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Extreme emission of N$_2$O from tropical wetland soil (Pantanal, South America)

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**INTRODUCTION**

The atmospheric concentration of nitrous oxide (N$_2$O) is increasing at an accelerating rate with anthropogenic sources estimated to account for ~38% of the current N$_2$O emission (IPCC, 2007). This is concerning because N$_2$O is a powerful greenhouse gas (IPCC, 2007) and the most important ozone depleter of the twenty-first century (Ravishankara et al., 2009). Modeling of the future global climate is dependent on our understanding of the mechanisms that control the atmospheric concentration of greenhouse gases (CO$_2$, CH$_4$, and N$_2$O) and our ability to obtain an accurate budget of anthropogenic and natural sources and sinks. Microbial processes play a major role in the global cycling of carbon and nitrogen (e.g., Gruber and Galloway, 2008) and while the cycling of both is closely linked (e.g., Schlesinger, 2010) most focus has been on carbon. Both CH$_4$ and N$_2$O are primarily biogenic (IPCC, 2007) and methanogens, nitrifiers, and denitrifiers are key players in regulating the global sources and sinks of CH$_4$ and N$_2$O. This underscores the need to better understand how environmental parameters and microorganisms interact to become sources or sinks of these greenhouse gases on a larger scale. In this study the focus is on tropical wetlands soils and we present in situ evidence that the world’s largest freshwater wetland (Pantanal, Brazil) acts as significant source of N$_2$O.

Nitrous oxide (N$_2$O) is an important greenhouse gas and ozone depleter, but the global budget of N$_2$O remains unbalanced. Currently, ~25% of the global N$_2$O emission is ascribed to uncultivated tropical soils, but the exact locations and controlling mechanisms are not clear. Here we present the first study of soil N$_2$O emission from the Pantanal indicating that this South American wetland may be a significant natural source of N$_2$O. At three sites, we repeatedly measured in situ fluxes of N$_2$O and sampled porewater nitrate (NO$_3^-$) during the low water season in 2008 and 2009. In 2010, 10 sites were screened for in situ fluxes of N$_2$O and soil NO$_3^-$ content. The in situ fluxes of N$_2$O were comparable to fluxes from heavily fertilized forests or agricultural soils. An important parameter affecting N$_2$O emission rate was precipitation, inducing peak emissions of ~3 mmol N$_2$O m$^{-2}$ day$^{-1}$, while the mean daily flux was 0.43 ± 0.03 mmol N$_2$O m$^{-2}$ day$^{-1}$. Over 170 days of the drained period, we estimated non-wetted drained soil to contribute 70.0 mmol N$_2$O m$^{-2}$, while rain-induced peak events contributed 9.2 mmol N$_2$O m$^{-2}$, resulting in a total N$_2$O emission of 79.2 mmol N$_2$O m$^{-2}$. At the sites of repeated sampling, the pool of porewater nitrate varied (0.002 – 7.1 µmol NO$_3^-$ g dW$^{-1}$) with higher concentrations of NO$_3^-$ (p < 0.05) found in drained soil than in water-logged soil, indicating dynamic shifts between nitrification and denitrification. In the field, O$_2$ penetrated the upper 60 cm of drained soil, but was depleted in response to precipitation. Upon experimental wetting the soil showed rapid O$_2$ depletion followed by N$_2$O accumulation and a peak emission of N$_2$O (2.5 - 3.0 m mol N$_2$O m$^{-2}$ day$^{-1}$). Assuming that the observed emission of N$_2$O from these wetland soils is generally representative to the Pantanal, we suggest that this undisturbed tropical wetland potentially contributes ~1.7% to the global N$_2$O emission budget, a significant single source of N$_2$O.

**Keywords:** tropical wetland, natural greenhouse gas source, microbial nitrogen cycling, nitrous oxide emission, soil oxic-anoxic transition
an up to five times higher emission of N$_2$O than expected (Kort et al., 2011).

Several studies indicate that tropical forest ecosystems simultaneously accumulate, recycle, and lose nitrogen in far larger quantities than temperate ecosystems (Martinelli et al., 1999; Matson et al., 1999; Hedin et al., 2009). In spite of being rich in nitrogen, this recycling may include high bacterial N$_2$-fixation activity, a nitrogen paradox (Hedin et al., 2009) enabling tropical forest ecosystems to sustain large gaseous nitrogen losses (Houlton et al., 2006). Half of the world’s wetland areas are found in the tropics (Neue et al., 1997); and if the nitrogen paradox is similarly applicable in these ecosystems, tropical wetlands may have a major and yet unresolved role in the global budget of atmospheric N$_2$O.

In both natural and impacted environments, the same microbial processes are responsible for the production of N$_2$O, i.e., nitrification (NH$_4^+$ → O$_2$ → NO$_3^-$) and denitrification (NO$_3^-$ → NO → NO$_2^-$ → N$_2$). Nitrifiers in soil can be stimulated to release N$_2$O at low O$_2$ availability (~5% air sat.; Bollmann and Conrad, 1999), soil moisture content of up to ~60% WFPS (Bateman and Baggs, 2005) and low pH (Mørkved et al., 2007). Denitrifying microorganisms can be stimulated to release N$_2$O by sudden onset of anoxia (Morley et al., 2008), high concentrations of NO$_3^-$ (Blackmer and Bremner, 1978; Blicher-Mathiesen and Hoffmann, 1999), and low pH (Simek and Cooper, 2002). Both nitrification and denitrification can occur simultaneously in complex soil microsites with different access to O$_2$. This makes it difficult to associate a measured soil N$_2$O emission with a specific microbial process (Stevens et al., 1997). However, denitrification is often considered the main N$_2$O producing process in soils (Dobby et al., 1999; Abbasi and Adams, 2000).

The continuous cycle of flooding and draining of wetlands affects important environmental soil parameters such as their O$_2$ content, pH, redox potential and thereby modulates the biogeochemical processes involved in production and emission of N$_2$O (Baldwin and Mitchell, 2000). This hydrological pulse effect is well known in systems influenced by anthropogenic input of nitrogen, where hot spots or hot moments (McCain et al., 2003) of N$_2$O emission are induced by temporal or spatial exoxic-anoxic transitions in for example riparian marshes (Hernandez and Mitsch, 2006), agricultural soil (Markgofed et al., 2011), and mangrove sediment (Allen et al., 2007). Similar flooding effects in tropical freshwater systems are much less explored.

Here we present the first study of the in situ fluxes of N$_2$O, the dynamics of soil nitrogen pools and soil O$_2$ content in Pantanal wetland soils at different times and water status. Three sites were sampled repeatedly over a period of 23–42 days in the beginning of the low water season in 2008 and 2009. Additionally, in the end of the low water season in 2010 10 sites were screened for in situ surface flux of N$_2$O and soil NO$_3^-$ content.

MATERIALS AND METHODS

STUDY SITES

The Pantanal, a pristine tropical wetland in central South America, is shaped by the deposition of sediments into a tectonic depression in the Upper Paraguay River Basin, which formed during the last Andean compressive event (~2.5Ma; Assine and Soares, 2004). The Pantanal supports a lush vegetation of floating and emergged herbs and is dominated by an annual flooding and precipitation cycle, alternately inundating and draining ~140,000 km$^2$ of soil (Swarts, 2000), thus representing the world’s largest wetland (Figure 1A). Aquatic macrophytes and herbaceous plants colonize the entire gradient from permanently dry to permanently wet conditions. In the aquatic-terrestrial transition zone, the herbaceous plant communities die off when the water floods the area annually; this is often followed by an anoxic event in the river and flood water due to the massive decomposition of vegetation (Hamilton et al., 1997; Calheiros et al., 2000). During the flooded season, aquatic macrophytes like Eichhornia crassipes and Salvina auriculata dominate the ecosystem, but decompose when left on the draining wetland soil as the water level decreases (Junk et al., 2006). The flooding pulse thus leads to a regular set-back of community development maintaining the system in an immature, but highly productive stage (Junk and Wantzen, 2004). The annual flooding cycle is driven by a distinct dry/wet season in the ~500,000 km$^2$ watershed, but due to a North-South slope of only 2–3 cm km$^{-1}$, there is a lag period of up to several months between precipitation in the watershed and the flooding of the Pantanal (Junk et al., 2006). This explains why the Pantanal receives the highest precipitation in the low water (drained) season (Figure 2A).

During three field campaigns in 2008 (42 days), 2009 (23 days), and 2010 (14 days), we investigated the in situ flux of N$_2$O and the soil nitrogen pools from wetland soil at representative sites, near the retreating edge of typical water bodies with temporary connection to the main water courses in the Pantanal (Figure 1B). In 2008 and 2009 a main site (site A: 1901.16’S; 5732.99’W) was chosen for repeated sampling. An additional site was chosen for repeated sampling in 2008 only (site B: 1900.61’S; 5733.51’W) and in 2009 only (site C: 1859.28’S; 5725.17’W). In 2010, 10 sites (A, B, D–K) representing different soil and habitat types were screened (Figure 1B; Table 1). All sampling was conducted during the drained season: In 2008 and 2009 sampling was conducted during the period of retreating water, while the screening in 2010 was conducted during the period of rising water level (Figure 2A).
Liengaard et al. Emission of N\textsubscript{2}O from Pantanal

FIGURE 2 | Precipitation and the water level in Rio Paraguai during 2008–2010. (A) Monthly precipitation at Corumbá Airport is shown as gray bars (source: www.inmet.gov.br). Daily water level of Rio Paraguai at Ladário Naval Station is shown as blue line (source: www.cprm.gov.br). The period of each field campaign in 2008, 2009, and 2010 is indicated with black dashed boxes, while the dotted box indicates the period of precipitation, soil moisture, and soil temperature measurements at site A. (B) Precipitation and soil moisture content in 2009–2010 at site A, Level 2. Weekly precipitation is indicated as gray bars. Soil moisture at different depths below the soil surface is shown as lines; 5 cm (red), 10 cm (blue), 30 cm (green), and 60 cm (black) below the soil surface.

Precipitation and soil water content was measured for 170 days of the drained season 2009–2010 (Figure 2B).

Measurements and sampling were carried out along 10–25 m long transects ranging from the retreating water edge to \~1 m above the initial water edge at sites A, B, and C. At the beginning of each field campaign in 2008 and 2009, the elevated end of transects had been drained for \~1 month, while the lowermost end was initially waterlogged, but drained during the field campaign. Three levels were selected along each transect: Level 1 at the lowest end, Level 2 in the center, and Level 3 at the highest end, thus representing both an increase in elevation and a gradual decrease in soil moisture content from Level 1 to Level 3. Due to an excessive amount of rain on site C during the 2009 field campaign, this site was waterlogged during all measurements, while early flooding of site C in 2010 left it inaccessible.

**IN SITU FLUX MEASUREMENTS OF N\textsubscript{2}O**

At each site, the in situ flux of N\textsubscript{2}O was measured at the two lowest levels (Level 1 and 2) along the transect. Flux chambers (n = 5 at each level) made of PVC tubes (\(\varnothing = 24\) cm, height = 20 cm) were inserted \~15 cm into the soil. If any litter layer was present on the soil surface prior to inserting the chamber, it was replaced on the soil surface inside the chamber. In situ flux measurements were performed every 2–14 days by placing a lid on the tube and measuring the N\textsubscript{2}O concentration for 30 min in each chamber with a photo-acoustic gas monitor (INNOVA 1312, LumaSense, Inc., Ballerup, Denmark). Flux chambers were lined with reflective material on the outside and were shaded during measurements to minimize temperature variations. The closed-chamber technique is known to create a bias by altering the diffusion gradient between soil and chamber headspace (Anthony et al., 1995). However, several studies have shown that this bias can be overcome by applying a non-linear regression method to describe the gas exchange (e.g., Kroon et al., 2008; Forbrich et al., 2010). In our case, the flux in each chamber at \(t = 0\) was estimated by fitting the partial pressure increase to a three-parameter exponential function \([pp_t = pp_0 + a(1 - e^{-bt})]\) in Sigmaplot (Systat Software, Inc., Chicago, IL, USA), where \(pp_t\) is the partial pressure of the measured gas at time \(t\), \(pp_0\) is the initial partial pressure in the closed-chamber, \(t\) is time, and \(a\) and \(b\) are constants. Integrated emissions of N\textsubscript{2}O were calculated for each level at each site, assuming linearity between subsequent measurements.
Table 1 | Screening of in situ N₂O flux, pH, and porewater NO₃⁻ in 2010 at 10 sites.

<table>
<thead>
<tr>
<th>Location</th>
<th>Position (WGS 84)</th>
<th>Level</th>
<th>In situ N₂O flux (mmol N₂O cm⁻² day⁻¹)</th>
<th>pH</th>
<th>NO₃⁻ (µmol NO₃⁻ cm⁻³ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site A</td>
<td>19°01′.16″S; 57°32.99″W</td>
<td>1</td>
<td>0.41 ± 0.05</td>
<td>5.32</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.31 ± 0.02</td>
<td>4.96</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.61 ± 0.29</td>
<td>4.43</td>
<td>2.69</td>
</tr>
<tr>
<td>Site B</td>
<td>19°00′.61″S; 57°33.51″W</td>
<td>1</td>
<td>0.21 ± 0.03</td>
<td>4.37</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.48 ± 0.12</td>
<td>4.13</td>
<td>3.45</td>
</tr>
<tr>
<td>Site D</td>
<td>18°43′.56″S; 57°32.12″W</td>
<td>-</td>
<td>0.27 ± 0.02</td>
<td>4.50 ± 0.12</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Site E</td>
<td>18°44′.08″S; 57°32.38″W</td>
<td>1</td>
<td>0.77 ± 0.18</td>
<td>5.08 ± 0.11</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.08 ± 0.17</td>
<td>4.75 ± 0.06</td>
<td>5.38 ± 2.94</td>
</tr>
<tr>
<td>Site F</td>
<td>19°04′.28″S; 57°20.08″W</td>
<td>1</td>
<td>0.22 ± 0.01</td>
<td>6.90 ± 0.06</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.41 ± 0.02</td>
<td>6.25 ± 0.03</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Site G</td>
<td>19°06′.03″S; 57°16.85″W</td>
<td>-</td>
<td>0.18 ± 0.04</td>
<td>5.33 ± 0.06</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td>Site H</td>
<td>19°15′.15″S; 57°04.83″W</td>
<td>-</td>
<td>0.20 ± 0.02</td>
<td>5.98 ± 0.10</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Site I</td>
<td>19°15′.03″S; 57°04.04″W</td>
<td>-</td>
<td>0.45 ± 0.09</td>
<td>5.18 ± 0.08</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Site J</td>
<td>19°18′.33″S; 57°03.29″W</td>
<td>-</td>
<td>0.09 ± 0.01</td>
<td>7.48 ± 0.08</td>
<td>0.29 ± 0.11</td>
</tr>
<tr>
<td>Site K</td>
<td>19°34′.50″S; 57°01.22″W</td>
<td>1</td>
<td>0.30 ± 0.01</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.30 ± 0.01</td>
<td>-</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 5).

During the 2010 screening, the sites had to the best of our knowledge not received precipitation in the preceding days, and the in situ measurements are thus assumed to represent drained soil fluxes.

SOIL PARAMETERS

Porewater NO₃⁻

Whole soil cores (Ø = 5.5 cm, length = 15 cm) were collected at all levels (n = 3 at each level) on every sample occasion at site A, site B, and site C (2008: three times at site A, three times at site B, 2009: five times at site A, four times at site C) and soil porewater immediately extracted in situ by inserting 0.2 µm Rhizon filters (Rhizosphere Research Products, Wageningen, Netherlands) into the side of the whole soil cores at 1.5, 3.5, and 6.5 cm below the soil surface. Samples of ~0.5 ml of porewater were extracted at each depth by suction with a 60 ml syringe. Additional water samples were taken from rivers and water bodies and filtered (0.2 µm filter, Sartorius AG, Göttingen, Germany). Extracted porewater samples and water samples were immediately transferred to 1.5 ml tubes and stored on ice until return to the field laboratory, where they were stored at −20°C until further analysis. Nitrate analysis (sample size 5 µl) was performed using the vanadium chloride reduction method (Braman and Hendrix, 1989) in combination with a chemoluminescence detector (CLD 86, Eco Physics AG, Dürnten, Switzerland), calibrated (r² = 0.99) at six different concentrations of NO₃⁻ (0, 20, 40, 60, 260, 660 µM). Soil samples (n = 1–3) from the mixed upper ~5 cm soil were collected by inserting a 50 ml screw cap centrifuge tube (Starstedt AG, Nürnbrecht, Germany) directly into the soil. Soil samples were refrigerated up to 48 h until NO₃⁻ was measured in a 1% (wt/wt) NaCl solution in the field laboratory. The reason for adding NaCl was primarily a higher stability of the NO₃⁻ biosensor reading in a saline solution.

Means of porewater NO₃⁻ at site A, B, and C were analyzed with a GLM (two-way-ANOVA) with time and levels as factors. A Tukey’s test was run for comparisons among means. Results were tested at a significance level of 95%. Analyses were performed using SAS 9.2 (SAS Institute, Inc., Cary, NC, USA).

Distribution of O₂ in soil

In 2009, the depth distribution of O₂ concentration was measured at site A and C at Level 2 (center of transect) with custom-built fiber-optic O₂ optodes (Ø = 2 mm; Rickelt et al., under review). The optical fibers were calibrated in an O₂-free solution (0.2 M ascorbate, pH 12) and in water equilibrated with atmospheric air prior to installation in the soil at 13 fixed depths (2.5, 5, 10, 15, and 20–100 cm with 10 cm intervals). The optodes were connected to a four-channel fiber-optic O₂ detector system (OXY-4, Presens GmbH, Regensburg, Germany) at each visit to the sites (site A: n = 22, site C, n = 9).

Soil moisture and precipitation

Precipitation, soil temperature, and seasonal changes in volumetric soil water content were measured for a period of 170 days (2009–2010) at site A, Level 2. The volumetric soil water content was logged using soil moisture sensors (Theta-Probes ML2x, Delta-T Devices, Ltd., Cambridge, UK) installed in four depths; 5, 10, 30, and 60 cm below the soil surface in one profile. Each probe was calibrated in the laboratory using depth-specific soil data.
samples from the site. Precipitation was measured using a “Tipping Bucket” rain gage. Soil temperature and precipitation sensors were logged at 10 min intervals, while the Theta-probes were logged every 6 h (CR10X Datalogger, Campbell Scientific, Loughborough, UK).

**N₂O DYNAMICS IN SOIL WETTED EITHER EXPERIMENTALLY OR BY NATURAL PRECIPITATION**

Dry soil cores (d = 5.5 cm, length = 15 cm) for experimental wetting were collected from level 3 at site A and site B. Further soil cores were collected immediately after a natural, moderate precipitation event of short duration (<15 min). Optical O₂ microsensors were constructed according to Klimant et al. (1995) and mounted in hypodermic needles and connected to a fiber-optic O₂ meter (Microx TX3, Presens GmbH, Regensburg, Germany). Electrochemical N₂O microsensors were constructed according to Andersen et al. (2001) with a fortified outer casing to avoid breaking the sensor in the coarse wetland soil (Markfoged et al., 2011) and connected to a picocammeter (PA2000, Unisense A/S, Aarhus, Denmark). Both types of sensors had tip diameters of ~100 µm and a detection limit of ~5 Pa. The O₂ sensors were linearly calibrated from a two-point calibration in O₂-free solution (20% ascorbic acid, pH 11), and in water equilibrated with atmospheric air. The N₂O sensors were linearly calibrated from a two-point calibration in a 0 and 1% solution of N₂O produced by mixing 0.5 ml of saturated N₂O water into 49.5 ml water. The sensors were mounted on a motorized micromanipulator and both sensor positions and data collection were controlled by a PC running SensorTrace Pro software (Unisense A/S, Denmark). Retrieved dry soil cores were wetted from below with aerated river water and concentration profiles (n = 20–30) of O₂ and N₂O were obtained over the following 56–72 h. Additional profile measurements were done in a soil core retrieved after wetting by a natural rain event. The N₂O flux, J, was calculated from the concentration gradient in the water layer on the soil surface using Fick's first law (\( J = -D \frac{\partial C}{\partial x} \)), where D is the N₂O diffusivity in water at experimental temperature (2.41 × 10⁻⁵ cm² s⁻¹), \( C \) is the N₂O concentration in µmol⁻¹ calculated from the measured partial pressure and the experimental temperature according to Weiss and Price (1980), and x is the vertical distance in cm.

**RESULTS**

**IN SITU FLUX MEASUREMENTS OF N₂O**

The *in situ* flux of N₂O at the sites of repeated sampling varied considerably over time in both 2008 (Figure 3A) and 2009 (Figure 3B) the mean daily flux of N₂O varying between 0.04 and 1.37 mmol N₂O m⁻² day⁻¹ at site C, Level 1 and site A, Level 2 respectively (Table 2). *In situ* fluxes of N₂O during the 2010 screening (Figure 3C) varied between 0.09 mmol N₂O m⁻² day⁻¹ (site J) and 1.08 mmol N₂O m⁻² day⁻¹ (site E; Table 1). Peak events of *in situ* N₂O flux were apparently closely associated with sudden and heavy precipitation causing increasing soil water content in the upper 10 cm soil layer which was followed by an increased flux of N₂O measurable 6–12 h later (Figure 4).

**SOIL PARAMETERS**

**Porewater NO⁻3**

At site A (2008) the porewater NO⁻3 content increased significantly (p < 0.05), going from wet soil at Level 1 to drained soil at Level 3 (Figure 5A). In addition, over time the porewater NO⁻3 content increased significantly (p < 0.05) at Level 1 as the soil drained, while a significant decrease (p < 0.05) was observed at Level 2 and 3 (Figure 5A). In 2009 the porewater NO⁻3 content at site A similarly increased significantly (p < 0.05) from wet soil at Level 1 to drained soil at Level 3 (Figure 5A). In addition, a significant increase (p < 0.05) over time in porewater NO⁻3 content was observed at Level 1. The same trend was observed between levels...
Table 2 | Integrated flux and mean daily flux of N\textsubscript{2}O at each Level at the sites of repeated sampling.

<table>
<thead>
<tr>
<th>Site</th>
<th>Level</th>
<th>days</th>
<th>Integrated (N_2O) flux (\text{mmol} N_2O \text{ m}^{-2})</th>
<th>Mean (N_2O) flux (\text{mmol} N_2O \text{ m}^{-2} \text{day}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1(^\dagger)</td>
<td>42</td>
<td>22.0 ± 5.6</td>
<td>0.52 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>2(^\dagger)</td>
<td>42</td>
<td>14.0 ± 3.4</td>
<td>0.33 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>3(^\dagger)</td>
<td>23</td>
<td>12.9 ± 1.7</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2(^\dagger)</td>
<td>23</td>
<td>32.0 ± 5.0</td>
<td>1.37 ± 0.21</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>29</td>
<td>178 ± 1.8</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29</td>
<td>267 ± 4.0</td>
<td>0.92 ± 0.14</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>23</td>
<td>1.0 ± 0.1</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23</td>
<td>2.1 ± 0.4</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

Mean ± SE \((n = 5)\).

N.B. site C was completely water-logged during the entire field campaign 2008–2009.

Figure 4 | In situ \(N_2O\) flux, precipitation and soil moisture content at site A in 2010. Precipitation (gray bars; daily precipitation) caused a sudden increase in soil water content (5 cm; red line; 10 cm; blue line) in the upper 10 cm soil layer and triggered an increase in the in situ flux of \(N_2O\) [(Mean + SE \((n = 5)\) at Level 1 (▲) and Level 2 (▲)).

The integrated emission from the experimentally flooded soil at site A in 2010, with porewater \(NO_3^-\) content increasing from Level 0 to Level 3 (Figure 5A).

At site B in 2008 (Figure 5B) the porewater \(NO_3^-\) content at Level 3 was significantly higher \((p < 0.05)\) than at Level 1 and Level 2. In addition, a significant increase \((p < 0.05)\) over time in porewater \(NO_3^-\) content was observed at Level 1 and Level 3 (Figure 5B). The same trend was observed between levels at site B in 2010, with porewater \(NO_3^-\) content increasing from Level 0 to Level 2 (Figure 5B).

In 2008 site C (Figure 5C) soil samples \((n = 3)\) collected on a single occasion showed a soil \(NO_3^-\) content of 0.73 ± 0.09 \(\mu\text{mol} NO_3^- \text{ g dW}^{-1}\). However, in 2009 (Figure 5C) the overall porewater \(NO_3^-\) content at site C was much lower than at site A or B, presumably due to the water-logging of the soil. Comparing between levels at site C in 2009 (Figure 5C, small insert) the porewater \(NO_3^-\) content at Level 1 was significantly lower \((p < 0.05)\) than at Level 2, but not compared to Level 3.

The soil characteristics from the 2010 screening of 10 sites are shown in Table 1. Soil \(NO_3^-\) content varied between 0.06 \(\mu\text{mol} \text{ cm}^{-2}\) soil (site K) and 5.38 \(\mu\text{mol} \text{ cm}^{-2}\) soil (site E).

The \(NH_4^+\) concentration in river waters ranged between 0.6 ± 0.5 and 10.6 ± 4.8 \(\mu\text{mol} \text{ NH}_4^+ \text{ l}^{-1}\) (Figure 6A) and ranged between 1.0 ± 0.1 and 8.7 ± 2.6 \(\mu\text{mol} \text{ NH}_4^+ \text{ l}^{-1}\) in the other water bodies (Figure 6B). The \(NO_3^-\) concentration in rivers ranged between 0.1 and 12.6 ± 0.8 \(\mu\text{mol} NO_3^- \text{ l}^{-1}\) (Figure 6A) and ranged between 0.1 and 3.4 ± 1.2 \(\mu\text{mol} NO_3^- \text{ l}^{-1}\) in the other investigated water bodies (Figure 6B).

Depth distribution of \(O_2\) in soil
At site A, Level 2, \(O_2\) penetrated to a depth of ~60 cm (37–97% air sat.) but fluctuated throughout the field campaign in response to precipitation (Figure 7A). At site C, Level 2, \(O_2\) was not detected in the soil at Level 2 except the first measurement (Figure 7B) in consistency with the soil being water-logged during the field campaign.

Soil moisture and precipitation
Precipitation measured at Level 2 at site A resulted in an increase in soil moisture. This was most pronounced near the soil surface (5 cm) and less in the deeper soil layers. A subsequent decrease in soil moisture occurred after each rain event, most rapidly at the surface due to evaporation and drainage (Figure 2B).

Microsensor Measurements in Wetted Soils
Upon wetting of drained soil cores collected from Level 3 at site A and site B, porewater \(O_2\) depletion occurred within a few hours (Figures 8A–D), followed by a rapid accumulation of \(N_2O\). The accumulation of \(N_2O\) within the soil persisted for 2–3 days (Figures 9A,B). During this period, \(N_2O\) diffused into the overlying air causing peak emissions of 3.02 mmol \(N_2O \text{ m}^{-2} \text{ day}^{-1}\) from the Site A core (Figure 9C) and 2.53 mmol \(N_2O \text{ m}^{-2} \text{ day}^{-1}\) from the Site B core (Figure 9D), i.e., peak events similar in size and timing to the emission peaks found by in situ flux measurements.

The integrated emission from the experimentally flooded soil of 3.7 mmol \(N_2O \text{ m}^{-2}\) over 3 days (soil core from site A) and 2.92 mmol \(N_2O \text{ m}^{-2}\) over 2.3 days (soil core from site B) represented only a small fraction (35–38%) of the net production of \(N_2O\) in the upper 4 cm of the soil matrix, the difference being consumed within the water-logged soil 2–3 days after the wetting event (Figures 9E,F).

Discussion
Our study represents the first study of soil \(N_2O\) emission and the dynamics of the porewater nitrate in the Pantanal and revealed a large and hitherto unknown source of \(N_2O\) in the largest wetland of the world. In this discussion, we relate our observed in situ \(N_2O\) fluxes to the soil porewater nitrate and soil moisture and precipitation data and compare these results with those found for other tropical systems. Therefore, we discuss our findings of \(N_2O\) production, accumulation, and emission in experimentally wetted soil cores in relation to studies of parameters influencing the reduction
of N\textsubscript{2}O in soil. Finally we speculate on how the observed emission of N\textsubscript{2}O could be supported in a natural system like the Pantanal and how this might link into the cycling of carbon.

**IN SITU FLUX OF N\textsubscript{2}O**

The measured fluxes of N\textsubscript{2}O from Pantanal wetland soils had a high temporal variability (Figure 3) and were generally high (Tables 1 and 2). The average N\textsubscript{2}O emission from the Pantanal wetland soils was 10–390 times higher when compared to other unfertilized tropical systems (Matson and Vitousek, 1987), and 4–6 times higher when compared to fertilizer-induced N\textsubscript{2}O emission peaks in tropical forest soils (Hall and Matson, 1999). The fluxes of N\textsubscript{2}O from the Pantanal wetland soil were thus comparable to fluxes reported from heavily fertilized forest or agricultural soils receiving regular inputs of nitrogen (e.g., Hall and Matson, 1999; Ruser et al., 2006). In soil fertilized with high NO\textsubscript{3} concentrations the primary end product of denitrification upon wetting is often N\textsubscript{2}O (Ruser et al., 2006), and although not fertilized, the drained Pantanal wetland soils similarly contained high concentrations of NO\textsubscript{3}. The high soil NO\textsubscript{3} content thus explains the high emission of N\textsubscript{2}O from the drained Pantanal wetland soils.

Disturbance of tropical soil (e.g., by conversion from forest to pasture or cyclic flooding) has also been shown to increase the emission of N\textsubscript{2}O (Keller et al., 1993; Kern et al., 1996; Veldkamp

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**FIGURE 5** | Soil porewater NO\textsubscript{3} per soil volume (µmol N cm\textsuperscript{-3}) soil) measured along a transect from Level 1 to Level 3 at each site. (A) site A, (B) site B, and (C) site C and site D–K (one or two levels only) in 2008, 2009, and 2010. Mean ± SE (n = 9). At site A and site B a Level 0 was introduced in 2010 because the water level was much lower by the end of the drained season. Level 0 was situated close to the water edge and therefore equivalent to Level 1 with regard to distance to the water edge in 2008 and 2009. Please note scale break on y-axis on (A and D) and insert figure with different scale on (C).

**FIGURE 6** | Inorganic nitrogen (NH\textsubscript{4} and NO\textsubscript{3}) in the water phase in (A) Rio Paraguai and Rio Presa and in (B) water bodies near Site A, B, and C.
et al., 1999). In the Pantanal the development of the plant community is continuously disturbed by the alternating flooding and draining of the soil converting the almost exclusively terrestrial system to an almost exclusively aquatic system (Junk and Wantzen, 2004). Such disturbance by seasonal flooding, combined with a cyclic high biomass input and massive microbial decomposition, inevitably influences the transformation and storage of nitrogen compounds in the soil.

**DYNAMICS OF POREWATER NITRATE**

The concentration of soil porewater $\text{NO}_3^-$ in our study shifted dramatically from being undetectable in water-logged soil to $>1000\ \mu\text{mol NO}_3^-\ l^{-1}$ in drained soil indicating dynamic shifts between nitrate production and consumption in the soil. Extreme *in situ* concentrations of $10 - 30\ \text{mmol NO}_3^-\ l^{-1}$ observed in some samples might be ascribed to high evaporation and capillary forcing drawing nitrate-rich water up from deeper layers of soil and resulting condensation of nitrate near the surface (Wetselaar, 1960). At the beginning of the drained season at the sites of repeated sampling, we found that still water-logged soil contained no $\text{NO}_3^-$, but after 3–6 weeks of draining $\text{NO}_3^-$ could be found and increased further, presumably due to nitrification. This is supported by the $\text{O}_2$ profiles (Figure 7) showing that drained soil was aerated to a depth of 20–50 cm interrupted only by short anaerobic spells caused by a precipitation-induced increase in soil moisture.

Periodically flooded soils in the Amazon have similarly been found to be rich in inorganic nitrogen (Koschorreck, 2005). However, most of the inorganic nitrogen was removed during the first weeks of drying due to coupled nitrification-denitrification (Koschorreck, 2005), whereas decaying plant material may have supplied a continuous input of inorganic nitrogen to the Pantanal wetland soil.

As the wetland soil was draining at site A and B, only sparse plant growth (typically *Panicum maximum*) was observed, and 2–3 months after the end of the flooded season most of the soil surface at site A, B, and C was still covered with decaying aquatic macrophytes (largely *E. crassipes*). In the absence of inorganic...
competition for inorganic nitrogen would likely decrease the availability of inorganic nitrogen for soil microbial N transformations and gaseous N loss.

**N₂O PRODUCTION AND REDUCTION PROCESSES IN THE SOIL**

It is well known that sudden onset of anoxia (Bollmann and Conrad, 1998) and high concentrations of NO₃⁻ (Blackmer and Bremner, 1978) can increase the emission of N₂O. The four step reduction pathway of NO₃⁻ to N₂ is governed by four specific enzymes. The genes encoding each enzyme has multiple transcriptional promoters that are activated by different environmental parameters, and each enzyme has different substrate requirements and inhibitors (Zumft, 1997). This leads to temporary differences in the production and consumption rates of each intermediate causing a temporary accumulation of these intermediates (Frunzke and Zumft, 1986; Cervantes et al., 1998; Zhou et al., 2008). The high NO₃⁻ content of the drained Pantanal wetland soil combined with sudden anoxia may have delayed or partly inhibited the reduction of N₂O, stimulating a temporary high accumulation of N₂O as seen in the experimentally flooded soil cores (Figures 8 and 9). The emitted N₂O represented 35–38% of the registered cumulated net production (cumulated soil + emitted N₂O, Figure 9) the rest being retained and reduced to N₂ within the soil. Any additional N₂O production balanced by simultaneous reduction to N₂ would not be registered in this experimental set up and therefore it is unknown how much larger the N₂ production was.

Microsensor measurements of O₂ and N₂O in soil cores sampled immediately after a natural, moderate rain event (Figure 10) revealed a heterogeneous distribution of O₂ in the soil, reflecting a complex soil matrix with a combination of water-filled pores and gas-filled cracks, root channels, and other macro pores. During this partial water-logging of the soil the transport of gas was dominated by a mix of diffusion in water phase and gas phase, thereby allowing the fast escape of N₂O produced several cm below the soil surface rather than being temporarily accumulated as a transient pool of N₂O until being finally reduced to N₂. Partial wetting of the soil by a moderate rain shower may thus favor hot moments (McClain et al., 2003) of shorter duration, but with higher N₂O emissions than during complete waterlogging after heavy rain. Such a mixed situation of water-filled and gas-filled pore space has previously been associated with high N₂O fluxes, even facilitating the efficient transport of N₂O to the atmosphere (Markfoged et al., 2011).

At site A, we observed O₂ penetration to a depth of 50–60 cm at Level 2, while oxidized iron was observed in soil layers at 80–90 cm depth in holes dug at Level 3. These observations, together with the observed increase in soil NO₃⁻ content over time, suggest that nitrifiers could contribute to the production of N₂O throughout the drained soil. Anaerobic microsites in the generally oxic layers may, however, cause locally intense denitrification (Smith, 1980). A relative increase of the anoxic volume in the soil, e.g., by temporary increased soil moisture as observed in the soil after a rain shower, would further favor denitrification temporarily.

Peak events of N₂O flux from the drained Pantanal wetland soil were apparently closely coupled to precipitation events and variations in water content in the soil matrix (Figure 4). Increased soil moisture by a rain shower did not result in a complete depletion of O₂ in the soil (Figure 7A), but rather increased the anoxic soil...
volume thus temporarily increasing the heterotrophic turnover of organic matter via denitrification. Fluctuations in the anoxic soil volume and fluctuating gas transport parameters thus, controlled the relative contribution of nitrification and denitrification to the total emission of N$_2$O from the Pantanal wetland soil.

**SUGGESTED N$_2$O SOURCE STRENGTH OF THE PANTANAL**

As the drained wetland soil was rich in NO$_3^-$ at both the beginning and end of the drained season we suggest that the observed N$_2$O emission continued throughout drained season. To calculate an estimate of the seasonal N$_2$O flux we therefore classified each flux measurement as either a drained soil flux or a precipitation-triggered peak event flux. Considering the 10 sites as pseudo-replicates a total of 116 in situ flux measurements (each representing a mean of five chambers) were performed from 2008 to 2010. We classified 94 measurements as drained soil fluxes (mean = 0.43 ± 0.03 mmol N$_2$O m$^{-2}$ day$^{-1}$), while 22 measurements were considered precipitation-triggered peak events (mean = 1.54 ± 0.24 mmol N$_2$O m$^{-2}$ day$^{-1}$). The experimental flooding of soils (Figure 8) and the in situ N$_2$O flux measurements (Figure 3) suggest that a typical peak event lasted ~1 day. Therefore, cumulative emissions were calculated assuming linear changes between subsequent measurements of drained soil fluxes, while precipitation-triggered peak event fluxes were assumed to last 1 day.

Precipitation and soil moisture data at site A showed that, during 170 days of the drained season 2009–2010, there were at least six events of heavy precipitation and increased soil moisture that likely triggered a peak N$_2$O emission event. We therefore assume that during the 170 day period precipitation-triggered peak events contributed 9.2 mmol N$_2$O m$^{-2}$, while non-wetted drained soil flux contributed 70.0 mmol N$_2$O m$^{-2}$ to the total emission of N$_2$O. Consequently, we suggest that the cumulated N$_2$O emission from the wetland soil during 170 days of the drained season was 79.3 mmol N$_2$O m$^{-2}$, with precipitation-triggered peak events contributing ~12% of the total N$_2$O emission. In contrast, wetting events in forest soil in Rondônia, Brazil were estimated by (Garcia-Montiel et al., 2003) to contribute <2% of the annual emissions.

For the purpose of estimating the N$_2$O source strength of all the seasonally flooded soils in the Pantanal during the drained season, we calculated that the N$_2$O flux from drained soil over a period of 170 days would be 0.30 Tg N (79.3 mmol N$_2$O m$^{-2} \times 10^8$ g N mol$^{-1} \times 1.4 \times 10^{11}$ m$^{-2}$ of seasonally flooded soil (Swarts, 2000). With an estimated global N$_2$O source strength of 17.7 Tg N year$^{-1}$ (IPCC, 2007) the Pantanal would thus contribute 1.7% to the global N$_2$O emission budget, a significant single source of N$_2$O.

Obviously, our calculations rely on extrapolation from a relatively small data set and need further confirmation by measurements over larger spatio-temporal scales. However, our findings are strongly supported by a recent analysis of the tropospheric distribution and variability of N$_2$O which showed that N$_2$O emissions are concentrated in the tropics and that South America has an up to five times higher emission of N$_2$O than expected (Kort et al., 2011). In addition, the analysis by Kort et al. (2011) demonstrated that global N$_2$O sources are concentrated in the tropics in November and January, thus coinciding with the drained season in the Pantanal and our findings of high N$_2$O emission.

**SOURCE OF NITROGEN**

During 170 days of the drained season the loss of nitrogen from the soil via emission of N$_2$O alone would be 158.5 mmol N m$^{-2}$,
requiring an annual input of at least 158.5 mmol N m\(^{-2}\) or 22.2 kg N ha\(^{-1}\) to balance this loss assuming that the system is in steady-state. So where does this nitrogen come from?

Several reports indicate that natural tropical systems may export very large quantities of nitrogen (e.g., Martinelli et al., 1999; Matson et al., 1999), deemed the “tropical nitrogen paradox”, because input of nitrogen, presumably by \(\text{N}_2\)-fixation, would have to occur in a nitrogen rich environment (Hedin et al., 2009). A spatial decoupling of the \(\text{N}_2\)-fixation and the nitrogen rich soil due to epiphytic \(\text{N}_2\)-fixers has been proposed to solve this paradox for tropical forest systems (Hedin et al., 2009). Could the seasonal production of aquatic macrophytes in the Pantanal be the natural nitrogen source driving nitrification and incomplete denitrification and \(\text{N}_2\)O emission (Figure 11)?

Floating mats of *E. crassipes* have a seasonal biomass production of 10–20 t dry weight ha\(^{-1}\) with an estimated nitrogen content of 30–50 kg N t dry weight\(^{-1}\) and cover 5–100% of water bodies when the Pantanal is flooded (de Neiff et al., 2006). Such a decaying mat would supply the soil with 300–1,000 kg N ha\(^{-1}\) year\(^{-1}\) and ~10 times as much carbon (Abdo and Da Silva, 2002; Xie et al., 2004), which eventually must be mineralized. As the water phase in the Pantanal was generally poor in inorganic nitrogen (Figure 6), we suggest that \(\text{N}_2\)-fixing bacteria associated with the aquatic macrophytes (Iswaran et al., 1973; Purchase, 1977) are a major source of nitrogen to the system. Carignan and Neiff (1992) measured a total \(\text{N}_2\)-fixation of 2.88 mmol N m\(^{-2}\) day\(^{-1}\) in a floating *E. crassipes* mat suggesting an input of 65–85 kg N ha\(^{-1}\) year\(^{-1}\). Our calculated loss of nitrogen of 22.2 kg N ha\(^{-1}\) from the Pantanal wetland soil via \(\text{N}_2\)O would thus be in the range of 2–34% of the above estimated input of nitrogen to the soil via \(\text{N}_2\)-fixation and aquatic macrophytes. This estimate is comparable to managed tropical soil where up to 28% of the applied N was lost as \(\text{N}_2\)O (Veldkamp et al., 1998), but represents a much higher fraction than known from temperate soils. Fertilizer-induced \(\text{N}_2\)O emission from temperate agricultural soils are generally in the range of 0.3–7% of the applied nitrogen fertilizer (Bouwman, 1996; Velthof et al., 2009), roughly a fivefold lower fraction than from the Pantanal wetland soil. The seasonal input of nitrogen by aquatic macrophytes, like the regular application of fertilizer, may therefore be the major cause of the high emission of \(\text{N}_2\)O from the Pantanal wetland soil.

The carbon and nitrogen cycles are closely interlinked in wetlands and the large biomass input suggested above would imply a large input of carbon to the Pantanal wetlands. As the wetland soils of the Pantanal are not peat soils and have a C:N ratio of ~10–20 (data not shown), this input of carbon must be mineralized. Evidence for such mineralization can be found in studies of \(\text{CH}_4\) production and emission from lakes and flooded areas during the flooded season (Marani and Alvala, 2007) and the low water ammonium, and obscure light, preventing growth of other plants. As the soil is drained and aerated, \(\text{O}_2\) becomes available for intense nitrification in the soil while rain showers frequently deplete the \(\text{O}_2\) and elicit denitrification with bursts of \(\text{N}_2\)O until the drained season ends with re-flooding of the soil.

**FIGURE 11** | A conceptual drawing of microbial nitrogen cycling and \(\text{N}_2\)O emission during a 1 year flood cycle of the Pantanal. During flooding, intense nitrogen fixation accompanies the growth of floating meadows dominated by water hyacinths (*Eichhornia crassipes*). As the water retreats, the dense, decaying mats release organic nitrogen (Org. N) to the soil. As the soil is aerated, \(\text{O}_2\) becomes available for intense nitrification in the soil while rain showers frequently deplete the \(\text{O}_2\) and elicit denitrification with bursts of \(\text{N}_2\)O until the drained season ends with re-flooding of the soil.
period (Bastviken et al., 2010) suggesting an annual loss of CH$_4$ to the atmosphere of 450–500 kg C ha$^{-1}$ year$^{-1}$, this also makes the Pantanal a significant source of the greenhouse gas CH$_4$.

CONCLUSION
Studies of nitrogen dynamics and N$_2$O emissions from tropical freshwater wetlands are noticeably scarce. Furthermore, the contribution of N$_2$O from tropical freshwater wetlands has largely been considered negligible (Matson and Vitousek, 1990). The six major tropical freshwater wetlands in South America are estimated to cover an area of 500,000 km$^2$ that is flooded annually (Hamilton et al., 2002), while globally tropical wetlands are estimated to cover 5,000,000 km$^2$ (Neue et al., 1997). Based on our measurements of the N$_2$O flux from wetland soil we suggest that the Pantanal may be contributing 1.7% to the annual global N$_2$O emission budget during the drained season; this is a significant hitherto ignored single source of N$_2$O.

It is currently unknown to what extent the Pantanal wetland system with its dynamic cycling of nitrogen can be compared to other wetlands. The global N$_2$O budget is not balanced (IPCC, 2007), which has been attributed to either a major unknown source or uncertainties in the quantification of one or more known sources (Smith, 1997). Kort et al. (2011) measured atmospheric concentrations of N$_2$O suggesting that South America has a much higher emission of N$_2$O than expected, supporting our observation of the Pantanal as a significant, but hitherto unknown source of N$_2$O.

Our study underscores the direct and indirect importance of flooding and precipitation patterns in tropical wetlands, and wetlands, where sudden natural wetting events can cause significant N$_2$O emission comparable to heavily fertilized agricultural soils. This first study of the dynamics of soil nitrogen pools and emission of N$_2$O from the world’s largest wetland thus emphasizes the current lack of knowledge about nitrogen cycling in undisturbed wetlands, and about how such systems may alter in response to a changing global climate. Lastly, it underscores the paramount importance of varying environmental boundary conditions modulating microbial mineralization processes in the carbon and nitrogen cycle of wetland soils.

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REFERENCES


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