Effects of sterilization techniques on chemodenitrification and N_2O production in tropical peat soil microcosms

Steffen Buessecker, Kaitlyn Tylor, Joshua Nye, Keith E. Holbert, Jose D. Urquiza-Muñoz, Jennifer B. Glass, Hilairy E. Hartnett, Hinsby Cadillo-Quiroz

Response to reviewers

Reviewer 1:

L23-25: suggest splitting into two sentences and making the second sentence a more concrete statement of the reactants/conditions necessary for chemodenitrification, similar to that of L84-87. Not all readers readily familiar with chemodenitrification. | The sentence was split accordingly. The parameters upon which chemodenitrification is dependent are now defined more clearly.

L34: Consider adding, "of NO2- consumption" to the sentence, "dominant process..." | It was added.

L34-35: Consider defining abiotic N2O production as one endpoint of chemodenitrification. | We added a defining statement.

L50-52: Example of use of 'non-enzymatic' and 'abiotic'. Please define and then be consistent with use of non-enzymatic vs abiotic. Suggest using just one term, abiotic is more common, I believe. | For clarity " Non-enzymatic" was removed and "abiotic" was used throughout.

L56-58: Check equations. Are H2O and H+ flipped? L60: Cu2+? Should this be Fe2+? | Thank you for catching the error on citing Eq 3 as with Cu when Fe was listed in Equation. We revised in depth the reports on Eq 3 (with Fe) or the possible alternative with Cu, and concluded that the evidence provided to support the feasibility of Fe based equation was limited, the conditions required for its occurrence in nature very unlikely and could cause some unintended confusion. As per Cu, it will not have the role as reductant in reaction either. Hence we removed equation 3, indicated the lack of knowledge on the potential abiotic reduction of N2O and the unlikelihood that this reaction catalyzed by Cu in peat soils. Now L 60-63. We believe this address in depth the point of reviewer.

L66-L72: Found this section a bit confusing. A sentence or two introducing and/or contextualizing these reactions would help readers who are not familiar with these processes. Suggest adding an explanation of how these processes could be affected by sterilization techniques, if indeed that is the point of this part of intro. . . | A sentence was added to clarify that organic functional groups are possible reactants to NOx– just like soil metals (now L67-68). Also, a sentence in L81-82 was extended to reflect that those pools are affected by the methods or techniques used for sterilization: "but affecting metals, organic matter or other pools".

L73-74: Equations absent | Eq 4 (we changed it to Reaction Scheme 1) is a scheme showing nitrite reaction with phenolic groups that perhaps pdf version did not show in reviewers copy. We have added it, we are making sure the correct technical name (Reaction Scheme) is used and we are checking that it appears in file. Now L76

L124: Were all treatments prepared in an anaerobic glove box prior to incubation? | Indeed, a clarifying sentence was added in L140 in initial MS and now L143-144.

L175: N2 or H2 glove box? Not sure if it would matter, just checking for consistency | This was corrected and specified as 0.5% H_2 in N_2. Now L 180-181

L268: Zn data is shown in Fig. 3, referring to another figure or a typo? | There is a small confusion here Zn data mentioned in line is in reference to changes in native Zn metal concentration in samples, while Figure 3 has a legend indicating Zn as the treatment (ZnCl2). As for correction, we included in the sentence a term of "metals in soil samples" (L 277); and all figure legends have received the inclusion of a sentence explaining that "X-axis represents treatments where Zn=ZnCl2, etc." Thank you for pointing this out, figures will be clearer with this info.

L292: increased the FI relative to what? Suggest reminding readers of what the baseline and controls were for Table 1, or maybe I missed this explanation earlier. Not sure if referring to a change in time or change relative to live or relative to +/- nitrite etc. | We added a clarifying statement indicating that the first value is from live soil baseline prior to NO2– incubation in now L296.

L307: where is r2 value coming from, was there a regression analysis done? | We added a clarifying statement in now L311-312. A linear regression over full experimental data range was conducted.

L327: However, to me many of the NO2- consumption lines do not look highly linear in first 48 hrs (Fig. 5). | That is correct, we stated now that this linearity is more reflected in the N_2O curve. Now L 337

L380: none -> no | Corrected.

L392: delete 'accompanied by'? or check grammar of this sentence | Corrected.

Reviewer 2:

L 60: explain that Cu is required for nitrous oxide reductase. | We revised the inclusion on the note of Cu in this section of the MS and clarify that its concentrations are unlikely to abiotically promote N2O reduction reaction. Although we acknowledge that Cu is required for nitrous oxide reductase we deemed that the mentioning of it did not change the point we were describing and decided not to include to avoid confusion indicating the need of Cu in a biotic process when describing abiotic processes. Now L 60-63.

L 73: Equation 4 is really more of a Figure, no? | That is correct, since we equated the number of elemental atoms albeit in different structures, we chose the "reaction scheme" term as the most appropriate one to describe this item. we changed the name to Reaction Scheme 1 now.

L155: Explain what a methanizer is. | We included an explanation in now L159: "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.

It may be pertinent to mention that decomposition of nitrous acid (HNO2) could be occurring at the especially low pH conditions of the HgCl2 addition. Might be useful to consider whether you think this could be contributing to any of the dynamics observed in this treatment, where pH was 3.6. See Park and Lee, 1988 (J. Phys. Chem. v92. p6294). | Thank you, we now made aware of that potential contribution in L 370-371.

L380: . . . since no enzymes. . . | Corrected.

L392: . . . accompanied by ICP-OES. . . | Corrected.

Effects of sterilization techniques on chemodenitrification and N_2O production in tropical peat soil microcosms

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Keywords: soil sterilization, chemodenitrification, abiotic N_2O production, tropical peatlands

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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 O_2, pH, Fe^{2+} concentration and organic matter composition, which are variable across peat soils. 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 of NO_2^- consumption in tropical peatland soils assayed in this study. N_2O is one possible product of chemodenitrification reactions. Abiotic N_2O 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 N_2O production potential in tropical peatland soil.

1 Introduction

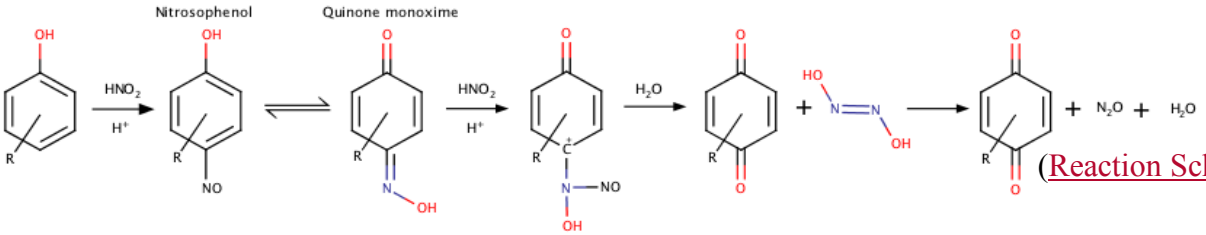
Across ecosystems, physical and chemical factors, such as solar radiation or redox gradients, can drive abiotic chemical transformations. The nitrogen (N) cycle, in particular, includes abiotic reactions that can affect the retention of nutrients or substrates (Clark, 1962; McCalley and Sparks, 2009; 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 step-wise reduction of nitrite (NO_2^-) to gaseous products, namely nitric oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2), often coupled to iron (Fe^{2+}) oxidation, as described in Eq. 1 and 2 (Davidson et al., 2003; Kampschreur et al., 2011; Zhu et al., 2013; Zhu-Barker et al., 2015).

$$NO_2^- + Fe^{2+} + 2\ H^+ \rightarrow NO + Fe^{3+} + H_2O \quad \text{(Equation 1)}$$

$$2\ NO + 2\ Fe^{2+} + 2\ H^+ \rightarrow N_2O + 2\ Fe^{3+} + H_2O \quad \text{(Equation 2)}$$

Eq. 1 and 2 are plausible in soils and sediments (Jones et al., 2015). The abiotic reduction of N_2O 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, NO_x^- is supplied by nitrification fueled by organic N mineralization or from external sources (fertilization, wet or dry deposition). Besides metals, reduction of NO_x^- 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^+, 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 ($H_2N_2O_2$) can be produced, which further decomposes to N_2O (e.g., Reaction Scheme 1; Porter 1969; Stevenson et al., 1970).



(Reaction Scheme 1)

Other OM-dependent NO_2^- reduction pathways can produce NO and N_2 (McKenney et al., 1990; Thorn et al., 2010) instead of N_2O.

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 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 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 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 $CHCl_3$ 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 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 NO_2^- depletion and N_2O 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-

115 V Total Organic Carbon Analyzer (Shimadzu Scientific Instruments, Columbia, MD). Inorganic
116 N species were quantified photometrically using an AQ2 Discrete Analyzer (Seal Analytical,
117 Southampton, UK) and method EPA-103-A Rev.10 for ammonium (NH_4^+; LoD 0.004 mg-N L^{-1},
118 range 0.02-2.0 mg-N L^{-1}) and method EPA-127-A for nitrate (NO_3^-) /nitrite (NO_2^-; LoD 0.003
119 mg-N L^{-1}, range 0.012-2 mg-N L^{-1}). Hydroxylamine was measured photometrically using the
120 iodate method (Afkhami et al., 2006).
121 ***2.2 Soil sterilization and slurry incubations***. Experiments were started within 6 weeks of soil
122 collection. For each sterilization procedure, anoxic wet soil was exposed to the chemical sterilant
123 48 hours prior to start of the NO_2^- incubation or sterilized by physical treatment and allowed to
124 equilibrate for at least 12 hours. The untreated/live control was incubated as a slurry without any
125 additions or treatments for 48 hours prior to start of the NO_2^- incubation. Anoxic vials filled with
126 wet soil were irradiated with a ^{60}Co source for 7 days, yielding a final radiation dose of 4 Mrad
127 (40 kGy). The irradiated soil was then prepared for incubation in an anoxic glove box (0.5% H_2 in
128 N_2) with disinfected surfaces and sterilized materials to prevent contamination. For autoclaved
129 samples, soil was prepared for incubation in closed vials and autoclaved at 121°C and 1.1 atm for
130 90 minutes. The $CHCl_3$-treated samples were fumigated for 48 hours under a 100% N_2
131 atmosphere. Because volatilized $CHCl_3$ corrodes electron capture detectors used for N_2O
132 detection (see below), $CHCl_3$ was removed by flushing the vials with N_2 for 5-7 minutes
133 immediately before the start of incubations.
134 In contrast to the physical sterilization treatments, soil samples were continuously
135 exposed to the chemical inhibitors throughout their incubation. Sodium azide (NaN_3, Eastman
136 Organic Chemicals), zinc chloride ($ZnCl_2$, Fisher Scientific) or mercuric chloride ($HgCl_2$, 99.5%,
137 Acros Organics) were added from anoxic stock solutions to final concentrations of 150, 87.5, and
138 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. All microcosms were prepared in an anaerobic glove box (0.5% H_2 in N_2) prior to incubation. 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_2O 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 (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 N_2O 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,

163 respectively. Gas accumulation in the incubation vials was monitored over time. Gas
164 concentrations were corrected using Henry's law and the dimensionless concentration constants
165 $k_H^{cc}(N_2O) = 0.6112$ and $k_H^{cc}(CO_2) = 0.8313$ (Stumm and Morgan, 2012) to account for gas
166 partitioning into the aqueous phase at 25°C.
167 ***2.4 Live/dead cell staining.*** To assess the efficacy of sterilants or inhibitors visually, the bacterial
168 viability kit LIVE/DEAD BacLight L7012 (Molecular Probes, Invitrogen) containing SYTO9
169 and propidium iodide dyes was used to stain and distinguish dead and living cells on the basis of
170 intact cell walls. The green (live) and red (dead) signals were counted at 60x magnification from
171 10 squares of 0.01 mm^2 randomly distributed in the center of a 5 µL Neubauer chamber, using an
172 Olympus BX-61 microscope with the FITC/Cy5 filter set. Photographs were taken with an
173 Olympus DP-70 camera attached to the microscope. Particles were counted with ImageJ software
174 version 1.50i (Abràmoff et al., 2004).
175 ***2.5 Fe extraction and quantification.*** Dissolved Fe species were extracted from peat soil
176 incubations following the protocol of (Veverica et al., 2016). The method is based on an ionic
177 liquid extraction using *bis*-2-ethylhexyl phosphoric acid (Pepper et al., 2010), which was shown
178 to be more suitable for extraction of Fe from humic-rich matrices than the traditional ferrozine or
179 phenanthroline methods. Briefly, 2.5 mL of soil slurry was filtered (0.2 µm nylon filter; Celltreat
180 Scientific Products) and mixed with 7.5 mL of HCl (0.67 N) in an extraction vial in an 0.5% H_2
181 in N_2 glove box. The O_2 concentration in the glove box was continuously monitored and remained
182 <10 ppm. To separate Fe^{3+} from Fe^{2+}, 10 mL of 0.1 M *bis*-2-ethylhexyl phosphate (95%, Alfa
183 Aesar) in *n*-heptane (99.5%, Acros Organics) was added to the acidified sample. Next, the
184 organo-aqueous emulsion was shaken at 250 rpm in closed extraction vials for 2 hours. The *bis*-2-
185 ethylhexyl phosphate chelates Fe^{3+} more effectively than it chelates Fe^{2+}. The Fe^{2+}-containing
186 aqueous phase was sampled into a 3-fold HCl-washed HDPE vial (Nalgene) in the glove box.

187 The Fe^{3+} fraction chelated in the organic phase was then back-extracted into an aqueous phase by
188 the addition of 10 mL 4N HCl and shaking at 250 rpm in closed extraction vials for 20 minutes.
189 Fe^{3+} and Fe^{2+} fractions were quantified separately in acidified aqueous solution by inductively
190 coupled plasma-optical emission spectrometry (ICP-OES; Thermo iCAP6300 at the Goldwater
191 Environmental Laboratory at Arizona State University). The ICP-OES pump rate for the Ar
192 carrier was set to 50 rpm and Fe2395 and Fe2599 lines were used for Fe quantification. Iron
193 concentrations were determined from a calibration curve (0.01-10 mg L^{-1}) by diluting a standard
194 solution (100 mg L^{-1}, VHG Labs, product # SM75B-500) in 0.02 N HNO_3.
195 ***2.6 Dissolved organic matter fluorescence analysis.*** 3D-fluorescence analysis was performed on
196 a Horiba Jobin-Yvon Fluoromax 4 spectrofluorometer. Excitation-emission matrices (EEMs)
197 were generated by obtaining emission spectra (λ_{Em} = 300-550 nm, at a step size of 2 nm) at
198 excitation wavelengths from 240-450 nm at a 10 nm step size. All EEMs were blank corrected
199 and normalized daily to the Raman peak of ultrapure water (deionized, carbon-free,18.2 MΩ·cm;
200 Barnstead^{tm} NanoPure). The samples were taken at the same time as those for Fe analysis. Prior
201 to analysis, soil slurries were filtered using a solvent-rinsed Whatman GF/F filter (nominal pore
202 size 0.7 μm) to obtain ~10 mL filtrate. Samples were diluted with ultrapure water if their UV
203 absorbance exceeded 0.3 so that inner-filter corrections could be made (Stedmon, 2003). We
204 calculated total fluorescence as the matrix sum of all signals in the EEM. Fluorescence indices
205 were used to characterize various classes of fluorophores in the dissolved organic matter (DOM)
206 pool. Fluorescence Index (FI) was calculated as the sum of the intensity signal in the emission
207 spectra from 470-520 nm collected at an excitation wavelength of 370 nm (Cory and McKnight,
208 2005). Humification index (HIX) was determined from the peak area under the emission
209 spectrum from 435–480 nm divided by the area from 300–445 nm, both collected at an excitation
210 wavelength of 254 nm (Ohno, 2002). The "freshness" was determined as β/α, 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 Fe^{3+} and 213±16 μM extractable Fe^{2+}. The native soil pore water had 13.2±1.2 mg L^{-1} DOC, 436±79 μg N L^{-1} NH_4^+, 9.7±1.3 μg N L^{-1} NO_3^-, and 3.9±0.2 μg N L^{-1} NO_2^-. 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 $CHCl_3$, respectively. Only N_3 treatment increased soil pH (to 6.4).

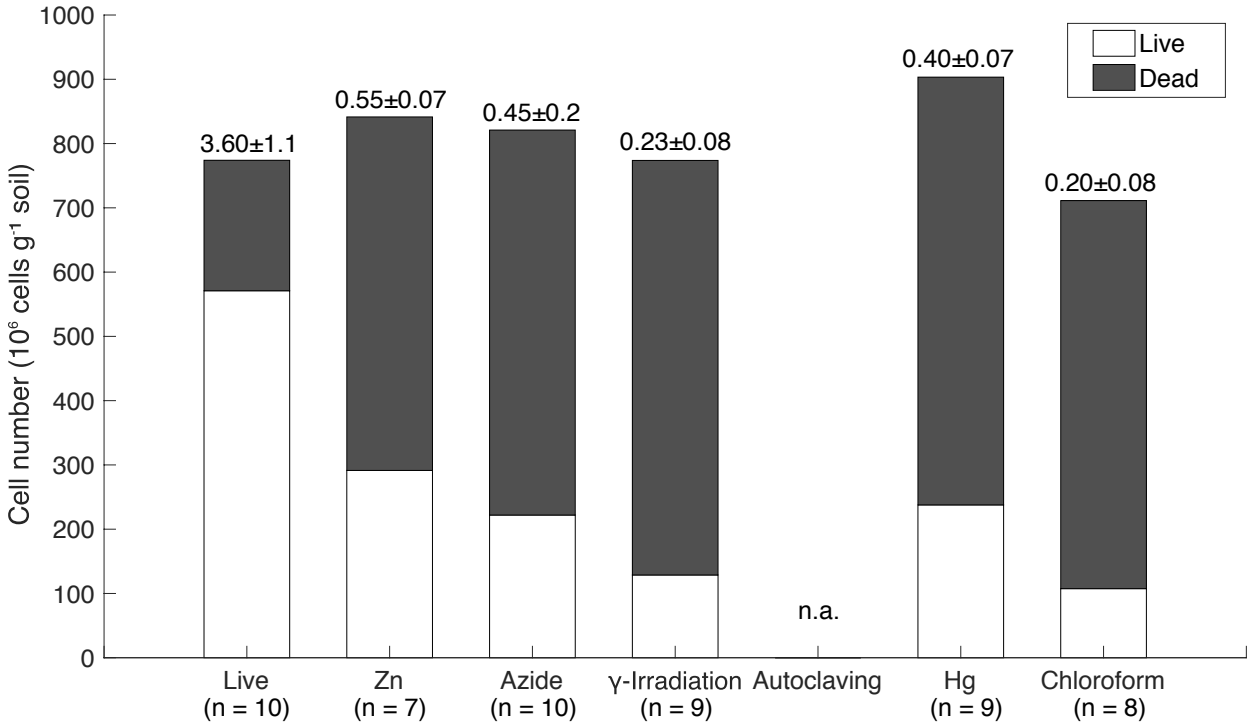


Figure 1. Live/dead microbial cell counts of tropical peatland soils. The numbers above the bars indicate the live to dead signal ratio ± SD. No detectable signal was observed in autoclaved samples. The x-axis represents treatments, including Live = no treatment, Zn = $ZnCl_2$, Hg = $HgCl_2$, Azide = NaN_3.

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 $CHCl_3$ and γ-irradiated treatments were most effective at reducing the number of viable cells (~15% intact membranes after sterilization). Chemical inhibitors (Hg, Zn, and N_3) were less effective at killing cells (~30% intact membranes after sterilization). Autoclaved samples did not fluoresce, likely due to cell lysis during steam pressurization.

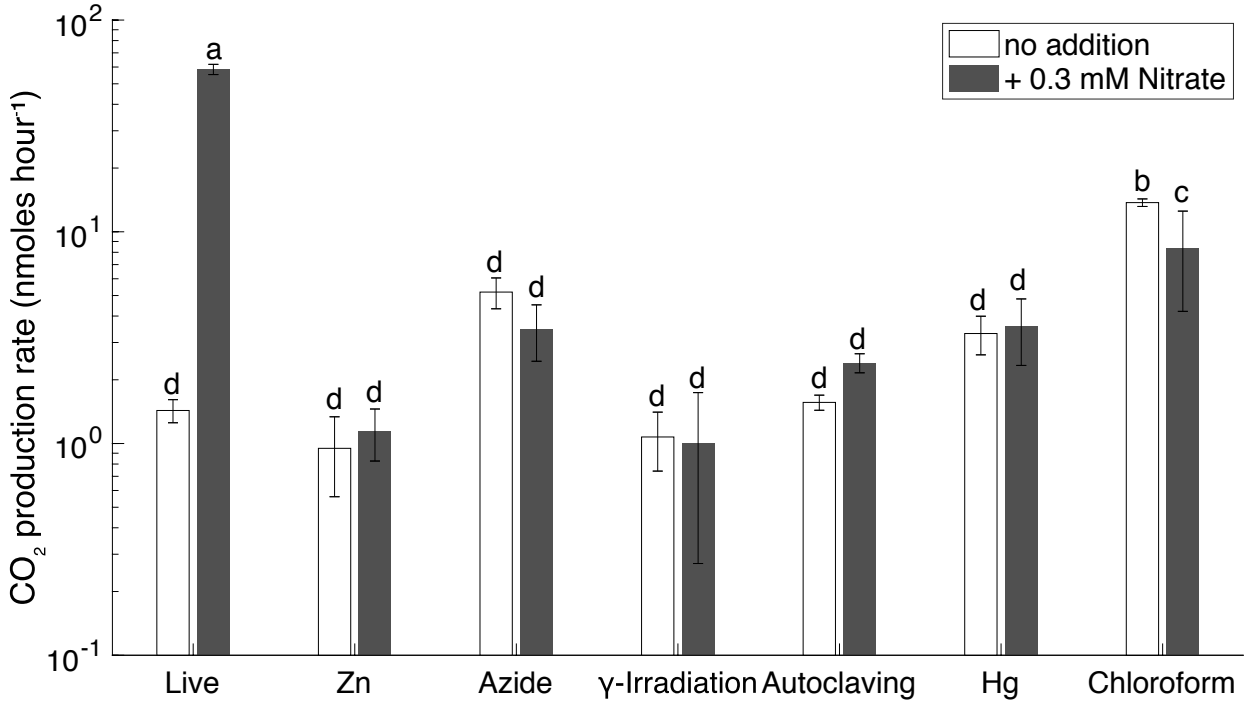
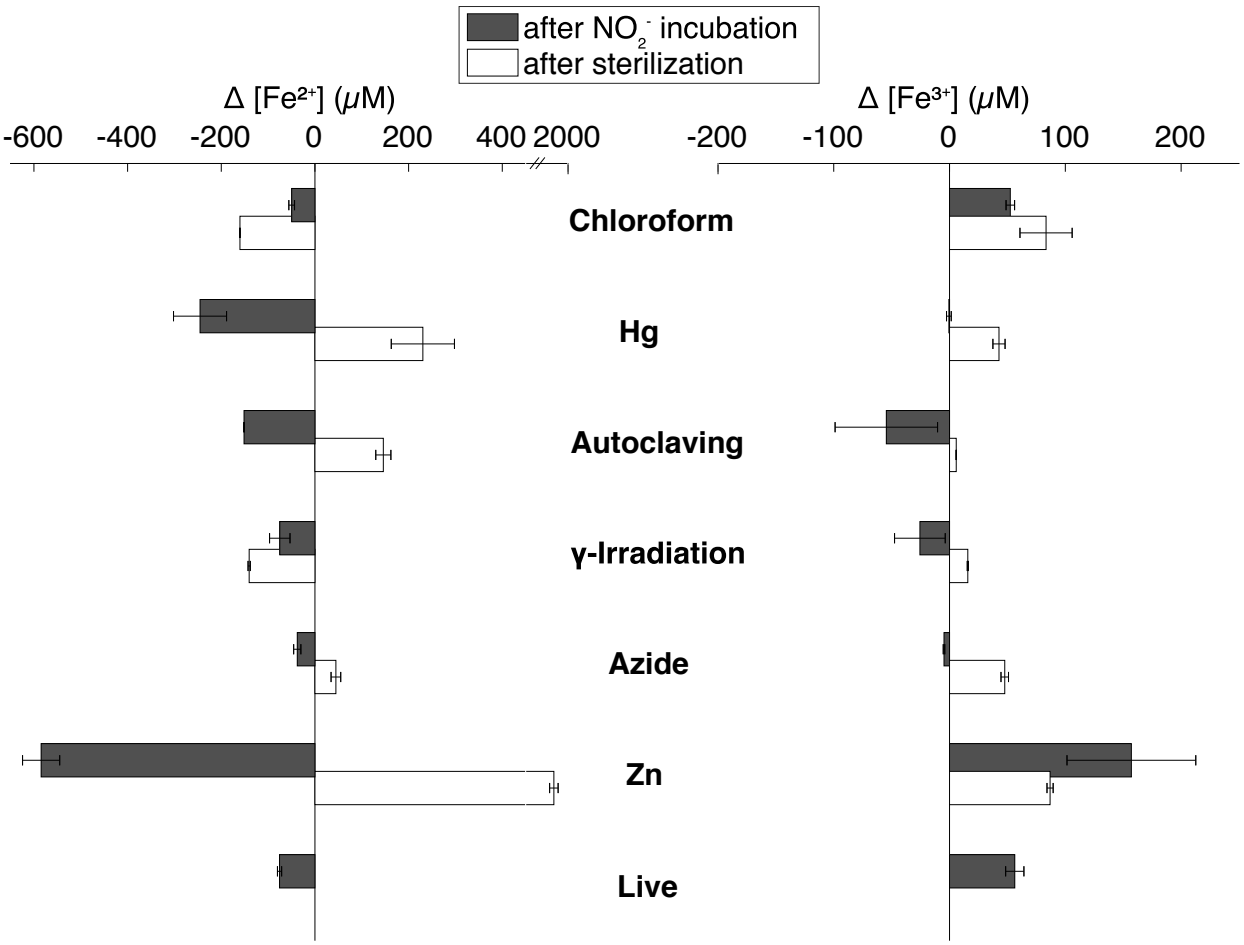


Figure 2. CO_2 production rates in 3-day soil slurry incubations of Quistococha peat soil amended with and without 0.3 mM NO_3^-. Error bars are one SD (n=3). Columns marked with the same letter are not statistically different from each other (Student's *t*, $p > 0.05$, n=3). The x-axis represents treatments as in the legend of Fig. 1.

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 γ-irradiated, Zn, Hg, N_3, or autoclaved incubations **(Fig. 2)**, indicating that residual cells in the sterilized treatments were not capable of denitrification.



255

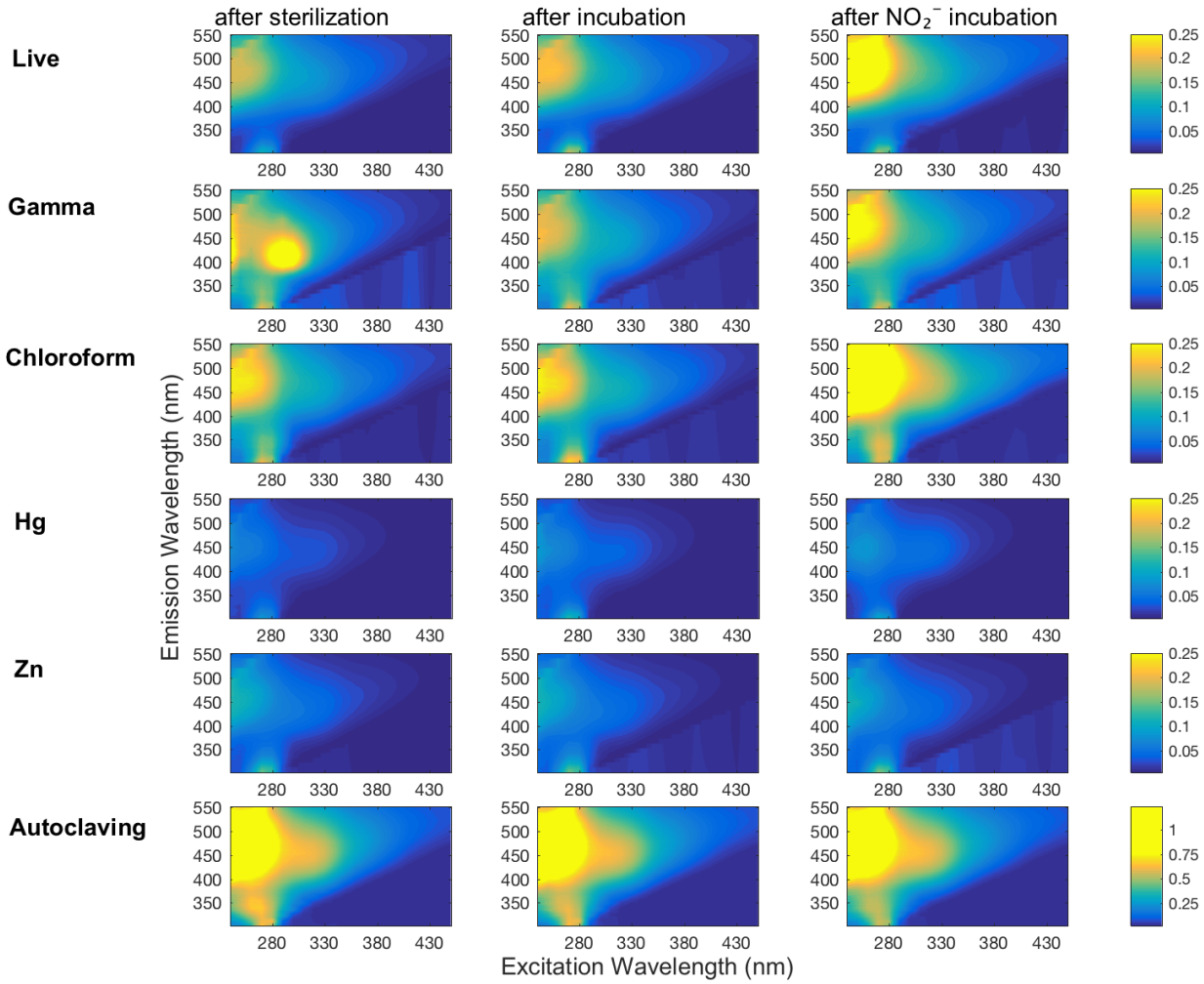
256

257 **Figure 3. Changes in extractable Fe^{2+} (left) and Fe^{3+} (right) concentration in Quistococha**
258 **peat soil incubations after sterilization (difference between sterilization baseline and live**
259 **baseline value) and after NO_2^- amendment and incubation (difference between NO_2^- and**
260 **control incubations).** Note the difference in scales. Values represent the extractable fraction of
261 both species. Error bars are one SD (n=2). The y-axis represents treatments as in the legend of
262 Fig. 1.

263

264 ***3.3 Effects of sterilants on soil chemistry***. In general, sterilization increased extractable Fe^{2+} and
265 Fe^{3+} relative to live controls (**Fig. 3**). This trend was particularly pronounced in Zn treatments,
266 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 in soil samples (e.g., Mn, Al, Cu, and Zn; data not shown).



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280 **Figure 4. Representative plots of DOM fluorescence in soil slurry incubations of**

281 **Quistococha peat soils.** DOM fluorescence is presented as excitation-emission matrices (EEMs)

282 collected for each treatment (rows) after the sterilization procedure or live control (left column),

283 after incubation with no amendment ("after incubation" control, middle column), and after

284 incubation with 300 µM NO_2^- (same time point as control, right column). The colored bar shows

285 the individual signal intensity. All but "autoclaving" treatment has same scale of signal intensity,

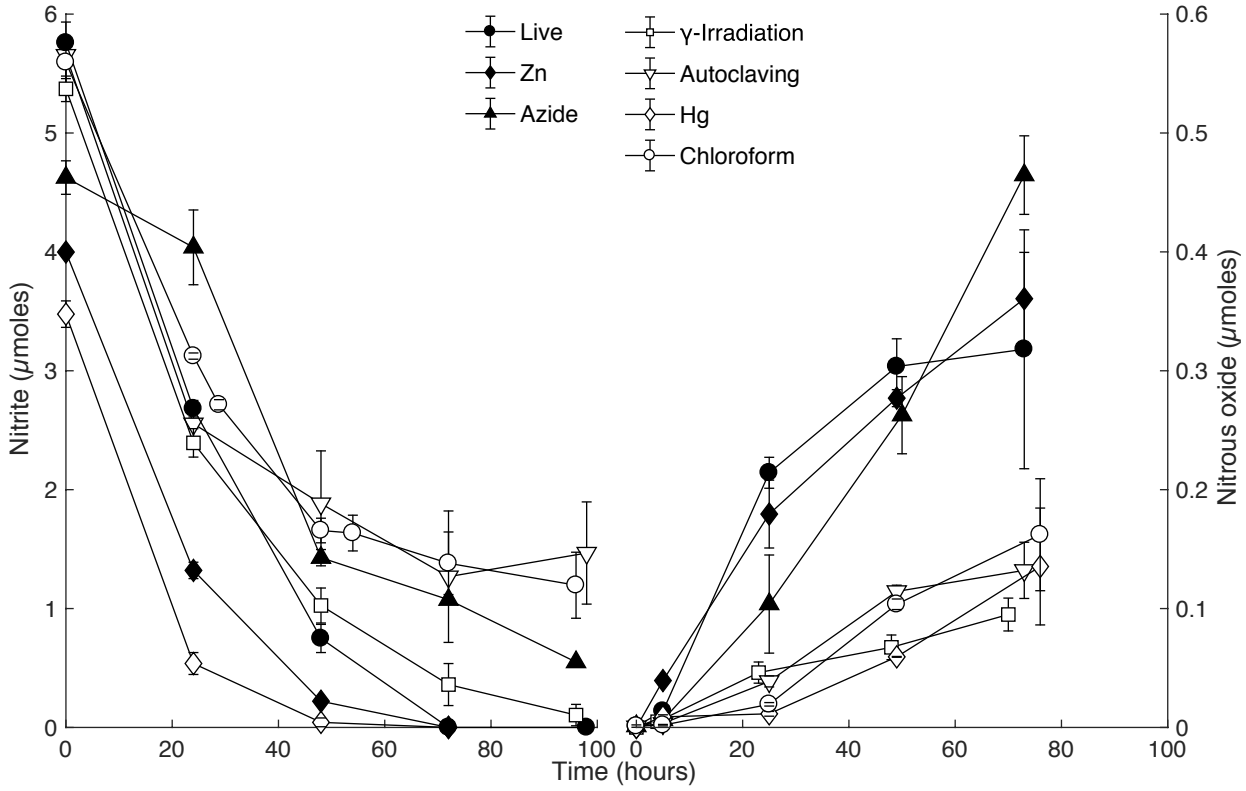
286 autoclaving effects increased about 5 times the signal intensity scale. Treatments as in the legend

287 of Fig. 1.

288

289 Fluorescence analysis of soil extracts using excitation-emission matrices (EEMs) was
290 used to evaluate changes in DOM containing aromatic moieties or conjugated double bonds
291 (Stedmon et al., 2003; **Fig. 4**). The N_3 treatment was excluded from this analysis due to an
292 interference with N_3 absorbance that prevented inner-filter corrections from being made. The
293 EEM signals showed the greatest change in the "humic" region (λ_{Ex} <240-270 nm, and λ_{Em} =
294 460-500 nm; (Fellman et al., 2010), especially in Zn and Hg treatments, which significantly
295 increased the FI from 1.20 (in live soil baseline, prior to NO_2^- incubation) to 1.49 (**Table 1**). Zn
296 and Hg may elicit direct fluorescence quenching by the formation of Zn and Hg metal complexes
297 (McKnight et al., 2001) or possibly due to indirect quenching by higher dissolved Fe^{2+}. Signal
298 strength in the humic region was enhanced by NO_2^- addition in the live, $CHCl_3$-fumigated, and γ-
299 irradiated treatments. All five sterilization treatments had lower aromaticity (HIX) than live
300 controls (**Table 1**). Autoclaved samples had tenfold higher total fluorescence compared to live
301 soils, suggesting that autoclaving degraded insoluble humics into more soluble and less
302 condensed OM.
303 ***3.4 Effects of sterilants on chemodenitrification and abiotic N_2O production***. In the first 48
304 hours, NO_2^- consumption rates were the highest in live soil (5.2 μM h^{-1}), closely followed by
305 irradiated samples (4.5 μM h^{-1}, **Fig. 5**). The major chemodenitrification pathway for N_2O
306 formation was likely NO_2^- reduction by Fe^{2+}, resulting in consumption of ~1.5 μmol Fe^{2+} and
307 accumulation of ~1.1 μmol Fe^{3+} in the live control (**Fig. 3**). After 48 hours, NO_2^- depletion
308 continued to completion in the live control but slowed in all treatments other than the metal
309 additions. After 72 hours of incubation, 3-16% of NO_2^--N was converted to N_2O-N across
310 treatments. N_2O production rates were assessed by linear regression of data points over the whole
311 duration of the experiment. Higher rates were observed in live, Zn^{2+}, and N_3^- treatments (0.5-0.7
312 nmol N_2O g^{-1} h^{-1}, $r^2 > 0.95$) than in γ-irradiated, $CHCl_3$-fumigated, autoclaved, and Hg treatments

313 (0.1-0.2 nmol N_2O g^{-1} h^{-1}, $r^2 > 0.9$). Production rates within treatments showing high or low rates
314 were not significantly different (Student's *t*, $p > 0.05$) although comparisons across treatments
315 with high or low rates were statistically different (Student's *t*, $p < 0.05$). Thus, we identified a
316 higher and lower group of sterilant-dependent N_2O production rates from the same soil samples.
317 The live control showed logarithmic N_2O accumulation while the sterilized treatments had linear
318 accumulation over time, the later as expectable in abiotic accumulation (**Fig. 5**).
319



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321
322 **Figure 5. NO_2^- consumption (left) and N_2O production (right) for different sterilant**
323 **treatments in soil slurry incubations of Quistococha peat soil.** Both N species were
324 simultaneously measured in all treatments. The product yield represents N_2O-N as molar fraction
325 of NO_2^--N. Note the difference in left and right y-axis scales. Error bars are one SD (n=3).
326 Treatments as in the legend of Fig. 1.

4 Discussion

4.1 Chemodenitrification is a dominant NO_2^- consumption process in slurry incubations of tropical peat soils. Similar NO_2^- consumption rates between live and irradiated treatments imply that NO_2^- 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). Linearity is more reflected in the N_2O curve than in the NO_2^- curve. The difference in linearity of N_2O 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_2^-, 0.5-8.1 mM Fe^{2+}, and a pH of 7-8 had similar rates of Fe^{2+} depletion but 10x higher rates of NO_2^- reduction, and higher (~10-50%) N_2O yields (Buchwald et al., 2016; Jones et al., 2015). In our peat incubations, reactive OM likely trapped NO_2^- 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, not N_2O (McKenney et al. 1990).

4.2 Artifacts due to sterilization methods for chemodenitrification assays. Azide and Zn exhibited enhanced NO_2^- conversion to N_2O, 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 N_3 treatments, elevated N_2O production could be explained by the reaction of protonated

351 NO_2^- with N_3 in a pH dependent manner (Stedman, 1959), plus other changes in soil solution
352 originated from the increase of pH. Nitrite reaction with N_3 has been characterized in marine and
353 freshwater solutions reaching its maximum at pH 4.5 and proceeding slowly yet significantly
354 (20% conversion in 1 hour) at pH > 5 (McIlvin and Altabet, 2005) as in our slurries. Moreover,
355 N_3's self-fluorescence impeded OM measurements, making N_3 an incompatible sterilizing agent
356 for chemodenitrification studies. Zn increased Fe availability and may have increased NO_2^-
357 affinity for reactive OM groups; both effects would lead to an abiotic increase in N_2O production
358 (Clark, 1962; McCalley and Sparks, 2009; Parton et al., 2007). Zinc treatment lowered the soil
359 pH, which may have promoted cation displacement and stability of dissolved Fe^{2+} (Hutchins et
360 al., 2007), thus enhancing N_2O production. Several studies have used Zn treatments as valuable
361 agent for field applications (Babbin et al., 2015; Ostrom et al., 2016). Zn is less hazardous to
362 humans than some of the other sterilants. We propose that the use of Zn could provide useful
363 information about abiotic *in-situ* rates as long as Zn-induced chemodenitrification is accounted
364 for. A correction could be applied if a complementary laboratory assessment (using the more
365 efficient γ-irradiation) were used to develop an ecosystem-specific correction factor.

366 Divalent Hg^{2+} can be abiotically methylated by fulvic acid-type substances (Rogers,
367 1977). The reaction oxidizes OM and can diminish its reducing power as indicated by decreased
368 reactivity of humic acid with NO_2^- (Gu et al., 2011; Zheng et al., 2011) thus interfering with the
369 abiotic assay. Given the pH effect of the Hg treatment, we cannot rule out that decomposition of
370 nitrous acid (HNO_2) contributed to NO_2^- consumption (Fig. 5, Park and Lee, 1988). Another
371 potential factor associated with the Hg treatments is metal sorption. At low pH (3.6), 98% of Hg
372 was sorbed to humic acids, whereas only 29% of Zn was sorbed at pH ~4.8 (Kerndorff and
373 Schnitzer, 1980). Full sorption capacity of peat is presumably reached in seconds (Bunzl et al.,
374 1976) and the differing sorption behavior of Hg and Zn may play a role in the reaction potential

375 of NO_2^- with OM. It has been demonstrated that Hg introduced into peat soil leads to sorption of
376 Hg ions to various functional groups, including phenols (Drexel et al., 2002; Xia et al., 1998).
377 Hence it is plausible that Hg sorbed to functional groups subject to electrophilic attack by
378 NO^+(e.g., nitrosophenol, Reaction Scheme 1) may hamper nitrosation, and therefore protect OM
379 from reacting with NO_2^-. This could lead to a selective suppression of the OM-dependent N_2O
380 production pathway.
381 Chloroform fumigation resulted in potential N_2O production rates within the lower
382 production range treatments with minor differences in Fe speciation and DOM fluorescence.
383 However, unlike the other sterilized samples, $CHCl_3$-fumigated samples showed enhanced CO_2
384 production stimulated by NO_3^- addition. Removal of $CHCl_3$ from our samples before substrate
385 addition could have provided an opportunity for a few surviving heterotrophs to re-grow and use
386 the easily-degradable organic material derived from dead cells. Indeed, chloroform can lyse cells,
387 providing substrates for growth to $CHCl_3$-resistant microorganisms (Zelles et al., 1997).
388 Continued exoenzyme activity has been also described as a CO_2 source: however, this would not
389 include denitrification enzymes, since no enzymes involved in the denitrification pathway are
390 exoenzymes (Blankinship et al., 2014; Jenkinson and Powlson, 1976a). Chlorination of natural
391 OM may prompt formation of quinones (Criquet et al., 2015), which are intermediates in the OM-
392 based abiotic N_2O production (Thorn and Mikita, 2000); indeed, regions of the EEMs
393 corresponding to hydroquinones (Cory and McKnight, 2005) appear to be slightly higher in
394 $CHCl_3$ treatments. The benzene derivative produced during nitrosophenol reaction with NO_2^-
395 leads to reduced π-electron delocalization (Reaction Scheme 1). Because excitation of π-electrons
396 produces fluorescence, reactions with NO_2^- might be expected to reduce OM fluorescence.
397 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 $CHCl_3$ might keep down the numbers of any potential denitrifiers improving the use of this method.

Autoclaved peat soil revealed abiotic N_2O production rates close to the average of the lower production range group, along with ICP-OES and fluorescence spectroscopy results that 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 N_2O 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 N_2O 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.

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		0.41	
	Nitrite added	1.16	*	7.11	*	0.40	12.1±6.1
Zn	*Baseline*	1.49	*b*	2.70	*b*	0.58	
	Control	1.50		2.27		0.59	
	Nitrite added	1.55	*	2.05		0.62	5.9±4.0
Autoclaving	*Baseline*	1.20	*a*	2.54	*b*	0.47	
	Control	1.20		2.83		0.46	
	Nitrite added	1.20		2.97		0.43	31.5±24.6
Chloroform	*Baseline*	1.23	*c*	2.79	*b*	0.43	
	Control	1.27		2.70		0.44	
	Nitrite added	1.14	*	4.12	*	0.40	13.5±6.4
γ-Irradiation	*Baseline*	1.30	*d*	1.90	*b*	0.57	
	Control	1.27		2.35		0.56	
	Nitrite added	1.21	*	2.95		0.52	2.4±0.8
Hg	*Baseline*	1.49	*b*	2.20	*b*	0.57	
	Control	1.50		1.60		0.56	
	Nitrite added	1.44	*	2.12		0.51	13.8±3.9

* 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_2O emissions occurs 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_2O conversion from nitrite amendment. We also demonstrated that γ-irradiation is the "gold standard" for chemodenitrification assays. The application of N_3 to quantify abiotic N_2O production is unsuitable because changes associated to fraction of the sterilant itself may react to form N_2O and effects increased pH. $CHCl_3$ and γ-rays have slightly reducing effects on the soil Fe pool and might lead to a weak discrimination against pathways involving Fe as reactant. $CHCl_3$ 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 $CHCl_3$ removal is its main drawback. Autoclaving seemed to have minor disadvantages on abiotic N_2O 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_2O 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_2O 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_2O fluxes from soil ecosystems.

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Competing Interests Statement

The authors have no competing interests to declare.