# 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

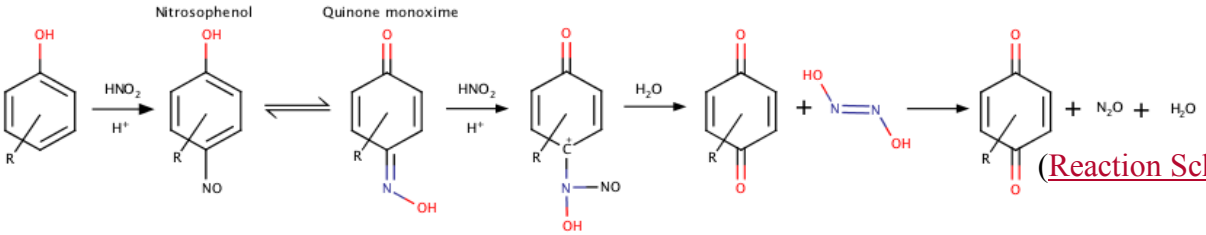
Across ecosystems, physical and chemical factors, such as solar radiation or redox gradients, can drive abiotic chemical transformations. The nitrogen (N) cycle, in particular, includes abiotic reactions that can affect the retention of nutrients or substrates (Clark, 1962; McCalley and Sparks, 2009; 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 ($\lambda_{Em}$ = 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 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).

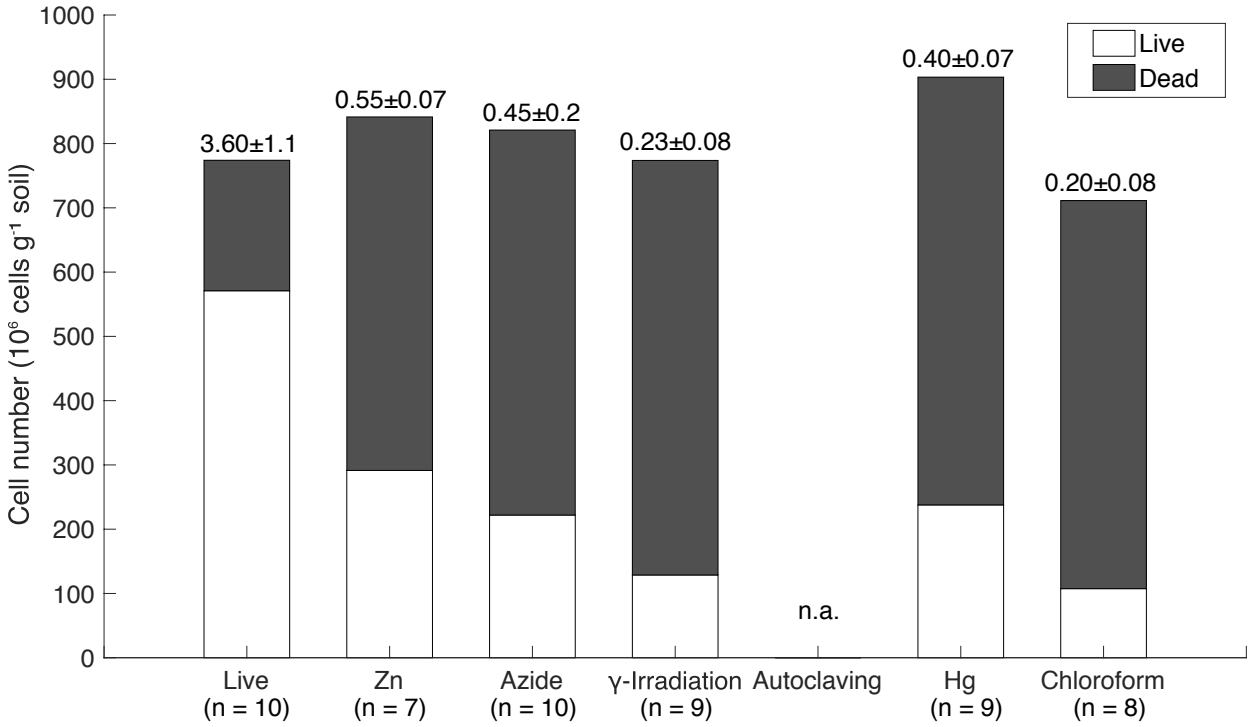



**Figure 1. Live/dead microbial cell counts of tropical peatland soils**. The numbers above the bars indicate the live to dead signal ratio ± SD. No detectable signal was observed in autoclaved samples. The x-axis represents treatments, including Live = no treatment, Zn = $ZnCl_2$, Hg = $HgCl_2$, Azide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

***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.

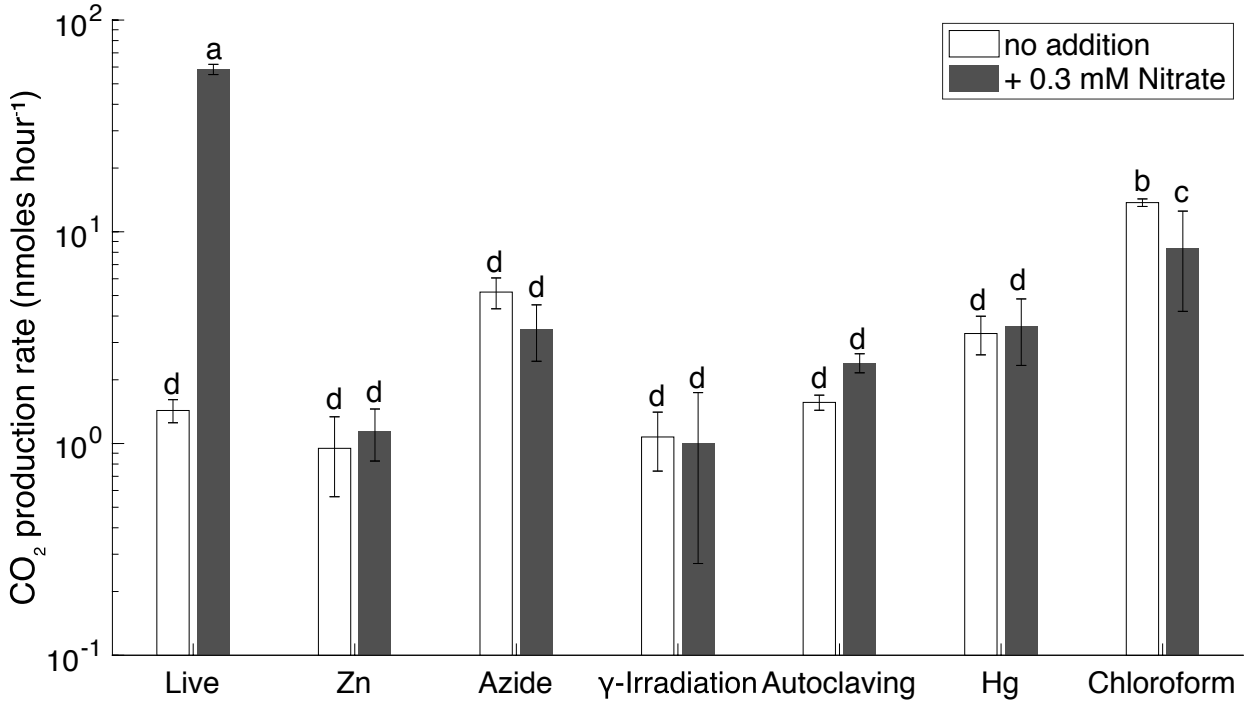
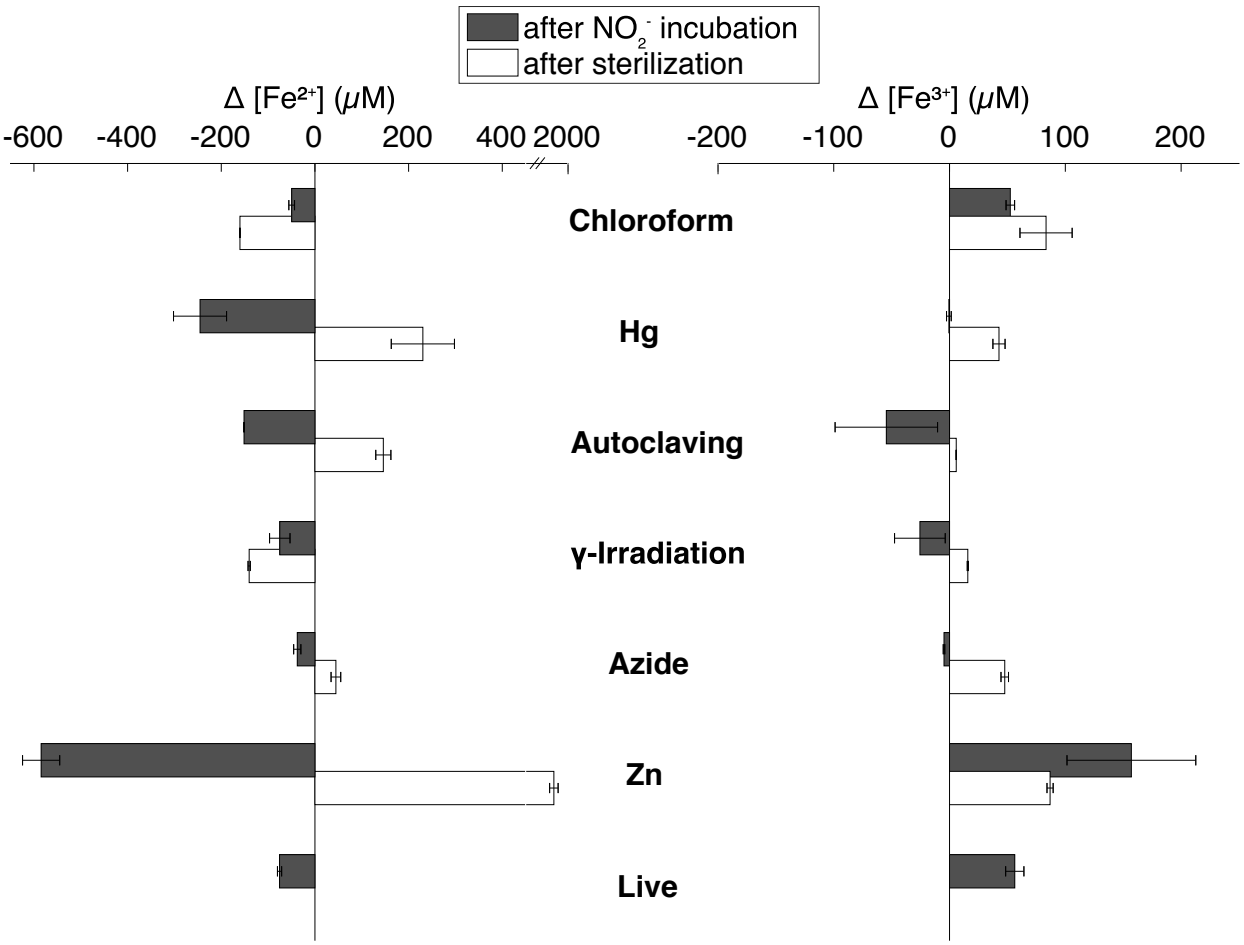



**Figure 2. $CO_2$ production rates in 3-day soil slurry incubations of Quistococha peat soil amended with and without 0.3 mM $NO_3^-$.** Error bars are one SD (n=3). Columns marked with the same letter are not statistically different from each other (Student's *t*, $p > 0.05$, n=3). The x-axis represents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

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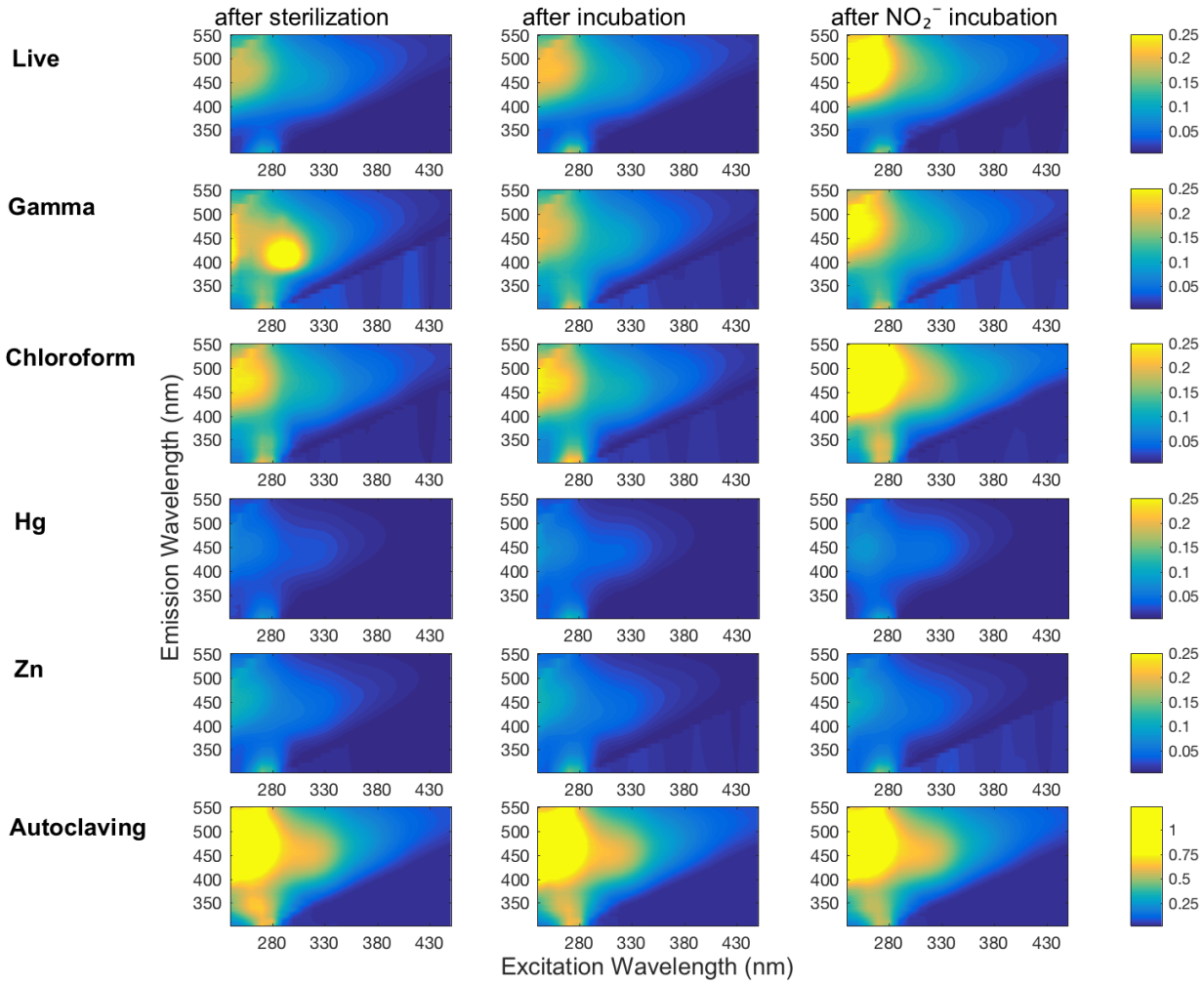
257 **Figure 3. Changes in extractable $Fe^{2+}$ (left) and $Fe^{3+}$ (right) concentration in Quistococha**
258 **peat soil incubations after sterilization (difference between sterilization baseline and live**
259 **baseline value) and after $NO_2^-$ amendment and incubation (difference between $NO_2^-$ 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 ($\lambda_{Ex}$ <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 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**).
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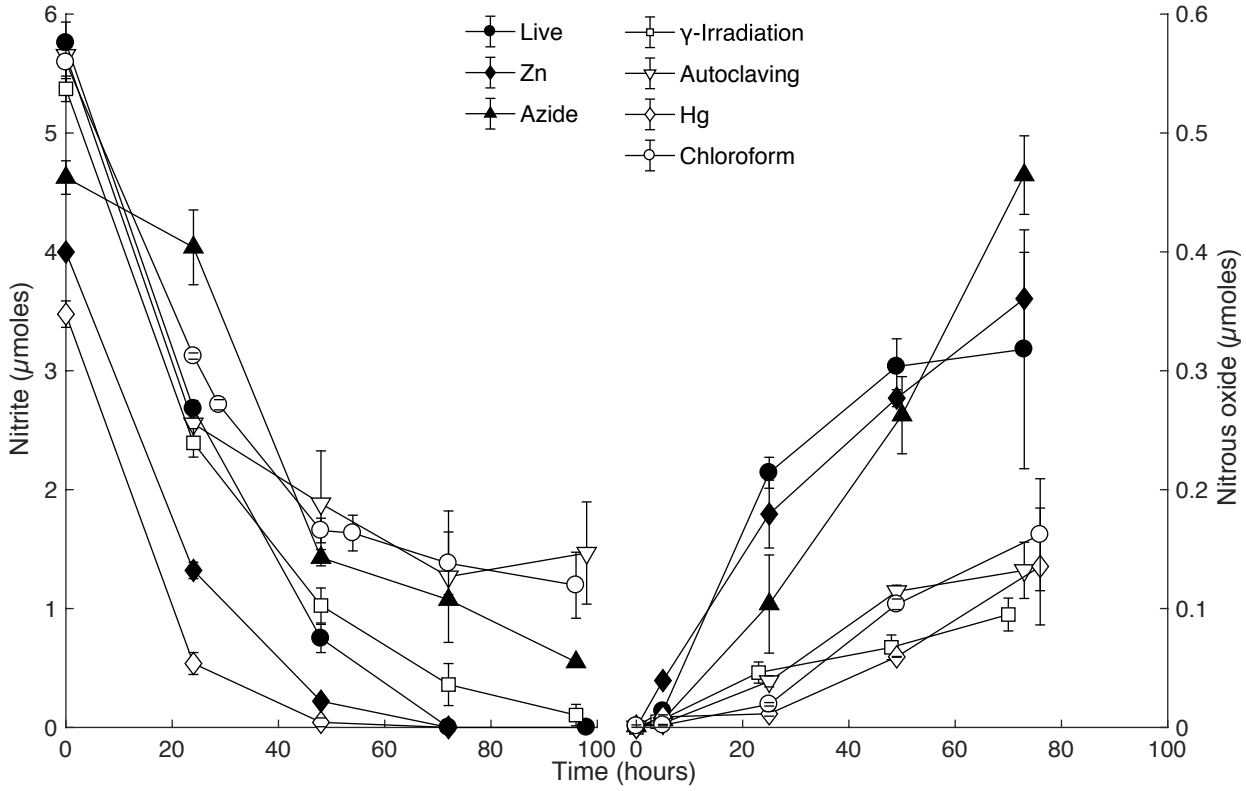


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321
322 **Figure 5. $NO_2^-$ consumption (left) and $N_2O$ production (right) for different sterilant**
323 **treatments in soil slurry incubations of Quistococha peat soil.** Both N species were
324 simultaneously measured in all treatments. The product 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.

**Acknowledgements**

We thank Chris Laurel, Roy Erickson, and Cathy Kochert for training and assistance with the ICP-OES analysis at ASU's Goldwater Environmental Laboratory, and Steven Hart for advice optimizing the epifluorescence microscopy. We also thank Nabil Fidai, Jaime Lopez, Analissa Sarno and Mark Reynolds of the Cadillo Lab for their enduring support during the experimental phase. This work was funded by an NSF-DEB award (#1355066) to H.C-Q and a NASA award (NNX15AD53G) to H.E.H and H.C-Q. The results reported herein also benefited from collaborations and/or information exchange within NASA's Nexus for Exoplanet System Science (NExSS) research coordination network sponsored by NASA's Science Mission Directorate. All data presented in this paper is available in the Dryad Digital Repository.

## References


Abràmoff, M. D., Magalhães, P. J. and Ram, S. J.: Image processing with ImageJ, Laurin Publishing. 2004.

Afkhami, A., Madrakian, T. and Maleki, A.: Indirect Kinetic Spectrophotometric Determination of Hydroxylamine Based on Its Reaction with Iodate, Analytical Sciences, 22(2), 329–331, doi:10.2116/analsci.22.329, 2006.

Babbin, A. R., Bianchi, D., Jayakumar, A. and Ward, B. B.: Rapid nitrous oxide cycling in the suboxic ocean, Science, 348(6239), 1127–1129, doi:10.1126/science.aaa8380, 2015.

Berns, A. E., Philipp, H., Narres, H. D., Burauel, P., Vereecken, H. and Tappe, W.: Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy, Journal of Soil Science, 59(3), 540–550, doi:10.1111/j.1365-2389.2008.01016.x, 2008.

Blankinship, J. C., Becerra, C. A., Schaeffer, S. M. and Schimel, J. P.: Separating cellular metabolism from exoenzyme activity in soil organic matter decomposition, Soil Biology and Biochemistry, 71, 68–75, doi:10.1016/j.soilbio.2014.01.010, 2014.

Bowler, M. W., Montgomery, M. G., Leslie, A. G. W. and Walker, J. E.: How azide inhibits ATP hydrolysis by the F-ATPases, Proc Natl Acad Sci USA, 103(23), 8646–8649, doi:10.1073/pnas.0602915103, 2006.

Buchwald, C., Grabb, K., Hansel, C. M. and Wankel, S. D.: Constraining the role of iron in environmental nitrogen transformations: Dual stable isotope systematics of abiotic $NO_2^-$ reduction by Fe(II) and its production of $N_2O$, Geochimica et Cosmochimica Acta, 186(C), 1–12, doi:10.1016/j.gca.2016.04.041, 2016.

Bunzl, K., Schmidt, W. and Sansoni, B.: Kinetics of Ion Exchange in Soil Organic Matter. Iv. Adsorption and Desorption of $Pb^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Ca^{2+}$ by Peat, Journal of Soil Science, 27(1), 32–41, doi:10.1111/j.1365-2389.1976.tb01972.x, 1976.

Clark, F. E.: Transactions of the International Society of Soil Science, Communications IV and V. 1962.

Cory, R. M. and McKnight, D. M.: Fluorescence Spectroscopy Reveals Ubiquitous Presence of Oxidized and Reduced Quinones in Dissolved Organic Matter, Environ. Sci. Technol., 39(21), 8142–8149, doi:10.1021/es0506962, 2005.

Criquet, J., Rodriguez, E. M., Allard, S., Wellauer, S., Salhi, E., Joll, C. A. and Gunten, von, U.: Reaction of bromine and chlorine with phenolic compounds and natural organic matter extracts – Electrophilic aromatic substitution and oxidation, Water Research, 85, 476–486, doi:10.1016/j.watres.2015.08.051, 2015.

Davidson, E. A., Chorover, J. and Dail, D. B.: A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis, Global Change Biology, 9(2), 228–236, doi:10.1046/j.1365-2486.2003.00592.x, 2003.

Drexel, T. R., Haitzer, M., Ryan, J. N., Aiken, G. R. and Nagy, K. L.: Mercury(II) Sorption to Two Florida Everglades Peats: Evidence for Strong and Weak Binding and Competition by Dissolved Organic Matter Released from the Peat, Environ. Sci. Technol., 36(19), 4058–4064, doi:10.1021/es0114005, 2002.

Duggleby, R. G.: [3] Analysis of enzyme progress curves by nonlinear regression, in Methods in Enzymology, vol. 249, pp. 61–90, Academic Press. 1995.

Fellman, J. B., Hood, E. and Spencer, R. G. M.: Fluorescence spectroscopy opens new windows into dissolved organic matter dynamics in freshwater ecosystems: A review, Limnology and Oceanography, 55(6), 2452–2462, doi:10.4319/lo.2010.55.6.2452, 2010.

Gu, B., Bian, Y., Miller, C. L., Dong, W., Jiang, X. and Liang, L.: Mercury reduction and complexation by natural organic matter in anoxic environments, Proc Natl Acad Sci USA, 108(4), 1–5, doi:10.1073/pnas.1008747108, 2011.

Hall, S. J. and Silver, W. L.: Iron oxidation stimulates organic matter decomposition in humid tropical forest soils, Global Change Biology, 19(9), 2804–2813, doi:10.1111/gcb.12229, 2013.

Hutchins, C. M., Teasdale, P. R., Lee, J. and Simpson, S. L.: The effect of manipulating sediment pH on the porewater chemistry of copper- and zinc-spiked sediments, Chemosphere, 69(7), 1089–1099, doi:10.1016/j.chemosphere.2007.04.029, 2007.

Jenkinson, D. S. and Powlson, D. S.: The effects of biocidal treatments on metabolism in soil—I. Fumigation with chloroform, Soil Biology and Biochemistry, 8(3), 167–177, doi:10.1016/0038-0717(76)90001-8, 1976a.

Jenkinson, D. S. and Powlson, D. S.: The effects of biocidal treatments on metabolism in soil—V: A method for measuring soil biomass, Soil Biology and Biochemistry, 8(3), 209–213, doi:10.1016/0038-0717(76)90005-5, 1976b.

Jones, L. C., Peters, B., Pacheco, J. S. L., Casciotti, K. L. and Fendorf, S.: Stable Isotopes and Iron Oxide Mineral Products as Markers of Chemodenitrification, Environ. Sci. Technol., 49(6), 3444–3452, doi:10.1021/es504862x, 2015.

Jun, L. A. B., Gilmour, C. M. and Bollen, W. B.: Non-biological Reduction of Nitrite in Soil, Nature, 225(5233), 664–664, doi:10.1038/225664a0, 1970.

Kampschreur, M. J., Kleerebezem, R., de Vet, W. W. J. M. and van Loosdrecht, M. C. M.: Reduced iron induced nitric oxide and nitrous oxide emission, Water Research, 45(18), 5945–5952, doi:10.1016/j.watres.2011.08.056, 2011.

Kappelmeyer, U., Kuschk, P. and Stottmeister, U.: Model Experiments on the Influence of Artificial Humic Compounds on Chemodenitrification, Water, Air, & Soil Pollution, 147(1-4), 317–330, doi:10.1023/A:1024518027312, 2003.

Kerndorff, H. and Schnitzer, M.: Sorption of metals on humic acid, Geochimica et Cosmochimica Acta, 44(11), 1701–1708, doi:10.1016/0016-7037(80)90221-5, 1980.

Lawson, I. T., Jones, T. D., Kelly, T. J., Coronado, E. N. H. and Roucoux, K. H.: The Geochemistry of Amazonian Peats, Wetlands, 34(5), 905–915, doi:10.1007/s13157-014-0552-z, 2014.

Lähteenoja, O., Ruokolainen, K., Schulman, L. and Alvarez, J.: Amazonian floodplains harbour minerotrophic and ombrotrophic peatlands, CATENA, 79(2), 140–145, doi:10.1016/j.catena.2009.06.006, 2009.

Liengaard, L., Figueiredo, V., Markfoged, R., Revsbech, N. P., Nielsen, L. P., Prast, A. E. and Kühl, M.: Hot moments of $N_2O$ transformation and emission in tropical soils from the Pantanal and the Amazon (Brazil), Soil Biology and Biochemistry, 75, 26–36, doi:10.1016/j.soilbio.2014.03.015, 2014.

McCalley, C. K. and Sparks, J. P.: Abiotic Gas Formation Drives Nitrogen Loss from a Desert Ecosystem, Science, 326(5954), 837–840, doi:10.1126/science.1178984, 2009.

McDevitt, C. A., Ogunniyi, A. D., Valkov, E., Lawrence, M. C., Kobe, B., McEwan, A. G. and Paton, J. C.: A Molecular Mechanism for Bacterial Susceptibility to Zinc, edited by J. Imlay, PLoS Pathog, 7(11), 1–9, doi:10.1371/journal.ppat.1002357, 2011.

McIlvin, M. R. and Altabet, M. A.: Chemical Conversion of Nitrate and Nitrite to Nitrous Oxide for Nitrogen and Oxygen Isotopic Analysis in Freshwater and Seawater, Anal. Chem., 77(17), 5589–5595, doi:10.1021/ac050528s, 2005.

McKenney, D. J., Lazar, C. and Findlay, W. J.: Kinetics of the Nitrite to Nitric Oxide Reaction in Peat, Soil Science Society of America Journal, 54(1), 106–112, doi:10.2136/sssaj1990.03615995005400010016x, 1990.

McKnight, D. M., Boyer, E. W., Westerhoff, P. K., Doran, P. T., Kulbe, T. and Andersen, D. T.: Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity, Limnology and Oceanography, 46(1), 38–48, 2001.

Moraghan, J. T. and Buresh, R. J.: Chemical Reduction of Nitrite and Nitrous Oxide by Ferrous Iron, Soil Science Society of America Journal, 41(1), 47–50, doi:10.2136/sssaj1977.03615995004100010017x, 1977.

Nelson, D. W. and Bremner, J. M.: Factors affecting chemical transformations of nitrite in soils, Soil Biology and Biochemistry, 1(3), 229–239, doi:10.1016/0038-0717(69)90023-6, 1969.

Ohno, T.: Fluorescence Inner-Filtering Correction for Determining the Humification Index of Dissolved Organic Matter, Environ. Sci. Technol., 36(4), 742–746, doi:10.1021/es0155276, 2002.

Ostrom, N. E., Gandhi, H., Trubl, G. and Murray, A. E.: Chemodenitrification in the cryoecosystem of Lake Vida, Victoria Valley, Antarctica, Geobiology, 14(6), 575–587, doi:10.1111/gbi.12190, 2016.

Park, J. Y. and Lee, Y. N.: Solubility and decomposition kinetics of nitrous acid in aqueous solution, J. Phys. Chem., 92(22), 6294–6302, doi:10.1021/j100333a025, 1988.

583 Park, S., Pérez, T., Boering, K. A., Trumbore, S. E., Gil, J., Marquina, S. and Tyler, S. C.: Can
584 $N_2O$ stable isotopes and isotopomers be useful tools to characterize sources and microbial
585 pathways of $N_2O$ production and consumption in tropical soils? Global Biogeochemical Cycles,
586 25(1), 1–16, doi:10.1029/2009GB003615, 2011.

587 Parton, W., Silver, W. L., Burke, I. C., Grassens, L., Harmon, M. E., Currie, W. S., King, J. Y.,
588 Adair, E. C., Brandt, L. A., Hart, S. C. and Fasth, B.: Global-Scale Similarities in Nitrogen
589 Release Patterns During Long-Term Decomposition, Science, 315(5810), 361–364,
590 doi:10.1126/science.1134853, 2007.

591 Pepper, S. E., Borkowski, M., Richmann, M. K. and Reed, D. T.: Determination of ferrous and
592 ferric iron in aqueous biological solutions, Analytica Chimica Acta, 663(2), 172–177,
593 doi:10.1016/j.aca.2010.01.056, 2010.

594 Pérez, T., Trumbore, S. E., Tyler, S. C., Matson, P. A., Monasterio, I. O., Rahn, T. and Griffith,
595 D. W. T.: Identifying the agricultural imprint on the global $N_2O$ budget using stable isotopes,
596 Journal of Geophysical Research: Atmospheres, 106(D9), 9869–9878,
597 doi:10.1029/2000JD900809, 2001.

598 Porter, L. K.: Gaseous Products Produced by Anaerobic Reaction of Sodium Nitrite with Oxime
599 Compounds and Oximes Synthesized from Organic Matter 1, Soil Science Society of America
600 Journal, 33(5), 696–702, doi:10.2136/sssaj1969.03615995003300050023x, 1969.

601 Raczyńska, E. D., Krygowski, T. M., Zachara, J. E., Ośmiałowski, B. and Gawinecki, R.:
602 Tautomeric equilibria, H-bonding and π-electron delocalization in o-nitrosophenol. A B3LYP/6-
603 311 + G(2df,2p) study, J. Phys. Org. Chem., 18(8), 892–897, doi:10.1002/poc.963, 2005.

604 Rogers, R. D.: Abiological Methylation of Mercury in Soil 1, Journal of Environmental Quality,
605 6(4), 463–467, doi:10.2134/jeq1977.00472425000600040029x, 1977.

606 Stedman, G.: Mechanism of the azide–nitrite reaction – Part II, J. Chem. Soc., 2949–2954, 1959.

607 Stedmon, C. A., Markager, S. and Bro, R.: Tracing dissolved organic matter in aquatic
608 environments using a new approach to fluorescence spectroscopy, Marine Chemistry, 82(3-4),
609 239–254, doi:10.1016/S0304-4203(03)00072-0, 2003.

610 Stevenson, F. J., Harrison, R. M., Wetselaar, R. and Leeper, R. A.: Nitrosation of Soil Organic
611 Matter: III. Nature of Gases Produced by Reaction of Nitrite with Lignins, Humic Substances,
612 and Phenolic Constituents Under Neutral and Slightly Acidic Conditions 1, Soil Science Society
613 of America Journal, 34(3), 430–435, doi:10.2136/sssaj1970.03615995003400030024x, 1970.

614 Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. and Ren, Q.: Critical aspects of using
615 bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide, BMC
616 Microbiology 2015 15:1, 15(1), 36, doi:10.1186/s12866-015-0376-x, 2015.

617 Stumm, W. and Morgan, J. J., Eds.: Aquatic Chemistry, 3rd ed., John Wiley & Sons. 2012.

618 Thorn, K. A. and Mikita, M. A.: Nitrite Fixation by Humic Substances Nitrogen-15 Nuclear
619 Magnetic Resonance Evidence for Potential Intermediates in Chemodenitrification, Soil Science

Society of America Journal, 64(2), 568–582, doi:10.2136/sssaj2000.642568x, 2000.

Thorn, K. A., Younger, S. J. and Cox, L. G.: Order of Functionality Loss during Photodegradation of Aquatic Humic Substances, Journal of Environmental Quality, 39(4), 1416–1428, doi:10.2134/jeq2009.0408, 2010.

Trevors, J. T.: Sterilization and inhibition of microbial activity in soil, Journal of Microbiological Methods, 26(1-2), 53–59, doi:10.1016/0167-7012(96)00843-3, 1996.

Tuominen, L., Kairesalo, T. and Hartikainen, H.: Comparison of Methods for Inhibiting Bacterial Activity in Sediment, Appl. Environ. Microbiol., 60(9), 3454–3457, 1994.

Van Cleemput, O., Patrick, W. H. and McIlhenny, R. C.: Nitrite Decomposition in Flooded Soil Under Different pH and Redox Potential Conditions, Soil Science Society of America Journal, 40(1), 55–60, doi:10.2136/sssaj1976.03615995004000010018x, 1976.

Veverica, T. J., Kane, E. S., Marcarelli, A. M. and Green, S. A.: Ionic Liquid Extraction Unveils Previously Occluded Humic-Bound Iron in Peat Soil Pore Water, Soil Science Society of America Journal, 80(3), 771–782, doi:10.2136/sssaj2015.10.0377, 2016.

Wilson, H. F. and Xenopoulos, M. A.: Effects of agricultural land use on the composition of fluvial dissolved organic matter, Nature Geoscience, 2(1), 37–41, doi:10.1038/ngeo391, 2009.

Wolf, D. C., Dao, T. H., Scott, H. D. and Lavy, T. L.: Influence of Sterilization Methods on Selected Soil Microbiological, Physical, and Chemical Properties, Journal of Environmental Quality, 18(1), 39–44, doi:10.2134/jeq1989.00472425001800010007x, 1989.

Wullstein, L. H. and Gilmour, C. M.: Non-enzymatic Formation of Nitrogen Gas, Nature, 210(5041), 1150–1151, doi:10.1038/2101150a0, 1966.

Xia, K., Skyllberg, U. L., Bleam, W. F., Bloom, P. R., Nater, E. A. and Helmke, P. A.: X-ray Absorption Spectroscopic Evidence for the Complexation of Hg(II) by Reduced Sulfur in Soil Humic Substances, Environ. Sci. Technol., 33(2), 257–261, doi:10.1021/es980433q, 1998.

Zelles, L., Palojärvi, A., Kandeler, E., Lützow, Von, M., Winter, K. and Bai, Q. Y.: Changes in soil microbial properties and phospholipid fatty acid fractions after chloroform fumigation, Soil Biology and Biochemistry, 29, 1325–1336, 1997.

Zheng, W., Liang, L. and Gu, B.: Mercury Reduction and Oxidation by Reduced Natural Organic Matter in Anoxic Environments, Environ. Sci. Technol., 46(1), 292–299, doi:10.1021/es203402p, 2011.

Zhu, X., Silva, L. C. R., Doane, T. A. and Horwath, W. R.: Iron: The Forgotten Driver of Nitrous Oxide Production in Agricultural Soil, edited by B. Bond-Lamberty, PLOS ONE, 8(3), e60146, doi:10.1371/journal.pone.0060146, 2013.

Zhu-Barker, X., Cavazos, A. R., Ostrom, N. E., Horwath, W. R. and Glass, J. B.: The importance of abiotic reactions for nitrous oxide production, Biogeochemistry, 126(3), 251–267, doi:10.1007/s10533-015-0166-4, 2015.

## Competing Interests Statement

The authors have no competing interests to declare.