

1 **Effects of sterilization techniques on chemodenitrification and N₂O production in tropical**
2 **peat soil microcosms**

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18 **Keywords:** soil sterilization, chemodenitrification, abiotic N₂O production, tropical peatlands

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22 **Abstract**

23 Chemodenitrification – the non-enzymatic process of nitrite reduction – may be an important sink
24 for fixed nitrogen in tropical peatlands. Rates and products of chemodenitrification are dependent
25 on O₂, pH, Fe²⁺ concentration and organic matter composition, which are variable across peat
26 soils. Assessing abiotic reaction pathways is difficult because sterilization/inhibition agents can
27 alter the availability of reactants by changing iron speciation and organic matter composition. We
28 compared six commonly used soil sterilization techniques – γ -irradiation, chloroform,
29 autoclaving, and chemical inhibitors (mercury, zinc, and azide) – for their compatibility with
30 chemodenitrification assays for tropical peatland soils (organic-rich low pH soil from the Eastern
31 Amazon). Out of the six techniques, γ -irradiation resulted in soil treatments with lowest cell
32 viability and denitrification activity, and least effect on pH, iron speciation, and organic matter
33 composition. Nitrite depletion rates in γ -irradiated soils were highly similar to untreated/live
34 soils, whereas other sterilization techniques showed deviations. Chemodenitrification was a
35 dominant process of NO₂⁻ consumption in tropical peatland soils assayed in this study. N₂O is
36 one possible product of chemodenitrification reactions. Abiotic N₂O production was low to
37 moderate (3-16% of converted nitrite), and different sterilization techniques lead to significant
38 variations on production rates due to inherent processes or potential artifacts. Our work represents
39 the first methodological basis for testing the abiotic denitrification and N₂O production potential
40 in tropical peatland soil.

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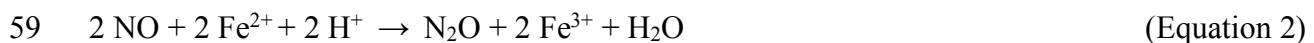
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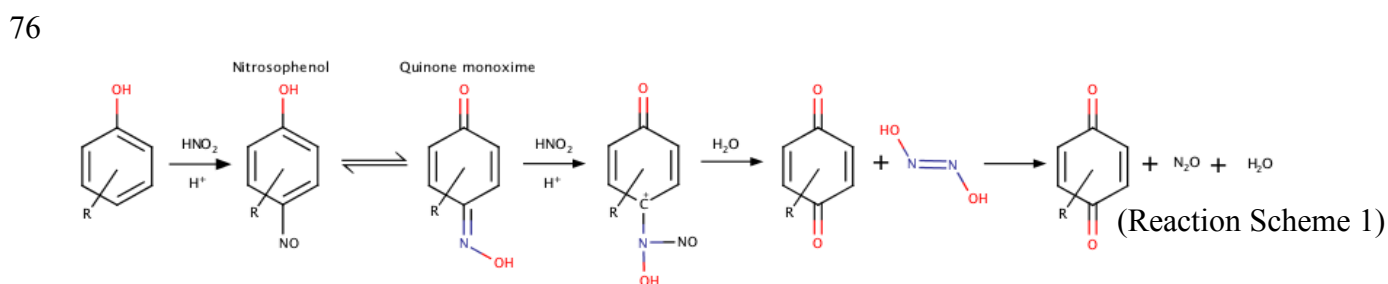
48 **1 Introduction**

49 Across ecosystems, physical and chemical factors, such as solar radiation or redox gradients, can
50 drive abiotic chemical transformations. The nitrogen (N) cycle, in particular, includes abiotic
51 reactions that can affect the retention of nutrients or substrates (Clark, 1962; McCalley and
52 Sparks, 2009; Parton et al., 2007). Abiotic formation of N-containing gases has long been known
53 (Jun et al., 1970; Wullstein and Gilmour, 1966). A major abiotic process in the N cycle is
54 chemodenitrification, the step-wise reduction of nitrite (NO_2^-) to gaseous products, namely nitric
55 oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2), often coupled to iron (Fe^{2+}) oxidation, as
56 described in Eq. 1 and 2 (Davidson et al., 2003; Kampschreur et al., 2011; Zhu et al., 2013; Zhu-
57 Barker et al., 2015).



60 Eq. 1 and 2 are plausible in soils and sediments (Jones et al., 2015). The abiotic reduction of N_2O
61 to N_2 is not well known. It has been associated with the presence of copper (Moraghan and
62 Buresh, 1977), but this species is unlikely to be present at sufficient levels in peat soils to
63 promote this reaction. Anoxic tropical peat soils are expected to have the ideal conditions for
64 chemodenitrification: low- O_2 , low pH, high organic matter (OM), and high Fe^{2+} (Kappelmeyer et
65 al., 2003; Nelson and Bremner, 1969; Porter, 1969; Van Cleemput et al., 1976). In these
66 ecosystems, NO_x^- is supplied by nitrification fueled by organic N mineralization or from external
67 sources (fertilization, wet or dry deposition). Besides metals, reduction of NO_x^- compounds can
68 also be mediated by organic functional groups found in soils. Abiotic phenol oxidation occurs at
69 oxic-anoxic interfaces in tropical soils, and may be linked to the N cycle (Hall and Silver, 2013).

70 In such reactions, NO_2^- can be reduced by phenolic groups to form the nitrosonium cation NO^+ ,
 71 which can either (1) remain fixed within the organic compound as nitrosophenol (Thorn and
 72 Mikita, 2000; Thorn et al., 2010), or (2) be emitted in gaseous form. After tautomerization to an
 73 oxime (Raczyńska et al., 2005) and reaction with NO^+ derived from a second NO_2^- ion,
 74 hyponitrous acid ($\text{H}_2\text{N}_2\text{O}_2$) can be produced, which further decomposes to N_2O (e.g., Reaction
 75 Scheme 1; Porter 1969; Stevenson et al., 1970).



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

79 The importance of abiotic N transformations in environmental samples has been
 80 notoriously difficult to quantify due to the artifacts emerging from physical or chemical “killing”
 81 methods intended to eliminate biological activity but affecting metals, organic matter, or other
 82 pools. In order to distinguish denitrification from chemodenitrification, enzymes contributing to
 83 gaseous N production must be inactivated, most commonly by addition of sterilants or inhibitors.
 84 An efficient sterilization treatment ideally: (1) contains a negligible number of live cells, (2)
 85 eliminates biological activity, and (3) has little or no effect, directly or indirectly, on abiotic
 86 reactions (e.g., it should not alter mineral structure, nor lyse cells because release of cellular
 87 contents could influence abiotic reactions). Because rates and products of chemodenitrification
 88 are dependent on O_2 , pH, Fe^{2+} concentration and OM composition, it is important to assess
 89 whether a sterilant/inhibitor elicits a physicochemical change that can affect the availability or
 90 interaction of these reactants.

91 Soil sterilization techniques include γ -irradiation, chloroform (CHCl_3) fumigation,
92 autoclaving, and addition of chemical inhibitors such as mercury (Hg), zinc (Zn), or azide (N_3).
93 Highly energetic γ -irradiation damages enzymes and cell components, rendering cells non-viable
94 and inactive, generally with minimal effect on soil chemistry (Trevors, 1996). Autoclaving with
95 high-pressure steam disrupts cell membranes, denatures proteins, and decreases aromaticity and
96 polycondensation of soil OM (Berns et al., 2008; Jenkinson and Powlson, 1976b; Trevors, 1996).
97 Fumigation with CHCl_3 induces cell lysis and has minimal effect on enzymes (Blankinship et al.,
98 2014). Chemicals like Hg, Zn, and N_3 do the opposite: they inhibit enzymes (Bowler et al., 2006;
99 McDevitt et al., 2011), but do not lyse cells (Wolf et al., 1989).

100 We evaluated the appropriateness of six sterilants (γ -irradiation, autoclaving, CHCl_3 , Hg,
101 Zn, and N_3) for chemodenitrification measurements in low- O_2 , low-pH, high-OM tropical peat
102 soils. First, we tested the effects of sterilants on cell membrane viability and biological
103 denitrification activity. Next, we evaluated the effects of sterilants on soil chemistry (pH, OM
104 composition, and extractable Fe). Finally, we assessed the effects of the six sterilants on
105 chemodenitrification measured by NO_2^- depletion and N_2O production.

106

107 **2 Materials and Methods**

108 **2.1 Sample characteristics.** Soil samples were collected in October 2015 from a tropical
109 peatland, locally known as Quistococha ($3^\circ 50' \text{S}$, $73^\circ 19' \text{W}$), near Iquitos (Loreto, Peru). The soil
110 geochemistry of this site has been described previously (Lawson et al., 2014; Lahteenoja et al.,
111 2009). The samples were obtained from depths of 15-30 cm below the water table and kept
112 strictly anoxic during transport and storage at 4°C in the dark. Water saturation and organic
113 carbon content were determined by oven drying and loss-on-ignition, respectively. Dissolved
114 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 (Afkhani 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 ⁶⁰Co 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% H₂ in
128 N₂) 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 CHCl₃-treated samples were fumigated for 48 hours under a 100% N₂
131 atmosphere. Because volatilized CHCl₃ corrodes electron capture detectors used for N₂O
132 detection (see below), CHCl₃ was removed by flushing the vials with N₂ 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

139 heterotrophic growth based on visual inspection of soil extract on agar plates exposed to 0.5 to
140 92.1 mg L⁻¹, which includes concentrations demonstrated to be effective previously (Tuominen et
141 al., 1994).

142 After the initial physical or chemical treatment, triplicate incubations were diluted
143 1:10 in 20 mL of autoclaved 18.2 MΩ-cm water in 60 mL glass serum vials. All microcosms
144 were prepared in an anaerobic glove box (0.5% H₂ in N₂) prior to incubation. Triplicate soil
145 slurries were amended from anoxic, sterile stock solution to a final concentration of 300 μM
146 NO₂⁻ (6 μmoles in 20 mL) and sealed with thick butyl rubber stoppers. A parallel set of samples
147 was amended with 300 μM NO₃⁻ to evaluate denitrification potential with CO₂ measurements.
148 Control incubations received an equivalent volume of autoclaved 18.2 MΩ-cm water without
149 NO_x⁻. Soil microcosms were incubated in the dark at a constant temperature of 25°C. NO₂⁻ was
150 quantified in all soil treatments using the Griess assay (Promega, Kit G2930; e.g., Griess 1879).
151 pH measurements were taken with an Orion 3 Star meter (Thermo Scientific) before and after
152 sterilization, and at the end of the experiment after 70-76 hours of incubation.

153 **2.3 Gas chromatography.** To quantify N₂O and CO₂ production, 200 μL of headspace gas was
154 sampled with a gas-tight syringe (VICI Precision Sampling) and injected onto a gas
155 chromatograph (GC, SRI Instruments) equipped with both an electron-capture detector (ECD)
156 and a flame-ionization detector (FID). Two continuous HayeSep-D columns were kept at 90°C
157 (oven temperature); N₂ (UHP grade 99.999%, Praxair Inc.) was used as carrier gas, and H₂ for
158 FID combustion was supplied by a H₂ generator (GCGS-7890, Parker Balston). For CO₂
159 measurements, a methanizer (which reduces CO₂ to the detectable CH₄ via a Ni catalyst at
160 355°C) was run in line before the FID. The ECD current was 250 mV and the ECD cell was kept
161 at 350°C. The N₂O and CO₂ measurements were calibrated using customized standard mixtures
162 (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 $k_H^{cc}(\text{N}_2\text{O}) = 0.6112$ and $k_H^{cc}(\text{CO}_2) = 0.8313$ (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 mm² 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% H₂
181 in N₂ glove box. The O₂ concentration in the glove box was continuously monitored and remained
182 <10 ppm. To separate Fe³⁺ from Fe²⁺, 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 Fe³⁺ more effectively than it chelates Fe²⁺. The Fe²⁺-containing
186 aqueous phase was sampled into a 3-fold HCl-washed HDPE vial (Nalgene) in the glove box.

187 The Fe³⁺ 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 Fe³⁺ and Fe²⁺ 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 HNO₃.

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 ($\lambda_{Em} = 300-550$ 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 M Ω -cm;
200 Barnsteadtm 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 μ m) 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

211 emission intensity at 380 nm to the emission intensity maximum between 420 and 435 nm, both
212 collected at an excitation wavelength of 310 nm (Wilson and Xenopoulos, 2009).

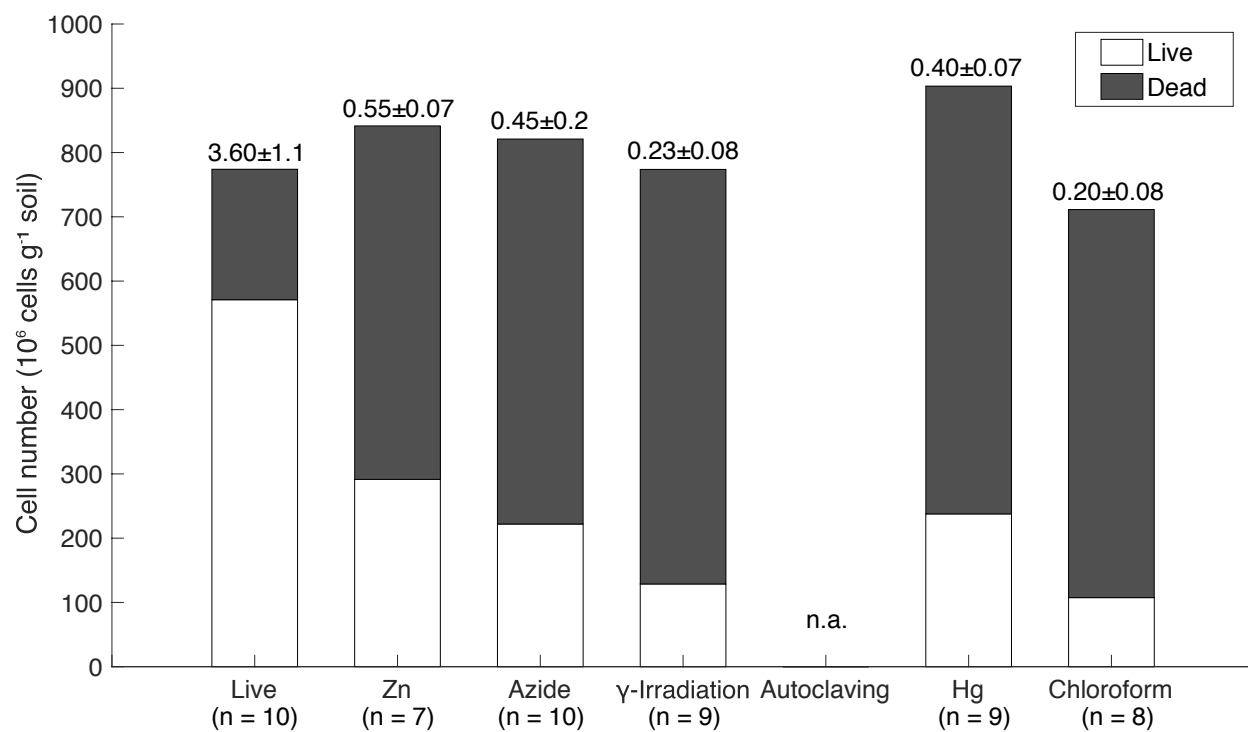
213 **2.7 Statistical Analyses.** All basic statistical tests were performed with JMP Pro software
214 (Version 13.1.0, SAS Institute Inc., Cary, NC, USA).

215

216 **3 Results**

217 **3.1 Composition of high-OM tropical soils.** The tropical peat soil used for the incubation
218 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
219 total Fe kg⁻¹ soil. The extractable iron fraction partitioned as 54±3 µM extractable Fe³⁺ and
220 213±16 µM extractable Fe²⁺. The native soil pore water had 13.2±1.2 mg L⁻¹ DOC, 436±79 µg N
221 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
222 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,
223 and 5.4 after treatment with Hg, Zn, γ-irradiation, autoclaving, and CHCl₃, respectively. Only N₃
224 treatment increased soil pH (to 6.4).

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226

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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 = ZnCl₂, Hg = HgCl₂, Azide = NaN₃.

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3.2 Effects of sterilants on cell integrity and potential of denitrifying activity. Live/dead dyes

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were used to assess microbial viability by means of membrane integrity, where a “dead” signal

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indicates disrupted or broken cell membranes (Stiefel et al., 2015). The majority (74%) of cells in

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the live incubation displayed the “live” signal (**Fig. 1**). The CHCl₃ and γ-irradiated treatments

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were most effective at reducing the number of viable cells (~15% intact membranes after

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sterilization). Chemical inhibitors (Hg, Zn, and N₃) were less effective at killing cells (~30%

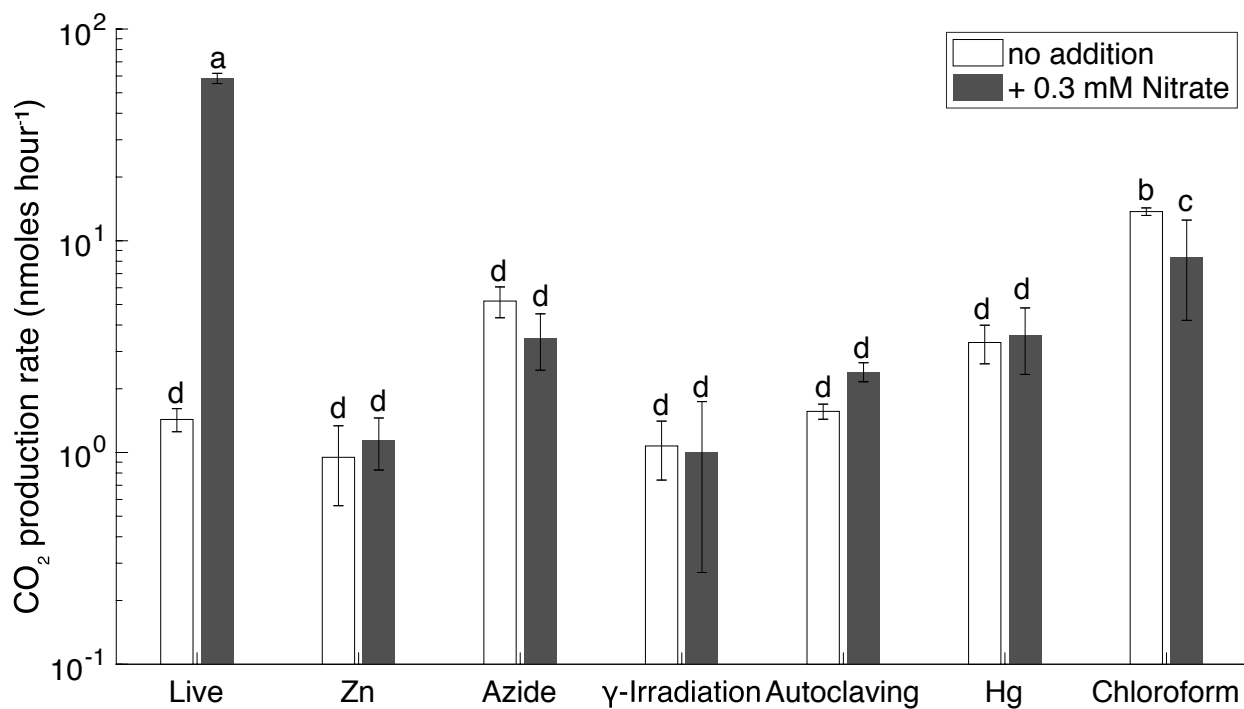
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intact membranes after sterilization). Autoclaved samples did not fluoresce, likely due to cell

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lysis during steam pressurization.

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243 **Figure 2. CO₂ production rates in 3-day soil slurry incubations of Quistococha peat soil**

244 **amended with and without 0.3 mM NO₃⁻.** Error bars are one SD (n=3). Columns marked with

245 the same letter are not statistically different from each other (Student's *t*, *p* > 0.05, n=3). The x-

246 axis represents treatments as in the legend of Fig. 1.

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248 Biological denitrification activity was measured over three days in live and sterilized soils

249 based on the difference in CO₂ production with and without added NO₃⁻. An efficient sterilization

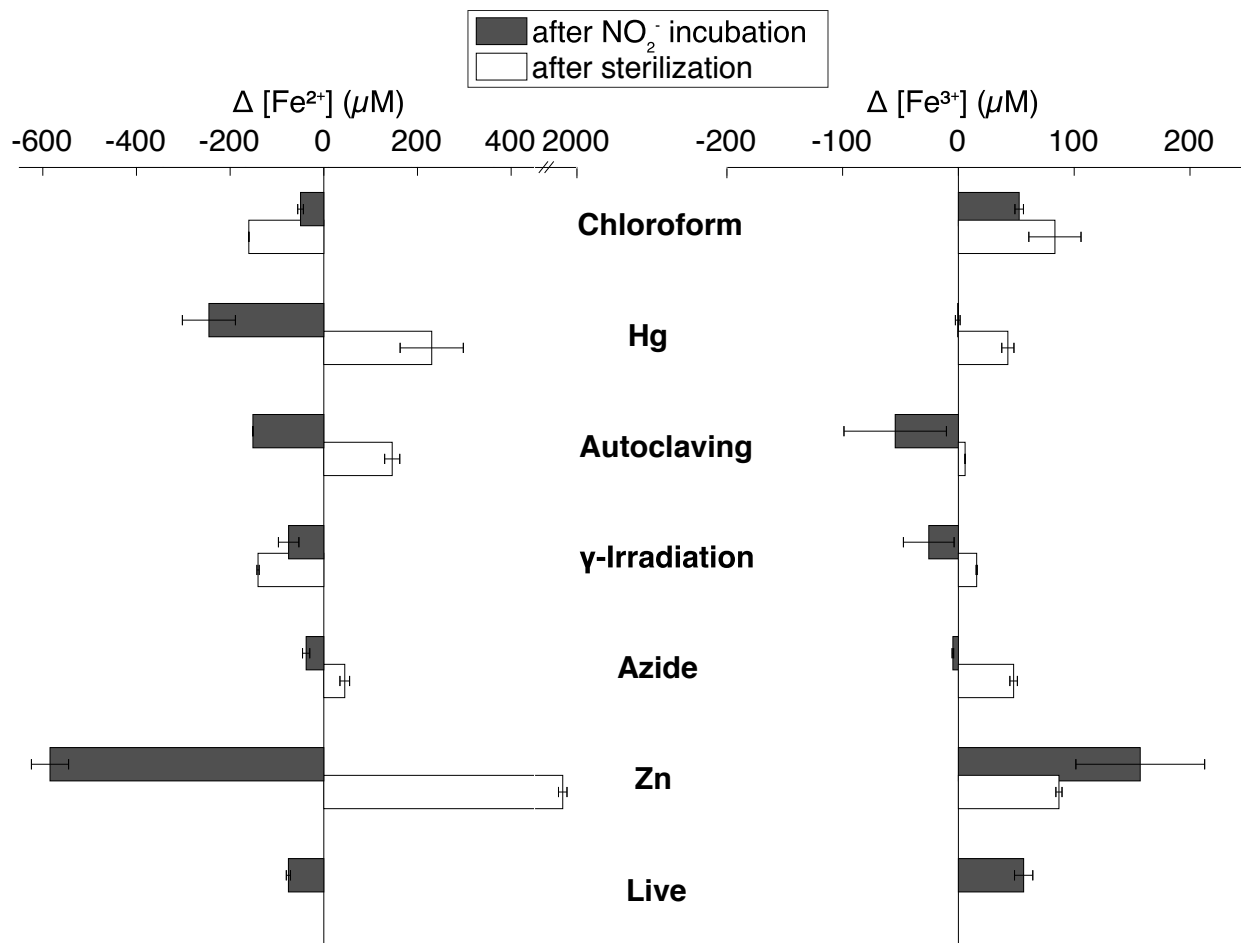
250 treatment would show no changes in CO₂ beyond that due to equilibration between the gas phase

251 and aqueous phase. Nitrate stimulated CO₂ production in live soil (ANOVA, *p* < 0.05) and not in

252 the γ-irradiated, Zn, Hg, N₃, or autoclaved incubations (**Fig. 2**), indicating that residual cells in

253 the sterilized treatments were not capable of denitrification.

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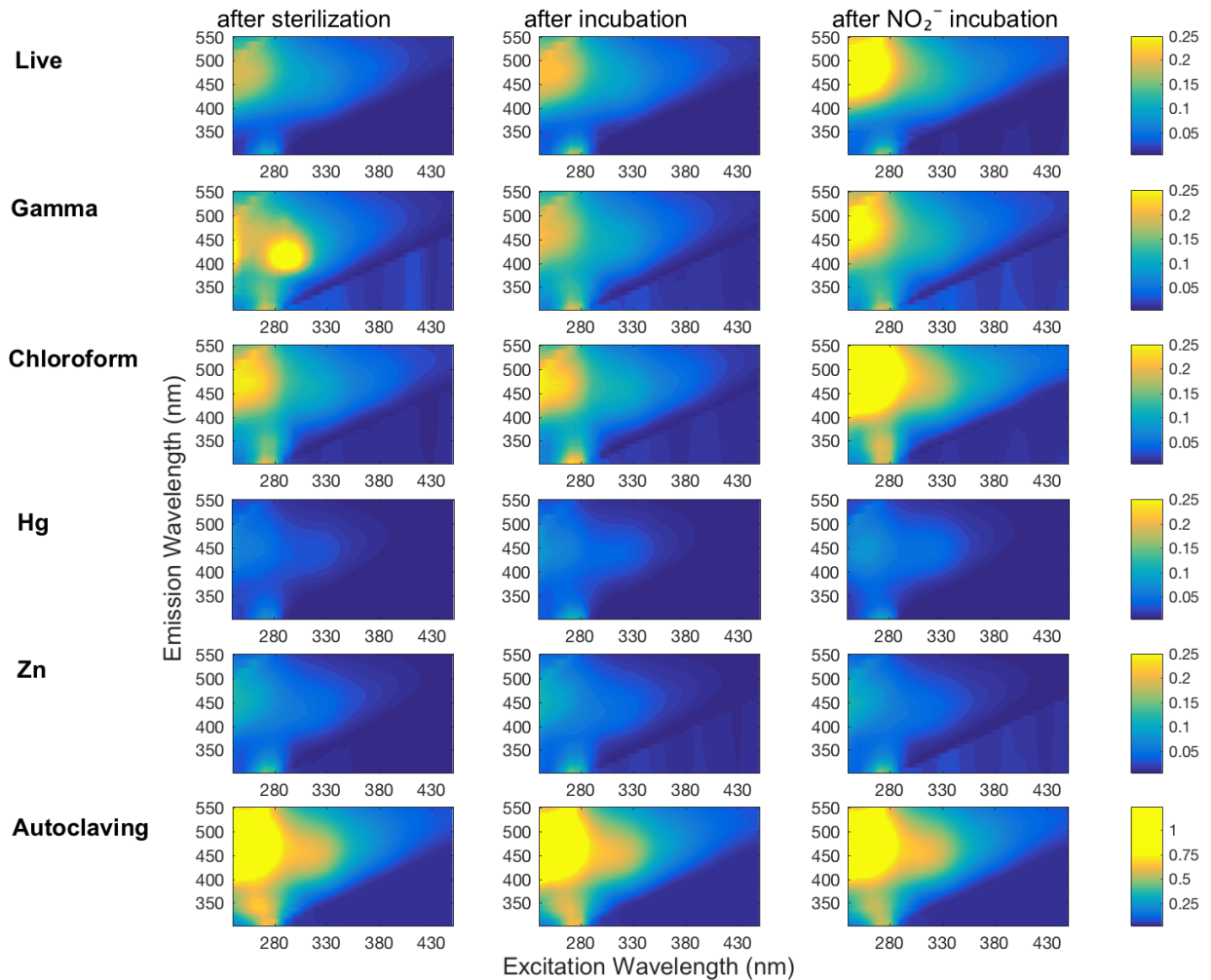
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257 **Figure 3. Changes in extractable Fe²⁺ (left) and Fe³⁺ (right) concentration in Quistococha**
 258 **peat soil incubations after sterilization (difference between sterilization baseline and live**
 259 **baseline value) and after NO₂⁻ amendment and incubation (difference between NO₂⁻ 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 Fe²⁺ and
 265 Fe³⁺ relative to live controls (**Fig. 3**). This trend was particularly pronounced in Zn treatments,
 266 which had 9x higher extractable Fe²⁺ (1915±26 μM) and 1.6x higher extractable Fe³⁺ (87±3 μM)

267 than live controls. The Hg treatment showed the second largest increases. In the presence of
268 NO_2^- , extractable Fe^{2+} decreased and extractable Fe^{3+} increased in live, Zn, and CHCl_3 -fumigated
269 treatments, as expected if Fe^{2+} was oxidized by NO_2^- during chemodenitrification. However,
270 autoclaving, γ -irradiation, and N_3 lowered Fe^{3+} concentrations, suggesting the influence of
271 unknown concomitant reactions. For instance, autoclaving (largest drop in Fe^{3+}) already showed
272 lower Fe^{3+} concentrations after sterilization. Production of Fe^{3+} -reduction artifacts in treatments
273 could lead to Fe^{3+} depletion and, hence, mask increase in Fe^{3+} due to chemodenitrification. NO_2^-
274 addition resulted in near-complete depletion of extractable Fe^{2+} in live, CHCl_3 -fumigated, and γ -
275 irradiated soils. Changes in Fe speciation with other sterilants were more moderate. Minimal
276 changes were observed for other metals in soil samples (e.g., Mn, Al, Cu, and Zn; data not
277 shown).

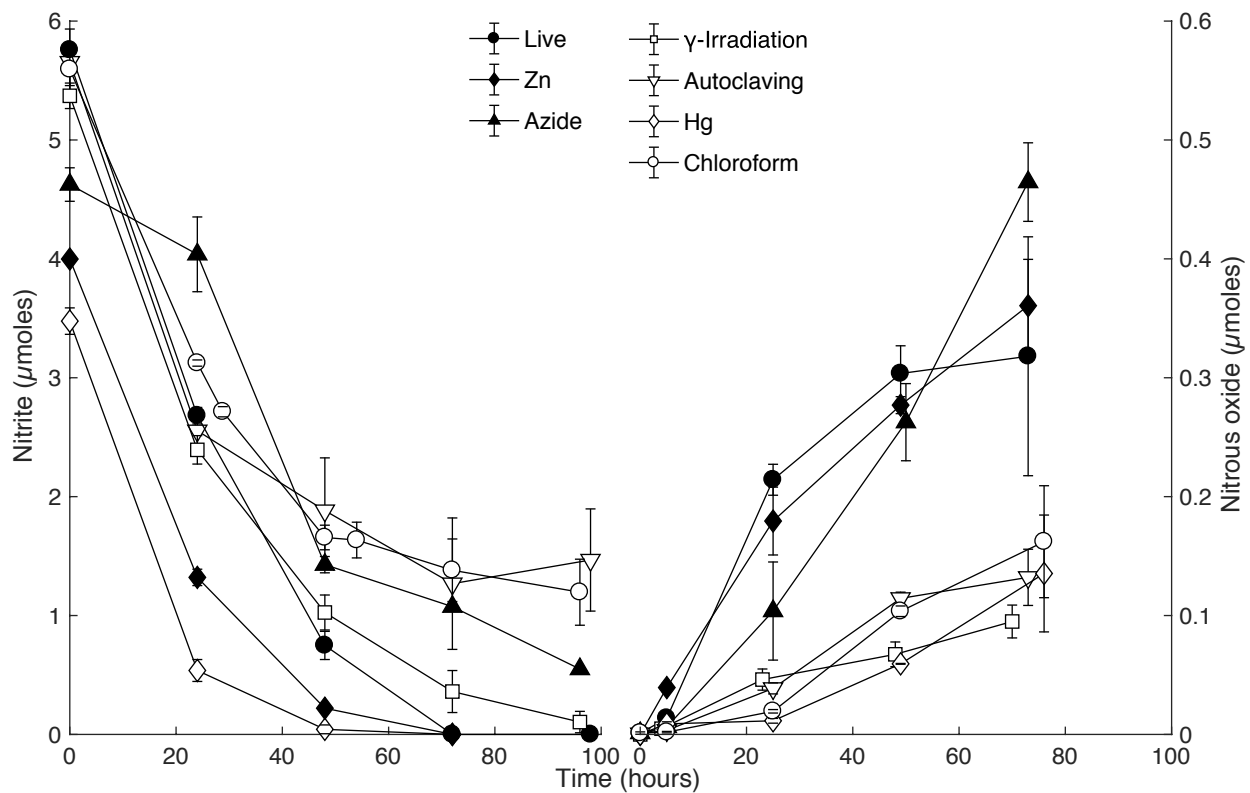


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 280 **Figure 4. Representative plots of DOM fluorescence in soil slurry incubations of**
 281 **Quistococha peat soils.** DOM fluorescence is presented as excitation-emission matrices (EEMs)
 282 collected for each treatment (rows) after the sterilization 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 ($\lambda_{Ex} < 240-270$ nm, and $\lambda_{Em} =$
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 ($5.2 \mu M h^{-1}$), closely followed by
305 irradiated samples ($4.5 \mu M h^{-1}$, **Fig. 5**). The major chemodenitrification pathway for N_2O
306 formation was likely NO_2^- reduction by Fe^{2+} , resulting in consumption of $\sim 1.5 \mu mol Fe^{2+}$ and
307 accumulation of $\sim 1.1 \mu mol Fe^{3+}$ 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 N₂O g⁻¹ h⁻¹, r² > 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 N₂O production rates from the same soil samples.
 317 The live control showed logarithmic N₂O accumulation while the sterilized treatments had linear
 318 accumulation over time, the later as expectable in abiotic accumulation (**Fig. 5**).
 319



320
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 322 **Figure 5. NO₂⁻ consumption (left) and N₂O 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 N₂O-N as molar fraction
 325 of NO₂⁻-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.

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330 **4 Discussion**
331 ***4.1 Chemodenitrification is a dominant NO₂⁻ consumption process in slurry incubations of***
332 ***tropical peat soils.*** Similar NO₂⁻ consumption rates between live and irradiated treatments imply
333 that NO₂⁻ depletion was dominated by abiotic processes over the first 48 hours. In general,
334 abiotic reactions tend to be linear processes, whereas microbially mediated reactions can be
335 affected by enhanced expression of genes or cell reproduction in a nonlinear fashion (Duggleby,
336 1995). Linearity is more reflected in the N₂O curve than in the NO₂⁻ curve. The difference in
337 linearity of N₂O production in sterilized vs. live treatments (**Fig. 5**) suggests that biological
338 denitrification did not occur in sterilized soils.

339 Compared to our study, incubations of artificial media with 200 μM NO₂⁻, 0.5-8.1
340 mM Fe²⁺, and a pH of 7-8 had similar rates of Fe²⁺ depletion but 10x higher rates of NO₂⁻
341 reduction, and higher (~10-50%) N₂O yields (Buchwald et al., 2016; Jones et al., 2015). In our
342 peat incubations, reactive OM likely trapped NO₂⁻ in the soil matrix via OM-bound nitrosation
343 reactions (Thorn and Mikita, 2000; Thorn et al., 2010) and the lower pH likely promoted
344 conversion of NO₂⁻ to NO (Kappelmeyer et al., 2003; Porter, 1969) or N₂ (Stevenson et al.,
345 1970). Studies in low pH northern temperate peat soils, have shown the primary product of
346 abiotic NO₂⁻ reduction was NO, not N₂O (McKenney et al. 1990).

347 ***4.2 Artifacts due to sterilization methods for chemodenitrification assays.*** Azide and Zn
348 exhibited enhanced NO₂⁻ conversion to N₂O, at rates at least twice to five times as high as those
349 measured for the other sterilants (**Fig 5**), likely due to higher pH and Fe availability, respectively.
350 In the N₃ treatments, elevated N₂O 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 (Babbitt 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

398 treatment, short experimental periods (e.g., hours or days) or reapplication of CHCl_3 might keep
399 down the numbers of any potential denitrifiers improving the use of this method.

400 Autoclaved peat soil revealed abiotic N_2O production rates close to the average of the
401 lower production range group, along with ICP-OES and fluorescence spectroscopy results that
402 showed significant changes in Fe speciation and DOM composition. EEMs demonstrate lower
403 values for the HIX in autoclaved peats (**Table 1**), consistent with fluorescence data from a study
404 that demonstrated a decrease in the aromaticity and polycondensation of soil extracts from
405 autoclaved soil (Berns et al., 2008). Autoclaving likely caused degradation and solubilization of
406 insoluble humic components. The direct effects of autoclaving are very much dependent on the
407 heat and pressure stability of the indigenous soil constituents, but the substantial soil structural
408 changes likely introduce chemical artifacts that are absent in the native live soil.

409 ***4.3 Gamma irradiation is the preferred sterilization method for chemodenitrification assays.***

410 The fewest chemical artifacts were observed in γ -irradiated samples. Soil that had been exposed
411 to γ -rays showed the lowest N_2O production rates, approximately one-fifth of those observed in
412 live samples. Irradiation also caused only very small changes in Fe speciation relative to live
413 controls and yielded EEMs that were remarkably similar to those obtained from live soil extracts.
414 Our measurements of sterility and respiratory activity indicated the lowest potential for biological
415 activity and hence, the least amount of interference for the time period tested. We therefore
416 confirmed γ -irradiation to be a preferred method for sterilizing soil (Trevors, 1996) and for
417 assessing abiotic N_2O production potentials. In practice, the long preparation time needed to
418 reach a sufficient dose (dependent on radiation source, see **Methods**) was compensated for by the
419 lack of chemical artifacts during the experiment and the reduced number of hazardous waste
420 products. Limited accessibility to irradiation facilities and the absence of a field portable option
421 remain the main challenges to wide distribution of this approach.

422

423 **Table 1. Characteristics of dissolved organic matter in soil extracts from incubations of peat**424 **from Quistococha, Peru.** FI, HIX, and freshness indices were calculated as described in the

425 methods section. The “tyrosine-like” region is defined at an excitation of 270-275 nm and an

426 emission of 304-312 nm (Fellman et al., 2010). The signal for that region was averaged across

427 replicates and expressed as percent difference between NO₂⁻ additions and controls ± standard

428 deviation of replicates. A drop in the signal intensity was consistently apparent, clear differences

429 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	<i>Baseline</i>	1.20	<i>a</i>	5.57	<i>a</i>	0.44	
	<i>Control</i>	1.21		4.72		0.41	
	<i>Nitrite added</i>	1.16	*	7.11	*	0.40	12.1±6.1
Zn	<i>Baseline</i>	1.49	<i>b</i>	2.70	<i>b</i>	0.58	
	<i>Control</i>	1.50		2.27		0.59	
	<i>Nitrite added</i>	1.55	*	2.05		0.62	5.9±4.0
Autoclaving	<i>Baseline</i>	1.20	<i>a</i>	2.54	<i>b</i>	0.47	
	<i>Control</i>	1.20		2.83		0.46	
	<i>Nitrite added</i>	1.20		2.97		0.43	31.5±24.6
Chloroform	<i>Baseline</i>	1.23	<i>c</i>	2.79	<i>b</i>	0.43	
	<i>Control</i>	1.27		2.70		0.44	
	<i>Nitrite added</i>	1.14	*	4.12	*	0.40	13.5±6.4
γ-Irradiation	<i>Baseline</i>	1.30	<i>d</i>	1.90	<i>b</i>	0.57	
	<i>Control</i>	1.27		2.35		0.56	
	<i>Nitrite added</i>	1.21	*	2.95		0.52	2.4±0.8
Hg	<i>Baseline</i>	1.49	<i>b</i>	2.20	<i>b</i>	0.57	
	<i>Control</i>	1.50		1.60		0.56	
	<i>Nitrite added</i>	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.

430

431 **5 Conclusion**

432 High N₂O emissions occurs in tropical regions with water-saturated soils (Lienggaard et al., 2014;
433 Park et al., 2011; Pérez et al., 2001). Whether these tropical N emissions are solely biotic or have
434 abiotic contributions is not well known, because rates of chemodenitrification are not commonly
435 evaluated. Abiotic processes in the N cycle remain overlooked, partly due to the lack of reliable
436 means of quantifying abiotic reactions. This study showed that chemodenitrification occurs in a
437 tropical peat soil, leading to a low to moderate fraction of N₂O conversion from nitrite
438 amendment. We also demonstrated that γ -irradiation is the “gold standard” for
439 chemodenitrification assays. The application of N₃ to quantify abiotic N₂O production is
440 unsuitable because changes associated to fraction of the sterilant itself may react to form N₂O and
441 effects increased pH. CHCl₃ and γ -rays have slightly reducing effects on the soil Fe pool and
442 might lead to a weak discrimination against pathways involving Fe as reactant. CHCl₃ fumigation
443 was another approach with limited effects on Fe chemistry that lowered the number of viable
444 cells greatly, however, the potential for microbial regrowth after CHCl₃ removal is its main
445 drawback. Autoclaving seemed to have minor disadvantages on abiotic N₂O production, despite
446 the substantial changes to soil OM.

447 Unlike other lab-intensive treatments, the application of Zn and Hg are amenable for field
448 experiments; however, we observed distinct chemical artifacts when using both of these options.

449 Care is warranted if using Zn and Hg chemical inhibitors, which can increase Fe availability and
450 may thus overestimate Fe-dependent abiotic N₂O production rate. A potential disadvantage of the
451 application of toxic metals is a decrease in soil pH. We cannot exclude pH-driven effects on N
452 intermediates; however, no major deviation in the final N₂O production rate related to
453 acidification was observed. With the methodological evaluation presented here, we determined
454 that a directed selection of approaches can allow for better constrained and more detailed studies
455 of the role of abiotic pathways and soil components shaping denitrification and N₂O fluxes from
456 soil ecosystems.

457

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659 **Competing Interests Statement**

660 The authors have no competing interests to declare.