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Discussions
EGU

1   **Effects of sterilization techniques on chemodenitrification and N$_2$O production in tropical**

2   **peat soil microcosms**

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18   **Keywords**: soil sterilization, chemodenitrification, abiotic N$_2$O production, tropical peatlands

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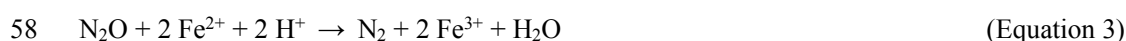21   hinsby@asu.edu

22   **Abstract**

23   Chemodenitrification – the non-enzymatic process of nitrite reduction – may be an important sink

24   for fixed nitrogen in tropical peatlands with low oxygen, low pH, high organic matter, and

25   variable ferrous iron concentrations. Assessing abiotic reaction pathways is difficult because

26   sterilization/inhibition agents can alter the availability of reactants by changing iron speciation

27   and organic matter composition. We compared six commonly used soil sterilization techniques –

28   γ-irradiation, chloroform, autoclaving, and chemical inhibitors (mercury, zinc, and azide) – for

29   their compatibility with chemodenitrification assays for tropical peatland soils (organic-rich low

30   pH soil from the Eastern Amazon). Out of the six techniques, γ-irradiation resulted in soil

31   treatments with lowest cell viability and denitrification activity, and least effect on pH, iron

32   speciation, and organic matter composition. Nitrite depletion rates in γ-irradiated soils were

33   highly similar to untreated/live soils, whereas other sterilization techniques showed deviations.

34   Chemodenitrification was a dominant process in tropical peatland soils assayed in this study.

35   Abiotic N_2O production was low to moderate (3-16% of converted nitrite), and different

36   sterilization techniques lead to significant variations on production rates due to inherent processes

37   or potential artifacts. Our work represents the first methodological basis for testing the abiotic

38   denitrification and N_2O production potential in tropical peatland soil.

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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 (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. 1-3 (Davidson et al., 2003; Kampschreur et al., 2011; Zhu et al., 2013; Zhu-Barker et al., 2015).

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

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

$$N_2O + 2\,Fe^{2+} + 2\,H^+ \rightarrow N_2 + 2\,Fe^{3+} + H_2O \qquad \text{(Equation 3)}$$

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_2O (Moraghan and Buresh, 1977) and relative inertness of N_2O.

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). 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

70   emitted in gaseous form. After tautomerization to an oxime (Raczyńska et al., 2005) and reaction

71   with NO^+ derived from a second NO_2^- ion, hyponitrous acid ($H_2N_2O_2$) can be produced, which

72   further decomposes to N_2O (Porter 1969; Stevenson et al., 1970) (Eq. 4).

(Equation 4)
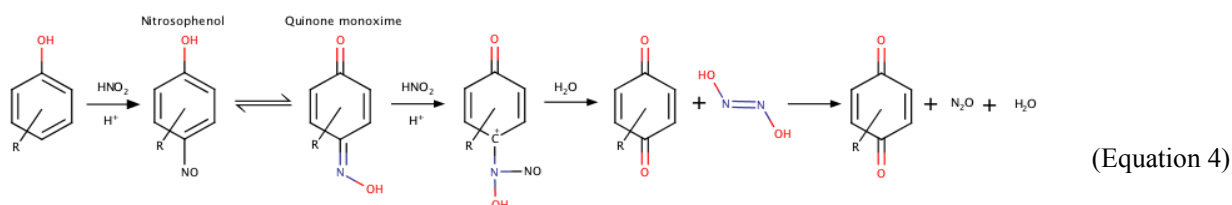
75   Other OM-dependent NO_2^- reduction pathways can produce NO and N_2 (McKenney et al., 1990;

76   Thorn et al., 2010) instead of N_2O.

77   The importance of abiotic N transformations in environmental samples has been

78   notoriously difficult to quantify due to the artifacts emerging from physical or chemical "killing"

79   methods intended to eliminate biological activity. In order to distinguish denitrification from

80   chemodenitrification, enzymes contributing to gaseous N production must be inactivated, most

81   commonly by addition of sterilants or inhibitors. An efficient sterilization treatment ideally: (1)

82   contains a negligible number of live cells, (2) eliminates biological activity, and (3) has little or

83   no effect, directly or indirectly, on abiotic reactions (e.g., it should not alter mineral structure, nor

84   lyse cells because release of cellular contents could influence abiotic reactions). Because rates

85   and products of chemodenitrification are dependent on O_2, pH, Fe^{2+} concentration and OM

86   composition, it is important to assess whether a sterilant/inhibitor elicits a physicochemical

87   change that can affect the availability or interaction of these reactants.

88   Soil sterilization techniques include γ-irradiation, chloroform ($CHCl_3$) fumigation,

89   autoclaving, and addition of chemical inhibitors such as mercury (Hg), zinc (Zn), or azide (N_3).

90   Highly energetic γ-irradiation damages enzymes and cell components, rendering cells non-viable

91   and inactive, generally with minimal effect on soil chemistry (Trevors, 1996). Autoclaving with

92   high-pressure steam disrupts cell membranes, denatures proteins, and decreases aromaticity and

93   polycondensation of soil OM (Berns et al., 2008; Jenkinson and Powlson, 1976b; Trevors, 1996).

94   Fumigation with $CHCl_3$ induces cell lysis and has minimal effect on enzymes (Blankinship et al.,

95   2014). Chemicals like Hg, Zn, and N_3 do the opposite: they inhibit enzymes (Bowler et al., 2006;

96   McDevitt et al., 2011), but do not lyse cells (Wolf et al., 1989).

97   We evaluated the appropriateness of six sterilants (γ-irradiation, autoclaving, $CHCl_3$, Hg,

98   Zn, and N_3) for chemodenitrification measurements in low-O_2, low-pH, high-OM tropical peat

99   soils. First, we tested the effects of sterilants on cell membrane viability and biological

100   denitrification activity. Next, we evaluated the effects of sterilants on soil chemistry (pH, OM

101   composition, and extractable Fe). Finally, we assessed the effects of the six sterilants on

102   chemodenitrification measured by NO_2^- depletion and N_2O production.

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104   **2 Materials and Methods**

105   *2.1 Sample characteristics.* Soil samples were collected in October 2015 from a tropical

106   peatland, locally known as Quistococha (3°50'S, 73°19'W), near Iquitos (Loreto, Peru). The soil

107   geochemistry of this site has been described previously (Lawson et al., 2014; Lähteenoja et al.,

108   2009). The samples were obtained from depths of 15-30 cm below the water table and kept

109   strictly anoxic during transport and storage at 4°C in the dark. Water saturation and organic

110   carbon content were determined by oven drying and loss-on-ignition, respectively. Dissolved

111   organic carbon (DOC) was determined by high-temperature combustion using a Shimadzu TOC-

112   V Total Organic Carbon Analyzer (Shimadzu Scientific Instruments, Columbia, MD). Inorganic

113   N species were quantified photometrically using an AQ2 Discrete Analyzer (Seal Analytical,

114   Southampton, UK) and method EPA-103-A Rev.10 for ammonium (NH_4^+; LoD 0.004 mg-N L^{-1},

115   range 0.02-2.0 mg-N L^{-1}) and method EPA-127-A for nitrate (NO_3^-) /nitrite (NO_2^-; LoD 0.003

116   mg-N L^{-1}, range 0.012-2 mg-N L^{-1}). Hydroxylamine was measured photometrically using the

117   iodate method (Afkhami et al., 2006).

118   **2.2 Soil sterilization and slurry incubations**. Experiments were started within 6 weeks of soil

119   collection. For each sterilization procedure, anoxic wet soil was exposed to the chemical sterilant

120   48 hours prior to start of the NO_2^- incubation or sterilized by physical treatment and allowed to

121   equilibrate for at least 12 hours. The untreated/live control was incubated as a slurry without any

122   additions or treatments for 48 hours prior to start of the NO_2^- incubation. Anoxic vials filled with

123   wet soil were irradiated with a ^{60}Co source for 7 days, yielding a final radiation dose of 4 Mrad

124   (40 kGy). The irradiated soil was then prepared for incubation in an anoxic glove box (0.5% H_2 in

125   N_2) with disinfected surfaces and sterilized materials to prevent contamination. For autoclaved

126   samples, soil was prepared for incubation in closed vials and autoclaved at 121°C and 1.1 atm for

127   90 minutes. The CHCl$_3$-treated samples were fumigated for 48 hours under a 100% N_2

128   atmosphere. Because volatilized CHCl$_3$ corrodes electron capture detectors used for N_2O

129   detection (see below), CHCl$_3$ was removed by flushing the vials with N_2 for 5-7 minutes

130   immediately before the start of incubations.

131   In contrast to the physical sterilization treatments, soil samples were continuously

132   exposed to the chemical inhibitors throughout their incubation. Sodium azide (NaN$_3$, Eastman

133   Organic Chemicals), zinc chloride (ZnCl$_2$, Fisher Scientific) or mercuric chloride (HgCl$_2$, 99.5%,

134   Acros Organics) were added from anoxic stock solutions to final concentrations of 150, 87.5, and

135   3.7 mM, respectively. The Hg concentration was the minimum needed to eliminate microbial

136   heterotrophic growth based on visual inspection of soil extract on agar plates exposed to 0.5 to

137   92.1 mg L^{-1}, which includes concentrations demonstrated to be effective previously (Tuominen et

138   al., 1994).

139   After the initial physical or chemical treatment, triplicate incubations were diluted

140   1:10 in 20 mL of autoclaved 18.2 MΩ·cm water in 60 mL glass serum vials. Triplicate soil

141   slurries were amended from anoxic, sterile stock solution to a final concentration of 300 µM

142   NO_2^- (6 µmoles in 20 mL) and sealed with thick butyl rubber stoppers. A parallel set of samples

143   was amended with 300 µM NO_3^- to evaluate denitrification potential with CO_2 measurements.

144   Control incubations received an equivalent volume of autoclaved 18.2 MΩ·cm water without

145   NO_x^-. Soil microcosms were incubated in the dark at a constant temperature of 25°C. NO_2^- was

146   quantified in all soil treatments using the Griess assay (Promega, Kit G2930; e.g., Griess 1879).

147   pH measurements were taken with an Orion 3 Star meter (Thermo Scientific) before and after

148   sterilization, and at the end of the experiment after 70-76 hours of incubation.

149   *2.3 Gas chromatography.* To quantify N_2O and CO_2 production, 200 µL of headspace gas was

150   sampled with a gas-tight syringe (VICI Precision Sampling) and injected onto a gas

151   chromatograph (GC, SRI Instruments) equipped with both an electron-capture detector (ECD)

152   and a flame-ionization detector (FID). Two continuous HayeSep-D columns were kept at 90°C

153   (oven temperature); N_2 (UHP grade 99.999%, Praxair Inc.) was used as carrier gas, and H_2 for

154   FID combustion was supplied by a H_2 generator (GCGS-7890, Parker Balston). For CO_2

155   measurements, a methanizer at 355°C was run in line before the FID. The ECD current was 250

156   mV and the ECD cell was kept at 350°C. The N_2O and CO_2 measurements were calibrated using

157   customized standard mixtures (Scott Specialty Gases, accuracy ±5%) over a range of 1-400 ppmv

158   and 5-5,000 ppmv, respectively. Gas accumulation in the incubation vials was monitored over

159   time. Gas concentrations were corrected using Henry's law and the dimensionless concentration

160   constants $k_H^{cc}(N_2O) = 0.6112$ and $k_H^{cc}(CO_2) = 0.8313$ (Stumm and Morgan, 2012) to account for

161   gas partitioning into the aqueous phase at 25°C.

162   *2.4 Live/dead cell staining.* To assess the efficacy of sterilants or inhibitors visually, the bacterial

163   viability kit LIVE/DEAD BacLight L7012 (Molecular Probes, Invitrogen) containing SYTO9

164   and propidium iodide dyes was used to stain and distinguish dead and living cells on the basis of

165   intact cell walls. The green (live) and red (dead) signals were counted at 60x magnification from

166   10 squares of 0.01 mm^2 randomly distributed in the center of a 5 µL Neubauer chamber, using an

167   Olympus BX-61 microscope with the FITC/Cy5 filter set. Photographs were taken with an

168   Olympus DP-70 camera attached to the microscope. Particles were counted with ImageJ software

169   version 1.50i (Abràmoff et al., 2004).

170   *2.5 Fe extraction and quantification.* Dissolved Fe species were extracted from peat soil

171   incubations following the protocol of (Veverica et al., 2016). The method is based on an ionic

172   liquid extraction using *bis*-2-ethylhexyl phosphoric acid (Pepper et al., 2010), which was shown

173   to be more suitable for extraction of Fe from humic-rich matrices than the traditional ferrozine or

174   phenanthroline methods. Briefly, 2.5 mL of soil slurry was filtered (0.2 µm nylon filter; Celltreat

175   Scientific Products) and mixed with 7.5 mL of HCl (0.67 N) in an extraction vial in an N_2 glove

176   box. The O_2 concentration in the glove box was continuously monitored and remained <10 ppm.

177   To separate Fe^{3+} from Fe^{2+}, 10 mL of 0.1 M *bis*-2-ethylhexyl phosphate (95%, Alfa Aesar) in *n*-

178   heptane (99.5%, Acros Organics) was added to the acidified sample. Next, the organo-aqueous

179   emulsion was shaken at 250 rpm in closed extraction vials for 2 hours. The *bis*-2-ethylhexyl

180   phosphate chelates Fe^{3+} more effectively than it chelates Fe^{2+}. The Fe^{2+}-containing aqueous phase

181   was sampled into a 3-fold HCl-washed HDPE vial (Nalgene) in the glove box. The Fe^{3+} fraction

182   chelated in the organic phase was then back-extracted into an aqueous phase by the addition of 10

183   mL 4N HCl and shaking at 250 rpm in closed extraction vials for 20 minutes. Fe^{3+} and Fe^{2+}

184   fractions were quantified separately in acidified aqueous solution by inductively coupled plasma-

185   optical emission spectrometry (ICP-OES; Thermo iCAP6300 at the Goldwater Environmental

186   Laboratory at Arizona State University). The ICP-OES pump rate for the Ar carrier was set to 50

187   rpm and Fe2395 and Fe2599 lines were used for Fe quantification. Iron concentrations were

188   determined from a calibration curve (0.01-10 mg L^{-1}) by diluting a standard solution (100 mg L^{-1},

189   VHG Labs, product # SM75B-500) in 0.02 N HNO_3.

190   **2.6 Dissolved organic matter fluorescence analysis.** 3D-fluorescence analysis was performed on

191   a Horiba Jobin-Yvon Fluoromax 4 spectrofluorometer. Excitation-emission matrices (EEMs)

192   were generated by obtaining emission spectra (λ_{Em} = 300-550 nm, at a step size of 2 nm) at

193   excitation wavelengths from 240-450 nm at a 10 nm step size. All EEMs were blank corrected

194   and normalized daily to the Raman peak of ultrapure water (deionized, carbon-free,18.2 MΩ·cm;

195   Barnstead^{tm} NanoPure). The samples were taken at the same time as those for Fe analysis. Prior

196   to analysis, soil slurries were filtered using a solvent-rinsed Whatman GF/F filter (nominal pore

197   size 0.7 μm) to obtain ~10 mL filtrate. Samples were diluted with ultrapure water if their UV

198   absorbance exceeded 0.3 so that inner-filter corrections could be made (Stedmon, 2003). We

199   calculated total fluorescence as the matrix sum of all signals in the EEM. Fluorescence indices

200   were used to characterize various classes of fluorophores in the dissolved organic matter (DOM)

201   pool. Fluorescence Index (FI) was calculated as the sum of the intensity signal in the emission

202   spectra from 470-520 nm collected at an excitation wavelength of 370 nm (Cory and McKnight,

203   2005). Humification index (HIX) was determined from the peak area under the emission

204   spectrum from 435–480 nm divided by the area from 300–445 nm, both collected at an excitation

205   wavelength of 254 nm (Ohno, 2002). The "freshness" was determined as β/α, the ratio of

206   emission intensity at 380 nm to the emission intensity maximum between 420 and 435 nm, both

207   collected at an excitation wavelength of 310 nm (Wilson and Xenopoulos, 2009).

208   **2.7 Statistical Analyses.** All basic statistical tests were performed with JMP Pro software

209   (Version 13.1.0, SAS Institute Inc., Cary, NC, USA).

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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).

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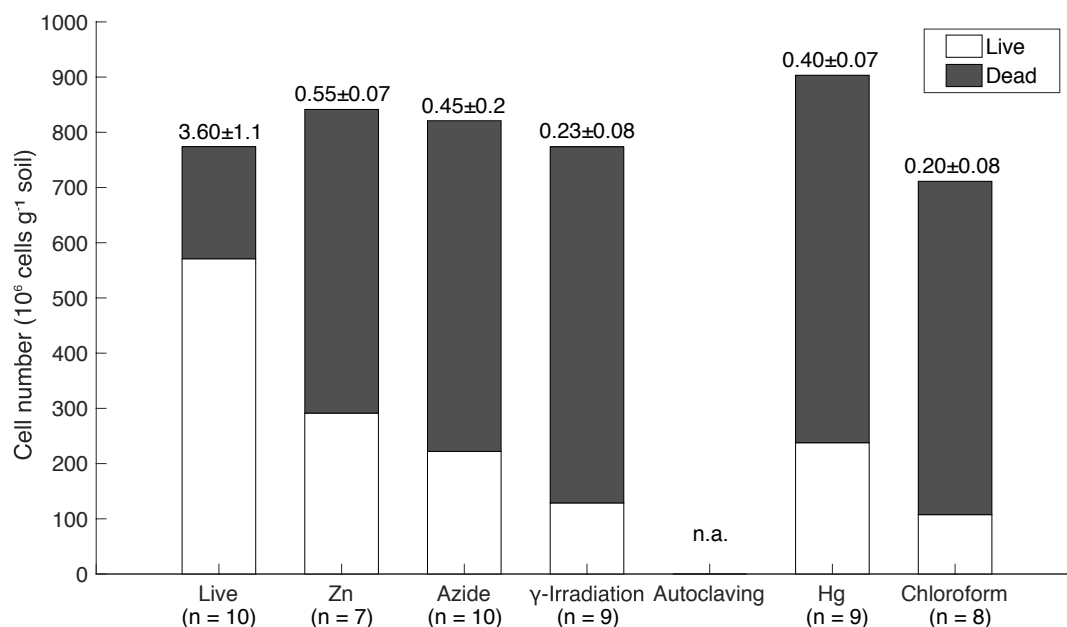
Figure 1. Live/dead microbial cell counts of tropical peatland soils. The numbers above the

bars indicate the live to dead signal ratio ± SD. No detectable signal was observed in autoclaved

samples.

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226   ***3.2 Effects of sterilants on cell integrity and potential of denitrifying activity.*** Live/dead dyes

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

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

229   the live incubation displayed the "live" signal (**Fig. 1**). The $CHCl_3$ and γ-irradiated treatments

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

231   sterilization). Chemical inhibitors (Hg, Zn, and N_3) were less effective at killing cells (~30%

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

233   lysis during steam pressurization.

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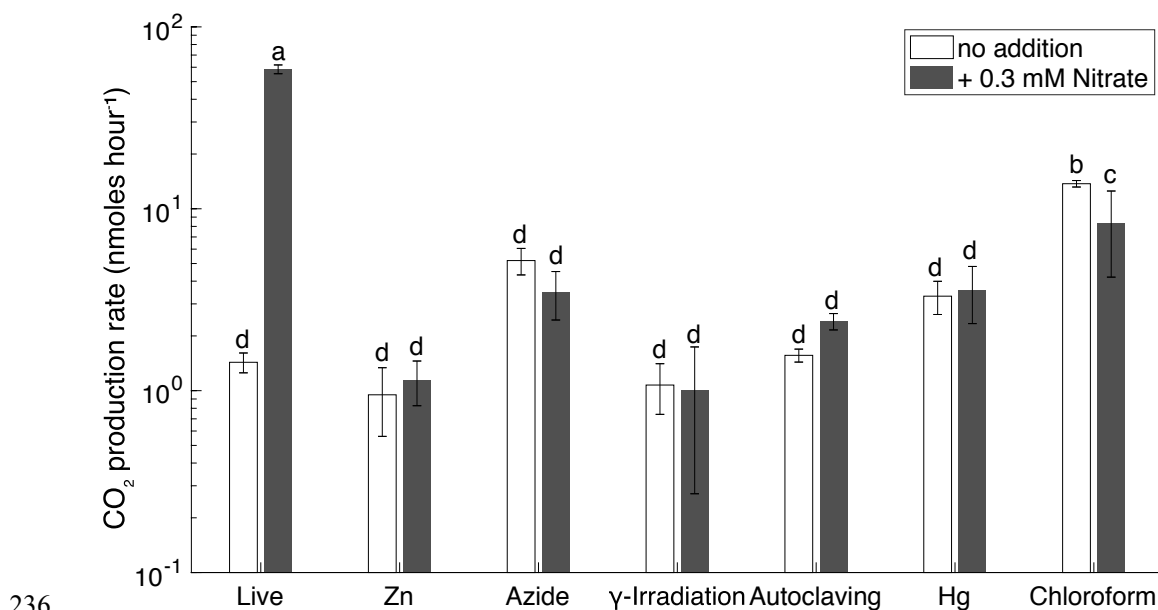
237   **Figure 2. CO_2 production rates in 3-day soil slurry incubations of Quistococha peat soil**

238   **amended with and without 0.3 mM NO_3^-.** Error bars are one SD (n=3). Columns marked with

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

240

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

242   based on the difference in CO_2 production with and without added NO_3^-. An efficient sterilization

243   treatment would show no changes in CO_2 beyond that due to equilibration between the gas phase

244   and aqueous phase. Nitrate stimulated CO_2 production in live soil (ANOVA, $p < 0.05$) and not in

245   the γ-irradiated, Zn, Hg, N_3, or autoclaved incubations **(Fig. 2)**, indicating that residual cells in

246   the sterilized treatments were not capable of denitrification.

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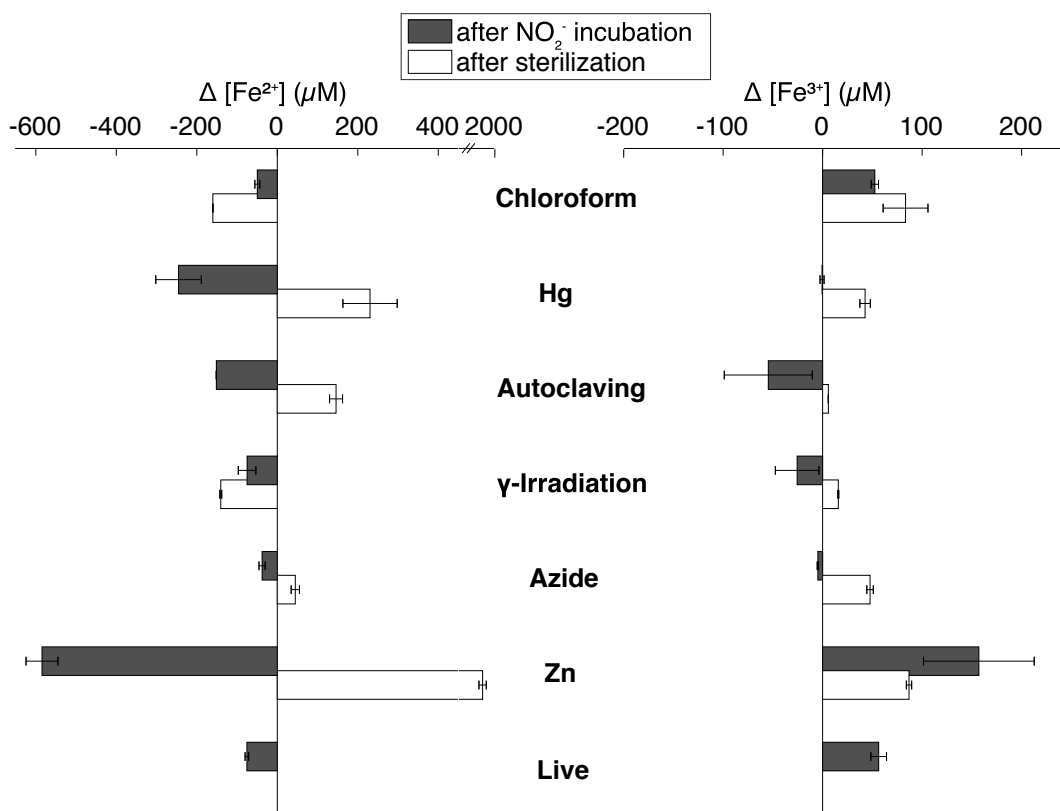
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250   **Figure 3. Changes in extractable Fe^{2+} (left) and Fe^{3+} (right) concentration in Quistococha**

251   **peat soil incubations after sterilization (difference between sterilization baseline and live**

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252 **baseline value) and after NO_2^- amendment and incubation (difference between NO_2^- and**

253 **control incubations).** Note the difference in scales. Values represent the extractable fraction of

254 both species. Error bars are one SD (n=2).

255

256 **_3.3 Effects of sterilants on soil chemistry_**. In general, sterilization increased extractable Fe^{2+} and

257 Fe^{3+} relative to live controls (**Fig. 3**). This trend was particularly pronounced in Zn treatments,

258 which had 9x higher extractable Fe^{2+} (1915±26 μM) and 1.6x higher extractable Fe^{3+} (87±3 μM)

259 than live controls. The Hg treatment showed the second largest increases. In the presence of

260 NO_2^-, extractable Fe^{2+} decreased and extractable Fe^{3+} increased in live, Zn, and $CHCl_3$-fumigated

261 treatments, as expected if Fe^{2+} was oxidized by NO_2^- during chemodenitrification. However,

262 autoclaving, γ-irradiation, and N_3 lowered Fe^{3+} concentrations, suggesting the influence of

263 unknown concomitant reactions. For instance, autoclaving (largest drop in Fe^{3+}) already showed

264 lower Fe^{3+} concentrations after sterilization. Production of Fe^{3+}-reduction artifacts in treatments

265 could lead to Fe^{3+} depletion and, hence, mask increase in Fe^{3+} due to chemodenitrification. NO_2^-

266 addition resulted in near-complete depletion of extractable Fe^{2+} in live, $CHCl_3$-fumigated, and γ-

267 irradiated soils. Changes in Fe speciation with other sterilants were more moderate. Minimal

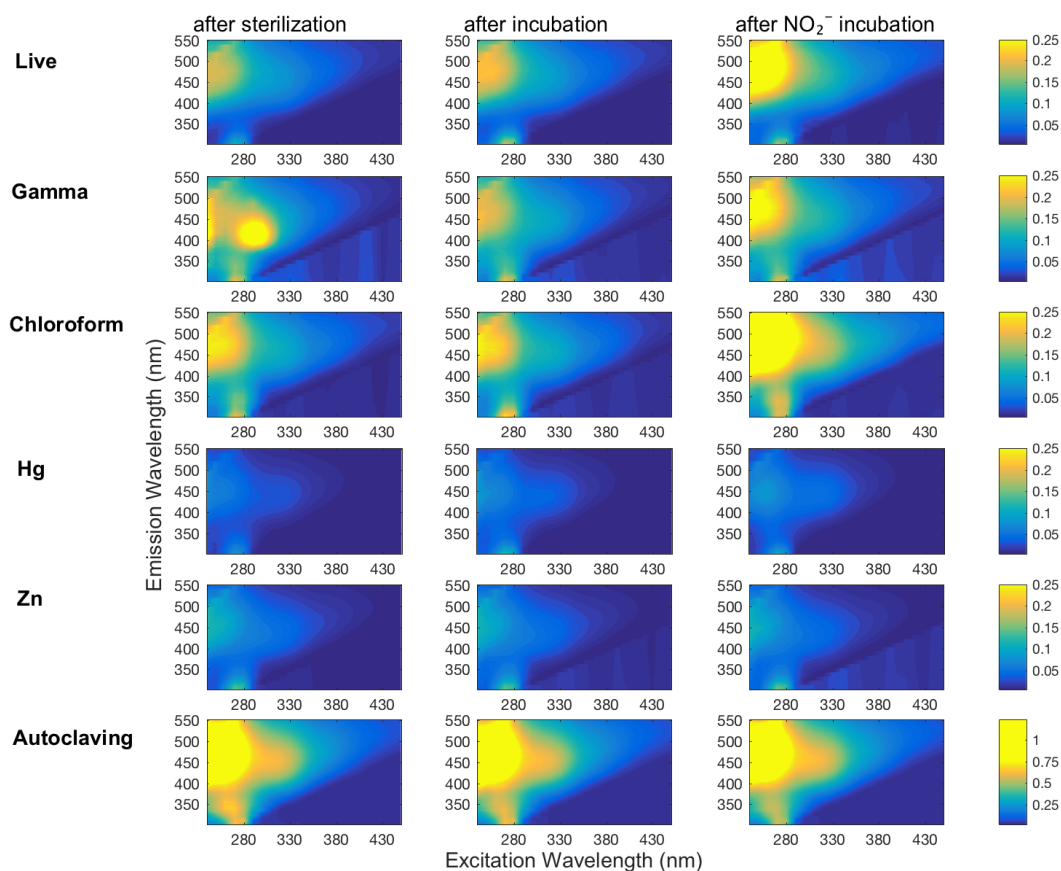268 changes were observed for other metals (e.g., Mn, Al, Cu, and Zn; data not shown).

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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 NO_2^- (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.

286   Fluorescence analysis of soil extracts using excitation-emission matrices (EEMs) was

287 used to evaluate changes in DOM containing aromatic moieties or conjugated double bonds

288 (Stedmon et al., 2003); **Fig. 4**). The N_3 treatment was excluded from this analysis due to an

289 interference with N_3 absorbance that prevented inner-filter corrections from being made. The

290 EEM signals showed the greatest change in the "humic" region (λ_{Ex} <240-270 nm, and λ_{Em} =

291 460-500 nm; (Fellman et al., 2010), especially in Zn and Hg treatments, which significantly

292 increased the FI to 1.49 (**Table 1**). Zn and Hg may elicit direct fluorescence quenching by the

293 formation of Zn and Hg metal complexes (McKnight et al., 2001) or possibly due to indirect

294 quenching by higher dissolved Fe^{2+}. Signal strength in the humic region was enhanced by NO_2^-

295 addition in the live, $CHCl_3$-fumigated, and γ-irradiated treatments. All five sterilization

296 treatments had lower aromaticity (HIX) than live controls (**Table 1**). Autoclaved samples had

297 tenfold higher total fluorescence compared to live soils, suggesting that autoclaving degraded

298 insoluble humics into more soluble and less condensed OM.

299 ***3.4 Effects of sterilants on chemodenitrification and abiotic N_2O production***. In the first 48

300 hours, NO_2^- consumption rates were the highest in live soil (5.2 µM h^{-1}), closely followed by

301 irradiated samples (4.5 µM h^{-1}, **Fig. 5**). The major chemodenitrification pathway for N_2O

302 formation was likely NO_2^- reduction by Fe^{2+}, resulting in consumption of ~1.5 µmol Fe^{2+} and

303 accumulation of ~1.1 µmol Fe^{3+} in the live control (**Fig. 3**). After 48 hours, NO_2^- depletion

304 continued to completion in the live control but slowed in all treatments other than the metal

305 additions. After 72 hours of incubation, 3-16% of NO_2^--N was converted to N_2O-N across

306 treatments. Higher N_2O production rates were observed in live, Zn^{2+}, and N_3^- treatments (0.5-0.7

307 nmol N_2O g^{-1} h^{-1}, r^2 > 0.95) than in γ-irradiated, $CHCl_3$-fumigated, autoclaved, and Hg treatments

308 (0.1-0.2 nmol N_2O g^{-1} h^{-1}, r^2 > 0.9). Production rates within treatments showing high or low rates

309 were not significantly different (Student's *t*, *p* >0.05) although comparisons across treatments

310 with high or low rates were statistically different (Student's t, $p <0.05$). Thus, we identified a

311 higher and lower group of sterilant-dependent N_2O production rates from the same soil samples.

312 The live control showed logarithmic N_2O accumulation while the sterilized treatments had linear

313 accumulation over time, the later as expectable in abiotic accumulation (**Fig. 5**).

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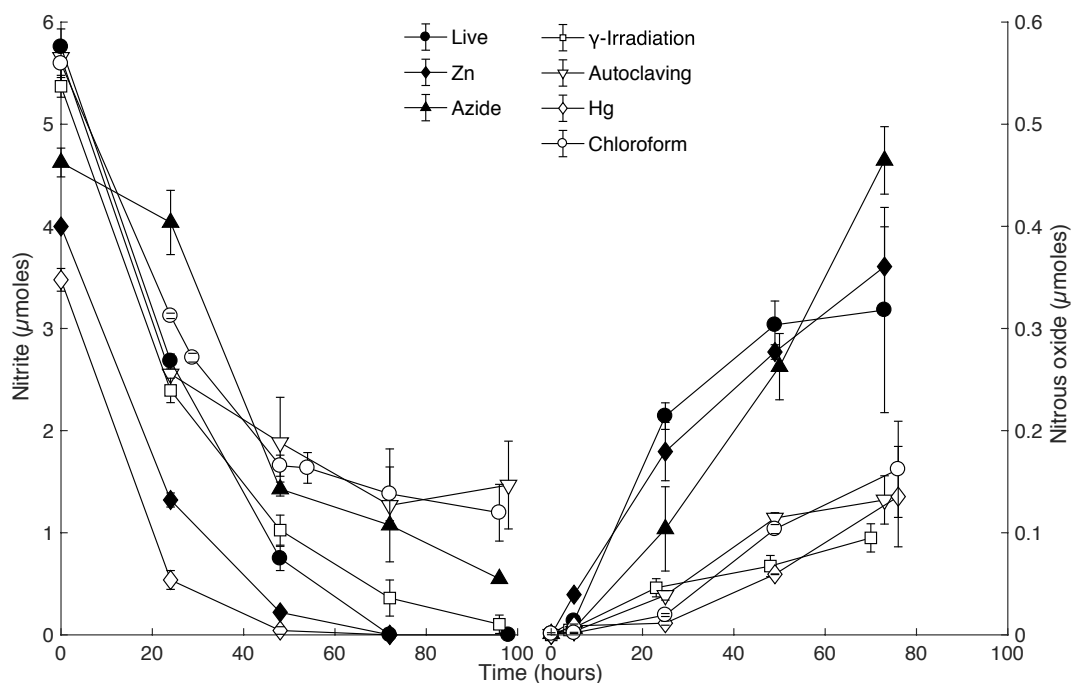
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317 **Figure 5. NO_2^- consumption (left) and N_2O production (right) for different sterilant**

318 **treatments in soil slurry incubations of Quistococha peat soil.** Both N species were

319 simultaneously measured in all treatments. The product yield represents N_2O-N as molar fraction

320 of NO_2^--N. Note the difference in left and right y-axis scales. Error bars are one SD (n=3).

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324 **4 Discussion**

325 ***4.1 Chemodenitrification is a dominant NO_2^- consumption process in slurry incubations of***

326 ***tropical peat soils.*** Similar NO_2^- consumption rates between live and irradiated treatments imply

327 that NO_2^- depletion was dominated by abiotic processes over the first 48 hours. In general,

328 abiotic reactions tend to be linear processes, whereas microbially mediated reactions can be

329 affected by enhanced expression of genes or cell reproduction in a nonlinear fashion (Duggleby,

330 1995). The difference in linearity of N_2O production in sterilized vs. live treatments (**Fig. 5**)

331 suggests that biological denitrification did not occur in sterilized soils.

332   Compared to our study, incubations of artificial media with 200 μM NO_2^-, 0.5-8.1

333 mM Fe^{2+}, and a pH of 7-8 had similar rates of Fe^{2+} depletion but 10x higher rates of NO_2^-

334 reduction, and higher (~10-50%) N_2O yields (Buchwald et al., 2016; Jones et al., 2015). In our

335 peat incubations, reactive OM likely trapped NO_2^- in the soil matrix via OM-bound nitrosation

336 reactions (Thorn and Mikita, 2000; Thorn et al., 2010) and the lower pH likely promoted

337 conversion of NO_2^- to NO (Kappelmeyer et al., 2003; Porter, 1969) or N_2 (Stevenson et al.,

338 1970). Studies in low pH northern temperate peat soils, have shown the primary product of

339 abiotic NO_2^- reduction was NO, not N_2O (McKenney et al. 1990).

340 ***4.2 Artifacts due to sterilization methods for chemodenitrification assays***. Azide and Zn

341 exhibited enhanced NO_2^- conversion to N_2O, at rates at least twice to five times as high as those

342 measured for the other sterilants (**Fig 5**), likely due to higher pH and Fe availability, respectively.

343 In the N_3 treatments, elevated N_2O production could be explained by the reaction of protonated

344 NO_2^- with N_3 in a pH dependent manner (Stedman, 1959), plus other changes in soil solution

345 originated from the increase of pH. Nitrite reaction with N_3 has been characterized in marine and

346 freshwater solutions reaching its maximum at pH 4.5 and proceeding slowly yet significantly

347 (20% conversion in 1 hour) at pH > 5 (McIlvin and Altabet, 2005) as in our slurries. Moreover,

348   N_3's self-fluorescence impeded OM measurements, making N_3 an incompatible sterilizing agent

349   for chemodenitrification studies. Zn increased Fe availability and may have increased NO_2^-

350   affinity for reactive OM groups; both effects would lead to an abiotic increase in N_2O production

351   (Clark, 1962; McCalley and Sparks, 2009; Parton et al., 2007). Zinc treatment lowered the soil

352   pH, which may have promoted cation displacement and stability of dissolved Fe^{2+} (Hutchins et

353   al., 2007), thus enhancing N_2O production. Several studies have used Zn treatments as valuable

354   agent for field applications (Babbin et al., 2015; Ostrom et al., 2016). Zn is less hazardous to

355   humans than some of the other sterilants. We propose that the use of Zn could provide useful

356   information about abiotic *in-situ* rates as long as Zn-induced chemodenitrification is accounted

357   for. A correction could be applied if a complementary laboratory assessment (using the more

358   efficient γ-irradiation) were used to develop an ecosystem-specific correction factor.

359   Divalent Hg^{2+} can be abiotically methylated by fulvic acid-type substances (Rogers,

360   1977). The reaction oxidizes OM and can diminish its reducing power as indicated by decreased

361   reactivity of humic acid with NO_2^- (Gu et al., 2011; Zheng et al., 2011) thus interfering with the

362   abiotic assay. Another potential factor associated with the Hg treatments is metal sorption. At

363   low pH (3.6), 98% of Hg was sorbed to humic acids, whereas only 29% of Zn was sorbed at pH

364   ~4.8 (Kerndorff and Schnitzer, 1980). Full sorption capacity of peat is presumably reached in

365   seconds (Bunzl et al., 1976) and the differing sorption behavior of Hg and Zn may play a role in

366   the reaction potential of NO_2^- with OM. It has been demonstrated that Hg introduced into peat

367   soil leads to sorption of Hg ions to various functional groups, including phenols (Drexel et al.,

368   2002; Xia et al., 1998). Hence it is plausible that Hg sorbed to functional groups subject to

369   electrophilic attack by NO^+(e.g., nitrosophenol, Eq. 3) may hamper nitrosation, and therefore

370   protect OM from reacting with NO_2^-. This could lead to a selective suppression of the OM-

371   dependent N_2O production pathway.

372   Chloroform fumigation resulted in potential N_2O production rates within the lower

373   production range treatments with minor differences in Fe speciation and DOM fluorescence.

374   However, unlike the other sterilized samples, $CHCl_3$-fumigated samples showed enhanced CO_2

375   production stimulated by NO_3^- addition. Removal of $CHCl_3$ from our samples before substrate

376   addition could have provided an opportunity for a few surviving heterotrophs to re-grow and use

377   the easily-degradable organic material derived from dead cells. Indeed, chloroform can lyse cells,

378   providing substrates for growth to $CHCl_3$-resistant microorganisms (Zelles et al., 1997).

379   Continued exoenzyme activity has been also described as a CO_2 source: however, this would not

380   include denitrification enzymes, since none enzymes involved in the denitrification pathway are

381   exoenzymes (Blankinship et al., 2014; Jenkinson and Powlson, 1976a). Chlorination of natural

382   OM may prompt formation of quinones (Criquet et al., 2015), which are intermediates in the OM-

383   based abiotic N_2O production (Thorn and Mikita, 2000); indeed, regions of the EEMs

384   corresponding to hydroquinones (Cory and McKnight, 2005) appear to be slightly higher in

385    $CHCl_3$ treatments. The benzene derivative produced during nitrosophenol reaction with NO_2^-

386   leads to reduced π-electron delocalization (Eq. 4). Because excitation of π-electrons produces

387   fluorescence, reactions with NO_2^- might be expected to reduce OM fluorescence. However, the

388   experiment duration is important and if indeed microbial cells reproduce after the treatment, short

389   experimental periods (e.g., hours or days) or reapplication of $CHCl_3$ might keep down the

390   numbers of any potential denitrifiers improving the use of this method.

391   Autoclaved peat soil revealed abiotic N_2O production rates close to the average of the

392   lower production range group, accompanied by but ICP-OES and fluorescence spectroscopy

393   results also showed significant changes in Fe speciation and DOM composition. EEMs

394   demonstrate lower values for the HIX in autoclaved peats (**Table 1**), consistent with fluorescence

395   data from a study that demonstrated a decrease in the aromaticity and polycondensation of soil

396   extracts from autoclaved soil (Berns et al., 2008). Autoclaving likely caused degradation and

397   solubilization of insoluble humic components. The direct effects of autoclaving are very much

398   dependent on the heat and pressure stability of the indigenous soil constituents, but the substantial

399   soil structural changes likely introduce chemical artifacts that are absent in the native live soil.

400   *4.3 Gamma irradiation is the preferred sterilization method for chemodenitrification assays.*

401   The fewest chemical artifacts were observed in γ-irradiated samples. Soil that had been exposed

402   to γ-rays showed the lowest N_2O production rates, approximately one-fifth of those observed in

403   live samples. Irradiation also caused only very small changes in Fe speciation relative to live

404   controls and yielded EEMs that were remarkably similar to those obtained from live soil extracts.

405   Our measurements of sterility and respiratory activity indicated the lowest potential for biological

406   activity and hence, the least amount of interference for the time period tested. We therefore

407   confirmed γ-irradiation to be a preferred method for sterilizing soil (Trevors, 1996) and for

408   assessing abiotic N_2O production potentials. In practice, the long preparation time needed to

409   reach a sufficient dose (dependent on radiation source, see **Methods**) was compensated for by the

410   lack of chemical artifacts during the experiment and the reduced number of hazardous waste

411   products. Limited accessibility to irradiation facilities and the absence of a field portable option

412   remain the main challenges to wide distribution of this approach.

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420 **Table 1. Characteristics of dissolved organic matter in soil extracts from incubations of peat**

421 **from Quistococha, Peru.** FI, HIX, and freshness indices were calculated as described in the

422 methods section. The "tyrosine-like" region is defined at an excitation of 270-275 nm and an

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

424 replicates and expressed as percent difference between NO_2^- additions and controls ± standard

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

426 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.

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428   *5 Conclusion*

429   High N_2O emissions occurs in tropical regions with water-saturated soils (Liengaard et al., 2014;

430   Park et al., 2011; Pérez et al., 2001). Whether these tropical N emissions are solely biotic or have

431   abiotic contributions is not well known, because rates of chemodenitrification are not commonly

432   evaluated. Abiotic processes in the N cycle remain overlooked, partly due to the lack of reliable

433   means of quantifying abiotic reactions. This study showed that chemodenitrification occurs in a

434   tropical peat soil, leading to a low to moderate fraction of N_2O conversion from nitrite

435   amendment. We also demonstrated that γ-irradiation is the "gold standard" for

436   chemodenitrification assays. The application of N_3 to quantify abiotic N_2O production is

437   unsuitable because changes associated to fraction of the sterilant itself may react to form N_2O and

438   effects increased pH. $CHCl_3$ and γ-rays have slightly reducing effects on the soil Fe pool and

439   might lead to a weak discrimination against pathways involving Fe as reactant. $CHCl_3$ fumigation

440   was another approach with limited effects on Fe chemistry that lowered the number of viable

441   cells greatly, however, the potential for microbial regrowth after $CHCl_3$ removal is its main

442   drawback. Autoclaving seemed to have minor disadvantages on abiotic N_2O production, despite

443   the substantial changes to soil OM.

444   Unlike other lab-intensive treatments, the application of Zn and Hg are amenable for field

445   experiments; however, we observed distinct chemical artifacts when using both of these options.

446   Care is warranted if using Zn and Hg chemical inhibitors, which can increase Fe availability and

447   may thus overestimate Fe-dependent abiotic N_2O production rate. A potential disadvantage of the

448   application of toxic metals is a decrease in soil pH. We cannot exclude pH-driven effects on N

449   intermediates; however, no major deviation in the final N_2O production rate related to

450   acidification was observed. With the methodological evaluation presented here, we determined

451   that a directed selection of approaches can allow for better constrained and more detailed studies

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452   of the role of abiotic pathways and soil components shaping denitrification and N_2O fluxes from

453   soil ecosystems.

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475   **References**

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659 **Competing Interests Statement**

660 The authors have no competing interests to declare.