated soils were highly similar to untreated/live soils, whereas other sterilization techniques showed deviations. Chemodenitrification was a dominant process in tropical peatland soils assayed in this study. Abiotic N₂O 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 N₂O production potential in tropical peatland soil.
1 Introduction

Across ecosystems, physical and chemical factors, such as solar radiation or redox gradients, can drive chemical transformations in the absence of enzymatic catalysis. 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). Non-enzymatic 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 step-wise 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 Eq. 1-3 (Davidson et al., 2003; Kampschreur et al., 2011; Zhu et al., 2013; Zhu-Barker et al., 2015).

$$\text{NO}_2^- + \text{Fe}^{2+} + 2 \text{H}^+ \rightarrow \text{NO} + \text{Fe}^{3+} + \text{H}_2\text{O} \quad \text{(Equation 1)}$$

$$2 \text{NO} + 2 \text{Fe}^{2+} + 2 \text{H}^+ \rightarrow \text{N}_2\text{O} + 2 \text{Fe}^{3+} + \text{H}_2\text{O} \quad \text{(Equation 2)}$$

$$\text{N}_2\text{O} + 2 \text{Fe}^{2+} + 2 \text{H}^+ \rightarrow \text{N}_2 + 2 \text{Fe}^{3+} + \text{H}_2\text{O} \quad \text{(Equation 3)}$$

Eq. 1-2 are plausible in soils and sediments (Jones et al., 2015), however Eq. 3 is likely negligible in most soil environments because of the unlikely availability of Cu$^{2+}$ at the required concentrations to reduce N$_2$O (Moraghan and Buresh, 1977) and relative inertness of N$_2$O.

Anoxic tropical peat soils are expected to have the ideal conditions for chemodenitrification: low-$\text{O}_2$, 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, NO$^-\text{N}$ is supplied by nitrification fueled by organic N mineralization or from external sources (fertilization, wet or dry deposition). 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$^-\text{N}$ can be reduced by phenolic groups to form the nitrosonium cation NO$^+\text{N}$, 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, hyponitrous acid (H\(_2\)N\(_2\)O\(_2\)) can be produced, which further decomposes to N\(_2\)O (Porter 1969; Stevenson et al., 1970) (Eq. 4).

\[
\begin{array}{c}
\text{Nitrosophenol} \\
\text{Quinone monoimine} \\
\text{Nitrosophenol} \\
\end{array}
\xrightarrow{\text{HNO}}
\begin{array}{c}
\text{Quinone monoimine} \\
\text{Hyponitrous acid} \\
\text{Hyponitrous acid} \\
\end{array}
\xrightarrow{\text{H}^+}
\begin{array}{c}
\text{Quinone monoimine} \\
\text{Hyponitrous acid} \\
\text{Nitrous oxide} \\
\text{Water} \\
\end{array}
\xrightarrow{\text{H}^+}
\begin{array}{c}
\text{Quinone monoimine} \\
\text{Hyponitrous acid} \\
\text{Nitrous oxide} \\
\end{array}
\xrightarrow{\text{H}^+}
\begin{array}{c}
\text{Quinone monoimine} \\
\text{Nitrous oxide} \\
\text{Water} \\
\end{array}
\]

(Equation 4)

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. 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 not 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/inhibitor elicits a physicochemical change that can affect the availability or interaction of these reactants.

Soil sterilization techniques include \(\gamma\)-irradiation, chloroform (CHCl\(_3\)) fumigation, autoclaving, and addition of chemical inhibitors such as mercury (Hg), zinc (Zn), or azide (N\(_3\)).

Highly energetic \(\gamma\)-irradiation damages enzymes and cell components, rendering cells non-viable 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 CHCl₃ induces cell lysis and has minimal effect on enzymes (Blankinship et al., 2014). Chemicals like Hg, Zn, and N₃ 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, CHCl₃, Hg, Zn, and N₃) for chemodenitrification measurements in low-O₂, low-pH, high-OM tropical peat soils. 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 NO₂⁻ depletion and N₂O production.

2 Materials and Methods

2.1 Sample characteristics. Soil samples were collected in October 2015 from a tropical peatland, locally known as Quistococha (3°50’S, 73°19’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 (NH₄⁺; LoD 0.004 mg-N L⁻¹, range 0.02-2.0 mg-N L⁻¹) and method EPA-127-A for nitrate (NO₃⁻) /nitrite (NO₂⁻; LoD 0.003
mg-N L\(^{-1}\), range 0.012–2 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 hours prior to start of the NO\(_2^–\) incubation or sterilized by physical treatment and allowed to equilibrate for at least 12 hours. The untreated/live control was incubated as a slurry without any additions or treatments for 48 hours prior to start of the NO\(_2^–\) incubation. Anoxic vials filled with wet soil were irradiated with a \(^{60}\)Co source for 7 days, 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% H\(_2\) in 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 minutes. The CHCl\(_3\)-treated samples were fumigated for 48 hours under a 100% N\(_2\) atmosphere. Because volatilized CHCl\(_3\) corrodes electron capture detectors used for N\(_2\)O detection (see below), CHCl\(_3\) was removed by flushing the vials with N\(_2\) for 5–7 minutes 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) or 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 mg 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. Triplicate soil slurries were amended from anoxic, sterile stock solution to a final concentration of 300 µM NO$_2^-$ (6 µmoles 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$_x^-$. Soil microcosms were incubated in the dark at a constant temperature of 25°C. NO$_2^-$ was quantified in all soil treatments using the Griess assay (Promega, Kit G2930; e.g., Griess 1879). 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 hours of incubation.

2.3 Gas chromatography. To quantify N$_2$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); N$_2$ (UHP grade 99.999%, Praxair Inc.) was used as carrier gas, and H$_2$ for FID combustion was supplied by a H$_2$ generator (GCGS-7890, Parker Balston). For CO$_2$ measurements, a methanizer 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 N$_2$O and CO$_2$ measurements were calibrated using customized standard mixtures (Scott Specialty Gases, accuracy ±5%) over a range of 1-400 ppmv and 5-5,000 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 $k_{H}^{cc}$(N$_2$O) = 0.6112 and $k_{H}^{cc}$(CO$_2$) = 0.8313 (Stumm and Morgan, 2012) to account for gas partitioning into the aqueous phase at 25°C.
2.4 Live/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 60x 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 (Abràmoff 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 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 an N$_2$ glove box. The O$_2$ concentration in the glove box was continuously monitored and remained <10 ppm. To separate Fe$^{3+}$ from Fe$^{2+}$, 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 hours. The bis-2-ethylhexyl phosphate chelates Fe$^{3+}$ more effectively than it chelates Fe$^{2+}$. The Fe$^{2+}$-containing aqueous phase was sampled into a 3-fold HCl-washed HDPE vial (Nalgene) in the glove box. The Fe$^{3+}$ fraction chelated in the organic phase was then back-extracted into an aqueous phase by the addition of 10 mL 4N HCl and shaking at 250 rpm in closed extraction vials for 20 minutes. Fe$^{3+}$ and Fe$^{2+}$ 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. Iron concentrations were determined from a calibration curve (0.01-10 mg L\(^{-1}\)) by diluting a standard solution (100 mg L\(^{-1}\), VHG Labs, product # SM75B-500) in 0.02 N HNO\(_3\).

### 2.6 Dissolved organic matter fluorescence analysis

Dissolved organic matter fluorescence analysis was performed on a Horiba Jobin-Yvon Fluoromax 4 spectrofluorometer. Excitation-emission matrices (EEMs) were generated by obtaining emission spectra (\(\lambda_{\text{Em}} = 300-550\) nm, at a step size of 2 nm) at excitation wavelengths from 240-450 nm at a 10 nm step size. All EEMs were blank corrected and normalized daily to the Raman peak of ultrapure water (deionized, carbon-free, 18.2 MΩ·cm; Barnstead\textsuperscript{tm} NanoPure). The samples were taken at the same time as those for Fe analysis. Prior to analysis, soil slurries were filtered using a solvent-rinsed Whatman GF/F filter (nominal pore size 0.7 µm) to obtain ~10 mL filtrate. Samples were diluted with ultrapure water if their UV absorbance exceeded 0.3 so that inner-filter corrections could be made (Stedmon, 2003). We calculated total fluorescence as the matrix sum of all signals in the EEM. Fluorescence indices were used to characterize various classes of fluorophores in the dissolved organic matter (DOM) pool. Fluorescence Index (FI) was calculated as the sum of the intensity signal in the emission spectra from 470-520 nm collected at an excitation wavelength of 370 nm (Cory and McKnight, 2005). Humification index (HIX) was determined from the peak area under the emission spectrum from 435–480 nm divided by the area from 300–445 nm, both collected at an excitation wavelength of 254 nm (Ohno, 2002). The “freshness” was determined as \(\beta/\alpha\), the ratio of emission intensity at 380 nm to the emission intensity maximum between 420 and 435 nm, both collected at an excitation wavelength of 310 nm (Wilson and Xenopoulos, 2009).

### 2.7 Statistical Analyses

All basic statistical tests were performed with JMP Pro software (Version 13.1.0, SAS Institute Inc., Cary, NC, USA).
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 N L⁻¹ NH₄⁺, 9.7±1.3 µg N L⁻¹ NO₃⁻, and 3.9±0.2 µg N L⁻¹ NO₂⁻. Hydroxylamine was below detection in all cases (<3 µM). Soil pH dropped from 5.5-5.8 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).

Figure 1. Live/dead microbial cell counts of tropical peatland soils. The numbers above the bars indicate the live to dead signal ratio ± SD. No detectable signal was observed in autoclaved samples.
3.2 Effects of sterilants on cell integrity and potential of denitrifying activity. Live/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.

Figure 2. CO₂ production rates in 3-day soil slurry incubations of Quistococha peat soil amended with and without 0.3 mM NO₃⁻. Error bars are one SD (n=3). Columns marked with the same letter are not statistically different from each other (Student’s t, p > 0.05, n=3).
Biological denitrification activity was measured over three days in live and sterilized soils based on the difference in CO$_2$ production with and without added NO$_3^-$ . An efficient sterilization treatment would show no changes in CO$_2$ beyond that due to equilibration between the gas phase and aqueous phase. Nitrate stimulated CO$_2$ production in live soil (ANOVA, $p < 0.05$) and not in the $\gamma$-irradiated, Zn, Hg, N$_3^-$, or autoclaved incubations (Fig. 2), indicating that residual cells in the sterilized treatments were not capable of denitrification.

Figure 3. Changes in extractable Fe$^{2+}$ (left) and Fe$^{3+}$ (right) concentration in Quistococha peat soil incubations after sterilization (difference between sterilization baseline and live
baseline value) and after NO$_2^-$ amendment and incubation (difference between NO$_2^-$ and control incubations). Note the difference in scales. Values represent the extractable fraction of both species. Error bars are one SD (n=2).

3.3 Effects of sterilants on soil chemistry. In general, sterilization increased extractable Fe$^{2+}$ and Fe$^{3+}$ relative to live controls (Fig. 3). This trend was particularly pronounced in Zn treatments, which had 9x higher extractable Fe$^{2+}$ (1915±26 µM) and 1.6x higher extractable Fe$^{3+}$ (87±3 µM) than live controls. The Hg treatment showed the second largest increases. In the presence of NO$_2^-$, extractable Fe$^{2+}$ decreased and extractable Fe$^{3+}$ increased in live, Zn, and CHCl$_3$-fumigated treatments, as expected if Fe$^{2+}$ was oxidized by NO$_2^-$ during chemodenitrification. However, autoclaving, γ-irradiation, and N$_3$ lowered Fe$^{3+}$ concentrations, suggesting the influence of unknown concomitant reactions. For instance, autoclaving (largest drop in Fe$^{3+}$) already showed lower Fe$^{3+}$ concentrations after sterilization. Production of Fe$^{3+}$-reduction artifacts in treatments could lead to Fe$^{3+}$ depletion and, hence, mask increase in Fe$^{3+}$ due to chemodenitrification. NO$_2^-$ addition resulted in near-complete depletion of extractable Fe$^{2+}$ in live, CHCl$_3$-fumigated, and γ-irradiated soils. Changes in Fe speciation with other sterilants were more moderate. Minimal changes were observed for other metals (e.g., Mn, Al, Cu, and Zn; data not shown).
Figure 4. Representative plots of DOM fluorescence in soil slurry incubations of Quistococha peat soils. DOM fluorescence is presented as excitation-emission matrices (EEMs) collected for each treatment (rows) after the sterilization procedure or live control (left column), after incubation with no amendment (“after incubation” control, middle column), and after incubation with 300 µM NO₂⁻ (same time point as control, right column). The colored bar shows the individual signal intensity. All but “autoclaving” treatment has same scale of signal intensity, autoclaving effects increased about 5 times the signal intensity scale.
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 N₃ 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 to 1.49 (Table 1). Zn and Hg may elicit direct fluorescence quenching by the formation of Zn and Hg metal complexes (McKnight et al., 2001) or possibly due to indirect quenching by higher dissolved Fe²⁺. Signal strength in the humic region was enhanced by NO₂⁻ 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 tenfold 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 hours, NO₂⁻ 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 NO₂⁻ 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 hours, NO₂⁻ depletion continued to completion in the live control but slowed in all treatments other than the metal additions. After 72 hours of incubation, 3-16% of NO₂⁻-N was converted to N₂O-N across treatments. Higher N₂O production rates were observed in live, Zn²⁺, and N₃⁻ treatments (0.5-0.7 nmol N₂O g⁻¹ h⁻¹, r² > 0.95) than in γ-irradiated, CHCl₃-fumigated, autoclaved, and Hg treatments (0.1-0.2 nmol N₂O g⁻¹ h⁻¹, r² > 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 were not possible.
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 N$_2$O production rates from the same soil samples. The live control showed logarithmic N$_2$O accumulation while the sterilized treatments had linear accumulation over time, the later as expectable in abiotic accumulation (Fig. 5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{NO$_2^-$ consumption (left) and N$_2$O production (right) for different sterilant treatments in soil slurry incubations of Quistococha peat soil. Both N species were simultaneously measured in all treatments. The product yield represents N$_2$O-N as molar fraction of NO$_2^-$-N. Note the difference in left and right y-axis scales. Error bars are one SD (n=3).}
\end{figure}
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 hours. In general, abiotic reactions tend to be linear processes, whereas microbially mediated reactions can be affected by enhanced expression of genes or cell reproduction in a nonlinear fashion (Duggleby, 1995). The difference in linearity of N₂O 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 NO₃⁻, 0.5-8.1 mM Fe²⁺, and a pH of 7-8 had similar rates of Fe²⁺ depletion but 10x higher rates of NO₃⁻ reduction, and higher (~10-50%) N₂O yields (Buchwald et al., 2016; Jones et al., 2015). In our peat incubations, reactive OM likely trapped NO₂⁻ 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 NO₂⁻ to NO (Kappelmeyer et al., 2003; Porter, 1969) or N₂ (Stevenson et al., 1970). Studies in low pH northern temperate peat soils, have shown the primary product of abiotic NO₂⁻ reduction was NO, not N₂O (McKenney et al. 1990).

4.2 Artifacts due to sterilization methods for chemodenitrification assays. Azide and Zn exhibited enhanced NO₂⁻ conversion to N₂O, at rates at least twice to five times as high as those measured for the other sterilants (Fig 5), likely due to higher pH and Fe availability, respectively. In the N₃ treatments, elevated N₂O production could be explained by the reaction of protonated NO₂⁻ with N₃ in a pH dependent manner (Stedman, 1959), plus other changes in soil solution originated from the increase of pH. Nitrite reaction with N₃ has been characterized in marine and freshwater solutions reaching its maximum at pH 4.5 and proceeding slowly yet significantly (20% conversion in 1 hour) 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_{2}^{-} 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 et al., 2007). Zinc treatment lowered the soil pH, which may have promoted cation displacement and stability of dissolved Fe^{2+} (Hutchins et al., 2007), thus enhancing N_{2}O production. Several studies have used Zn treatments as valuable agent for field applications (Babbin et al., 2015; Ostrom et al., 2016). Zn is less hazardous to humans than some of the other sterilants. We propose that the use of Zn could provide useful information about abiotic in-situ rates as long as Zn-induced chemodenitrification is accounted for. A correction could be applied if a complementary laboratory assessment (using the more efficient γ-irradiation) were used to develop an ecosystem-specific correction factor.

Divalent Hg^{2+} can be abiotically methylated by fulvic acid-type substances (Rogers, 1977). The reaction oxidizes OM and can diminish its reducing power as indicated by decreased reactivity of humic acid with NO_{2}^{-} (Gu et al., 2011; Zheng et al., 2011) thus interfering with the abiotic assay. Another potential factor associated with the Hg treatments is metal sorption. At low pH (3.6), 98% of Hg was sorbed to humic acids, whereas only 29% of Zn was sorbed at pH ~4.8 (Kerndorff and Schnitzer, 1980). Full sorption capacity of peat is presumably reached in seconds (Bunzl et al., 1976) and the differing sorption behavior of Hg and Zn may play a role in the reaction potential of NO_{2}^{-} with OM. It has been demonstrated that Hg introduced into peat soil leads to sorption of Hg ions to various functional groups, including phenols (Drexel et al., 2002; Xia et al., 1998). Hence it is plausible that Hg sorbed to functional groups subject to electrophilic attack by NO^{+}(e.g., nitrosophenol, Eq. 3) may hamper nitrosation, and therefore protect OM from reacting with NO_{2}^{-}. This could lead to a selective suppression of the OM-dependent N_{2}O production pathway.
Chloroform fumigation resulted in potential N\textsubscript{2}O production rates within the lower production range treatments with minor differences in Fe speciation and DOM fluorescence. However, unlike the other sterilized samples, CHCl\textsubscript{3}-fumigated samples showed enhanced CO\textsubscript{2} production stimulated by NO\textsuperscript{3}− addition. Removal of CHCl\textsubscript{3} from our samples before substrate addition could have provided an opportunity for a few surviving heterotrophs to re-grow and use the easily-degradable organic material derived from dead cells. Indeed, chloroform can lyse cells, providing substrates for growth to CHCl\textsubscript{3}-resistant microorganisms (Zelles et al., 1997).

Continued exoenzyme activity has been also described as a CO\textsubscript{2} source: however, this would not include denitrification enzymes, since none enzymes involved in the denitrification pathway are exoenzymes (Blankinship et al., 2014; Jenkinson and Powlson, 1976a). 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 π-electron delocalization (Eq. 4). Because excitation of π-electrons produces fluorescence, reactions with NO\textsubscript{2}− might be expected to reduce OM fluorescence. However, the experiment duration is important and if indeed microbial cells 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.

Autoclaved peat soil revealed abiotic N\textsubscript{2}O production rates close to the average of the lower production range group, accompanied by but ICP-OES and fluorescence spectroscopy results also showed significant changes in Fe speciation and DOM composition. EEMs demonstrate lower values for the HIX in autoclaved peats (Table 1), consistent with fluorescence data from a study that demonstrated a decrease in the aromaticity and polycondensation of soil.
extracts from autoclaved soil (Berns et al., 2008). Autoclaving likely caused degradation and solubilization of insoluble humic components. The direct effects of autoclaving are very much dependent on the heat and pressure stability of the indigenous soil constituents, but the substantial soil structural changes likely introduce chemical artifacts that are absent in the native live soil.

4.3 Gamma irradiation is the preferred sterilization method for chemodenitrification assays.

The fewest chemical artifacts were observed in γ-irradiated samples. Soil that had been exposed to γ-rays showed the lowest N₂O 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 potentials. 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.
Table 1. Characteristics of dissolved organic matter in soil extracts from incubations of peat from Quistococha, Peru. FI, 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$_2^-$ additions and controls ± standard deviation of replicates. A drop in the signal intensity was consistently apparent, clear differences between the treatments were not, due to high standard deviation of replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FI**</th>
<th>HIX***</th>
<th>Freshness</th>
<th>Drop in mean fluorescence of the &quot;Tyrosine-like&quot; region (% over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Live soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.20</td>
<td>a</td>
<td>5.57</td>
<td>a 0.44</td>
</tr>
<tr>
<td>Control</td>
<td>1.21</td>
<td></td>
<td>4.72</td>
<td>0.41</td>
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<tr>
<td>Nitrite added</td>
<td>1.16</td>
<td>*</td>
<td>7.11</td>
<td>* 0.40</td>
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<tr>
<td><strong>Zn</strong></td>
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<tr>
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<td>1.49</td>
<td>b</td>
<td>2.70</td>
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<tr>
<td>Control</td>
<td>1.50</td>
<td></td>
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<td>0.59</td>
</tr>
<tr>
<td>Nitrite added</td>
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<td>*</td>
<td>2.05</td>
<td>0.62</td>
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<tr>
<td><strong>Autoclaving</strong></td>
<td></td>
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<td></td>
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<tr>
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<td>a</td>
<td>2.54</td>
<td>a 0.47</td>
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<tr>
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<td></td>
<td>2.83</td>
<td>0.46</td>
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<tr>
<td>Nitrite added</td>
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<td>2.97</td>
<td>0.43</td>
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<td><strong>Chloroform</strong></td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>1.23</td>
<td>c</td>
<td>2.79</td>
<td>b 0.43</td>
</tr>
<tr>
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</tr>
<tr>
<td>Nitrite added</td>
<td>1.14</td>
<td>*</td>
<td>4.12</td>
<td>* 0.40</td>
</tr>
<tr>
<td><strong>γ-Irradiation</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Baseline</td>
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<td>0.56</td>
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<tr>
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<tr>
<td><strong>Hg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1.49</td>
<td>b</td>
<td>2.20</td>
<td>b 0.57</td>
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<tr>
<td>Control</td>
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<td>1.60</td>
<td>0.56</td>
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<tr>
<td>Nitrite added</td>
<td>1.44</td>
<td>*</td>
<td>2.12</td>
<td>0.51</td>
</tr>
</tbody>
</table>

* indicates significant difference to control.  
** Fluorescence index.  
*** Humification index.  
Mean values marked with the same letter are insignificantly different from each other.
5 Conclusion

High N\textsubscript{2}O 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 N\textsubscript{2}O conversion from nitrite amendment. We also demonstrated that $\gamma$-irradiation is the “gold standard” for chemodenitrification assays. The application of N\textsubscript{3} to quantify abiotic N\textsubscript{2}O production is unsuitable because changes associated to fraction of the sterilant itself may react to form N\textsubscript{2}O and effects increased pH. CHCl\textsubscript{3} and $\gamma$-rays have slightly reducing effects on the soil Fe pool and might lead to a weak discrimination against pathways involving Fe as reactant. CHCl\textsubscript{3} 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 CHCl\textsubscript{3} removal is its main drawback. Autoclaving seemed to have minor disadvantages on abiotic N\textsubscript{2}O 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 N\textsubscript{2}O 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 N\textsubscript{2}O 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 N$_2$O fluxes from soil ecosystems.

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Competing Interests Statement

The authors have no competing interests to declare.