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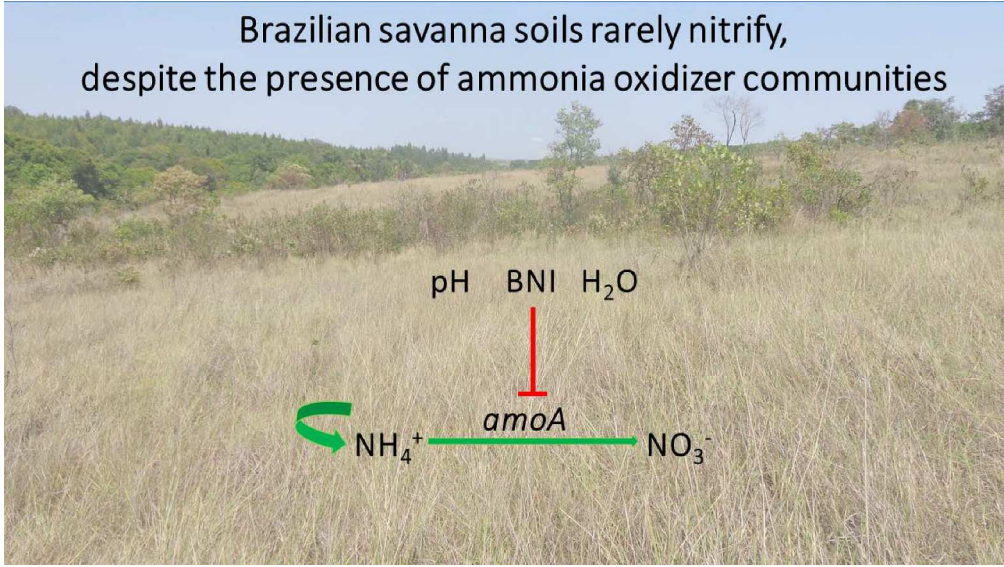
### Ammonia oxidisers in a non-nitrifying Brazilian savanna soil

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Keywords:	ammonia oxidisers, low nitrification, Brazilian savanna, inhibition, pH, soil moisture

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7 **Ammonia oxidisers in a non-nitrifying Brazilian savanna soil**

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11 Elisa C P Catão<sup>1,2</sup>, Cécile Thion<sup>2,3</sup>, R.H. Krüger<sup>1</sup> & James I Prosser<sup>2</sup>

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13 <sup>1</sup>Cellular Biology Department, Instituto Central de Ciencias Sul, Universidade de Brasilia  
14 (UnB), 700910-900 Brasilia, DF, Brazil

15  
16 <sup>2</sup>Institute of Biological and Environmental Sciences, University of Aberdeen, Cruickshank  
17 Building, St. Machar Drive, Aberdeen AB24 3UU, UK

18  
19 <sup>3</sup>Laboratoire Ampère, UMR CNRS 5005, École Centrale de Lyon, 34 avenue Guy de  
20 Collongue, 69130 Ecully, France

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25  
26 Corresponding author:

27  
28 Elisa Catão

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30 Max-Planck Institute for Biogeochemistry

31  
32 Department for Biogeochemical Processes

33  
34 Hans-Knöll Straße 10

35  
36 07745 Jena, Germany

37  
38 E-mail: [elisaccp@gmail.com](mailto:elisaccp@gmail.com)

39  
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41  
42 Phone: +49 3641 576106

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21 **Abstract**

22 Low nitrification rates in Brazilian savanna (Cerrado) soils ~~have~~ puzzled researchers for  
23 decades. Potential mechanisms include biological inhibitors, low pH, low microbial  
24 abundance and low soil moisture content, which hinders microbial activity, including  
25 ammonia oxidation. Two approaches were used to evaluate these potential mechanisms, (i)  
26 manipulation of soil moisture and pH in microcosms containing Cerrado soil and (ii)  
27 assessment of nitrification inhibition in slurries containing mixtures of Cerrado soil and an  
28 actively nitrifying agricultural soil. Despite high ammonium concentration in Cerrado soil  
29 microcosms, little  $\text{NO}_3^-$  accumulation was observed with increasing moisture or pH, but in  
30 some Cerrado soil slurries, AOA *amoA* transcripts were detected after 14 days. In mixed soil  
31 slurries, the final  $\text{NO}_3^-$  concentration ~~was comparative reflected to~~ the initial proportions of  
32 agricultural and Cerrado soils in the mixture, providing no evidence of nitrification inhibitors  
33 in Cerrado soil. AOA community denaturing gradient gel electrophoresis profiles ~~were was~~  
34 similar in the mixed and nitrifying soils. These results suggest that nitrification in Cerrado  
35 soils is not constrained by water availability, ammonium availability, low pH, or biological  
36 inhibitors ~~and alternative potential explanations for low nitrification levels are discussed.~~  
37 ~~The microbial community in Cerrado soil might be adapted to N retention possibly through~~  
38 ~~higher N immobilisation in organic matter rather than N loss through nitrification.~~  
39 Keywords: ammonia oxidisers, low nitrification, Brazilian savanna, inhibition, pH, soil  
40 moisture

## 42 Introduction

43 Autotrophic nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a major  
44 cause of N loss in terrestrial environments. In agricultural systems, nitrification is the main  
45 pathway of N transformation, and up to 95% of total N is present as  $\text{NO}_3^-$  transformed  
46 through nitrification, potentially leading to to nitrate ( $\text{NO}_3^-$ )-leaching and emission of nitric  
47 oxide (NO) and nitrous oxide ( $\text{N}_2\text{O}$ ) by nitrifiers and denitrifiers (Subbarao *et al.*, 2012).  
48 Inhibitors of nitrification can decrease nitrogen losses from these systems (Subbarao *et al.*,  
49 2006). These inhibitors target the first step in nitrification, ammonia oxidation, which is  
50 carried out by both bacterial and archaeal ammonia oxidisers. Some natural systems have  
51 lower nitrification rates and higher nitrogen fertiliser use efficiency than managed systems  
52 (Ste-Marie & Paré, 1999). For example, in soils of the tropical savanna biome in Central  
53 Brazil, also called ~~the~~-Cerrado,  $\text{NO}_3^-$  concentration is low or undetectable (Nardoto &  
54 Bustamante, 2003), the  $\text{NH}_4^+:\text{NO}_3^-$  ratio is high and the abundance of nitrifiers is low (Catão  
55 *et al.*, 2016). These ecosystems may therefore provide a model for greater and more  
56 sustainable crop productivity and decreased demand for nitrogen fertilisers.

57 There are several ~~several~~some potential explanations based on biological and  
58 physicochemical factors leading for to for low rates of nitrification in Cerrado soils, based on  
59 biological and physicochemical factors. Plants may decrease nitrification by competing for  
60  $\text{NH}_4^+$ -N and by increasing the C:N ratio through increased carbon supply, thereby promoting  
61 immobilisation, while some plants produce nitrification inhibitors in plant litter and root  
62 exudates (Subbarao *et al.*, 2006). These inhibitors target ammonia oxidation and can benefit  
63 plants by reducing competition for ammonium (Subbarao *et al.*, 2006, Subbarao *et al.*, 2015).  
64 The specific reasons for the low nitrification rates in the Cerrado biome are unclear, but Both  
65 ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) are both present  
66 in these soils (Catão *et al.*, 2016) but ~~However~~, the relatively high ammonium concentration

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7 67 in Cerrado soil [~~3~~ – 22  $\mu\text{g N g}^{-1}$  soil, (Nardoto & Bustamante, 2003)]; 5 – 49  $\mu\text{g N g}^{-1}$  soil;  
8  
9 68 (*Catão et al.*, 2016)] suggests that ammonia oxidisers are not limited by ammonia  
10  
11 69 concentration and low rates of nitrification in Cerrado soils may be better explained by  
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13 70 production of biological nitrification inhibitors.

14  
15 71 Low nitrification rates in acidic soils have been described for many years (De Boer &  
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17 72 Kowalchuk, 2001). Inhibition of ammonia oxidation in low pH soil was traditionally  
18  
19 73 considered to be due to the low availability of ammonia, through ionisation to  $\text{NH}_4^+$ , but may  
20  
21 74 be alleviated by growth in soil aggregates or on surfaces (De Boer *et al.*, 1991, Allison &  
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23 75 Prosser, 1993), urease activity (De Boer *et al.*, 1989, Burton & Prosser, 2001), or growth of  
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25 76 acidophilic archaeal ammonia oxidisers (Gubry-Rangin *et al.*, 2011, Lehtovirta-Morley *et al.*,  
26  
27 77 2011). Meta-analysis of net nitrification rates in a wide range of soils (Booth *et al.*, 2005)  
28  
29 78 suggests that pH limitation may not be widespread, but ~~increases-increased~~ nitrification  
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31 79 following amendment of Cerrado soil with calcium carbonate (Rosolem *et al.*, 2003) provides  
32  
33 80 evidence for pH limitation in soil.

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35 81 Low water availability decreases nitrification (Placella & Firestone, 2013, Thion &  
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37 82 Prosser, 2014) by increasing osmotic stress and reducing mobility of ammonia within the  
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39 83 soil. ~~In the The rainfall-seasonality dry in the~~ Cerrado biome, ~~and the reported increases in~~  
40  
41 84  $\text{N}_2\text{O}$  ~~production and 10-fold higher~~ NO emissions ~~increase after following~~ rainfall or addition  
42  
43 85 of artificial rainwater (Pinto *et al.*, 2002, Pinto *et al.*, 2006) provide~~ing~~ evidence for limitation  
44  
45 86 of nitrification during dry seasons ~~in this biome~~.

46  
47 87 ~~The specific reasons for the low nitrification rates in the Cerrado biome are unclear,~~  
48  
49 88 ~~but ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) are both~~  
50  
51 89 ~~present in these soils (Catão et al., 2016). Limited nitrification is alleviated by agriculture due~~  
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53 90 ~~to fertilisation, liming, tillage or plant community change. Considering the extensive~~  
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55 91 ~~conversion of Cerrado soils to agricultural production (Marris, 2005, Catão et al., 2016), it is~~

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7 92 ~~relevant~~important to understand adaptation of natural ecosystems ~~adaptation~~ to limit N loss.  
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9 93 The aim of this study was to test three hypotheses ~~which~~regarding ~~are~~ potential mechanisms  
10 94 ~~underlying~~for the low nitrification rates: presence of biological nitrification inhibitors, low  
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12 95 water availability and low pH. The presence of plant-derived nitrification inhibitors was  
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14 96 tested by analysing (i) the growth of AOB and AOA in the presence of aqueous extract from  
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16 97 Cerrado soil and (ii) the effect of Cerrado soil on ammonia oxidation by a nitrifying soil  
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18 98 (Craibstone) in soil slurries. The effects of low water availability and low pH on nitrification  
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20 99 were tested by manipulating Cerrado soil water content and pH in microcosms.  
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## 102 **Materials and methods**

### 103 *Soil sampling*

104 Cerrado soil was sampled from an undisturbed shrubland (Campo sujo), with some sparse  
105 shrubs over a continuous grass layer (Eiten, 1972) ~~described previously~~ (Catão *et al.*, 2016),  
106 where graminoids can account for around 45% of total aboveground biomass, leading to a  
107 contribution of 46% of relative abundance of fine roots (Castro & Kauffman, 1998). Campo  
108 sujo is dominated by plants from the families Asteraceae, Leguminosae, and Poaceae (Tannus  
109 & Assis, 2004). The average monthly precipitation and temperature, measured at the nearest  
110 meteorological centre in 2014 (~30 km from the farm; Pirenópolis – GO, Station 83376,  
111 15°50'60"S 48°57'36"W), were 143 mm (range 0 - 317 mm) and 23.4°C (range 21°C -  
112 25.6°C), respectively. Triplicate soil samples were obtained from the upper 10 cm of soil,  
113 pooled before sieving (2-mm mesh size) and then stored at 4°C. The climate in the Cerrado  
114 biome is tropical (Köppen Aw), and samples were collected at the beginning of the dry  
115 season (May 2014). Campo sujo and Cerrado *sensu stricto* are usually found on oxisols, with  
116 low nutrient content, low pH, and high content of aluminium (Reatto *et al.*, 1998). The soil,

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7 117 which was well aerated and well drained, is classified as sandy loam with 20.8% clay and had  
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9 118 an initial pH of 5.6 ( $\pm 0.04$ ). [Physicochemical parameters from the eCampo sujo sample were](#)  
10 119 [previously described](#) (Catão *et al.*, 2016): [organic matter content was 42.6 \( \$\pm 2.4\$ \) g kg<sup>-1</sup>](#)  
11 120 [cation exchange capacity 6 \( \$\pm 0.6\$ \) cmol<sub>c</sub> dm<sup>-3</sup>](#), [available phosphorus 1.8 \( \$\pm 0.13\$ \) mg dm<sup>-3</sup>](#)  
12 121 [aluminium 1.2 \( \$\pm 0.12\$ \) cmol<sub>c</sub> dm<sup>-3</sup>](#) and [Fe 165.4 \( \$\pm 41.04\$ \) mg dm<sup>-3</sup>](#). Craibstone soil, used in  
13 122 this study as a reference nitrifying soil, was sampled from an experimental agricultural field  
14 123 (Scottish Agricultural College, Craibstone, Scotland, Grid reference NJ872104) and  
15 124 maintained at pH 5.5 since 1961.  
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### 26 127 *Cultivation of ammonia oxidisers with soil extracts*

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28 128 Aqueous extracts of Craibstone and Campo sujo soils were prepared by blending 20 g soil in  
29 129 2 volumes of sterile distilled water for 40 s, rotating in 50-mL sterile tubes for 1 h,  
30 130 centrifuging (3,000×g for 15 min) and sterilising by progressive filtration through filters with  
31 131 10-mm, 5-mm, 0.45- $\mu$ m and 0.22- $\mu$ m pore size. NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations in the filtrates  
32 132 were below the level of detection (data not shown).  
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37 133 Pure cultures of AOA (*Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley  
38 134 *et al.*, 2016)) and AOB (*Nitrospira briensis*, #128, *Nitrospira tenuis*, #NV-12,  
39 135 *Nitrospira multiformis*, - NCIMB11849, ATCC25196 and *Nitrosomonas europaea*, -  
40 136 [NCIMB11850, ATCC25978](#)) were cultivated in inorganic growth medium in the dark  
41 137 without shaking. *Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley *et al.*, 2016)  
42 138 was cultivated at 40°C in medium described previously (Lehtovirta-Morley *et al.*, 2011) but  
43 139 modified by the addition of 1 mL L<sup>-1</sup> vitamin solution (Widdel & Bak, 1992), 1 mL L<sup>-1</sup>  
44 140 selenite-tungstate solution (Widdel & Bak, 1992) and 2 mM NH<sub>4</sub>Cl. The pH was maintained  
45 141 at ~7.5 by the addition of 10 mL L<sup>-1</sup> 1 M HEPES buffer. The AOB were grown in Skinner  
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7 142 and Walker medium (Skinner & Walker, 1961) and incubated at 30°C. Triplicate cultures  
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9 143 were prepared in 30-mL Universal tubes by adding 5 mL of the appropriate medium,  
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11 144 previously inoculated with exponentially growing cells (1 mL inoculum per 100 mL 2×  
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13 145 concentrated medium), to a 5-mL volume of sterile distilled water, Craibstone or Campo sujo  
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15 146 soil aqueous extract, or allylthiourea (100 µM final concentration), an ammonia oxidiser  
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17 147 inhibitor. The cultures were grown without agitation, and growth was monitored for 26 days  
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19 148 (AOA) and 13 days (AOB) by measuring nitrite accumulation (Shinn, 1941). The maximum  
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21 149 specific growth rate was estimated as the slope of semi-logarithmic plots of nitrite  
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23 150 concentration versus time.  
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#### 26 152 *Soil incubation in slurries*

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28 153 Soil slurries were established in 250-mL sterile Erlenmeyer flasks containing 20 g soil and  
29  
30 154 100 mL sterile distilled water, stirred at 100 rpm and maintained at 30°C in the dark.  
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32 155 Individual flasks contained Campo sujo soil, Craibstone soil or mixtures of Campo sujo and  
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34 156 Craibstone soils in 1:1 or 4:1 ratios. Before incubation and 1, 7, 14, and 21 days after  
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36 157 incubation, soil slurry aliquots (8 mL) were centrifuged at 3,000×g for 15 min. After  
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38 158 immediate measurement of pH in 2 mL of supernatant, the remaining supernatant (6 mL) was  
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40 159 stored at -20°C for quantification of inorganic N (see below). The soil pellet was frozen in  
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42 160 liquid nitrogen and stored at -80°C for [nucleic acid molecular](#) analysis.  
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#### 45 162 *Soil incubation in microcosms*

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47 163 ~~Cerrado~~ Campo sujo soil was incubated in sealed microcosms consisting of 140-mL sterile  
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49 164 serum glass bottles containing 10 g soil. The soil had an initial water content of  $24.9 \pm 0.03$  g  
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51 165 H<sub>2</sub>O [100](#) g<sup>-1</sup> dry soil, corresponding to a matric potential of  $-0.15 \pm 0.01$  MPa. Microcosms  
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53 166 were incubated for 4 days in the dark at 30°C (acclimation period) and then divided into two  
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7 167 groups. The 'dried soil' group was left to air dry, reaching a moisture content of 8.66 g H<sub>2</sub>O  
8 168 [100 g<sup>-1</sup>](#) dry soil (-6.34 ± 2.98 MPa matric potential). In the 'moist soil' group, the moisture  
9 169 content was adjusted to 37.9 ± 0.3 g H<sub>2</sub>O [100 g<sup>-1</sup>](#) dry soil by adding sterile distilled water. Soil  
10 170 in half of the dried soil microcosms was rewetted to 39.6 ± 1.92 g H<sub>2</sub>O [100 g<sup>-1</sup>](#) dry soil (-0.11  
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14 171 ± 0.02 MPa) ('Water Pulse' treatment), and the soil in the remaining dried soil microcosms  
15 172 was kept dry ('Dry' treatment). Finally, the pH of soil in half of the moist soil microcosms  
16 173 was increased to 6.34 ± 0.09 with CaCO<sub>3</sub> ('pH treatment'). The pH of soil in the remaining  
17 174 microcosms ('Dry', 'Water Pulse' and 'Moist' treatments) was 5.21 ± 0.02, which was  
18 175 slightly lower than the initial value of the sampled soil and was not adjusted. The four  
19 176 treatments were performed in triplicate, with or without the addition of the ammonia  
20 177 oxidation inhibitor acetylene (0.01% of headspace volume). The soil microcosms were  
21 178 incubated in the dark at 30°C, and aerobic conditions were maintained by removing the seals  
22 179 for 5 - 10 minutes twice weekly. The 'Moist' and 'Water Pulse' microcosms were watered  
23 180 weekly to maintain moisture content. The microcosms were sampled destructively after 6 h  
24 181 and 1, 3, 7, 14, and 21 days, with additional sampling after 28 days for the pH treatment). For  
25 182 each microcosm, half of the soil was used for chemical analysis and the remainder was stored  
26 183 at -80°C for molecular analysis.  
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#### 41 185 *Soil physicochemical analyses*

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43 186 Water matric potential was measured using a WP4C Dewpoint PotentialMeter (Decagon,  
44 187 Pullman, UK) and pH was determined in water. Soil NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub> (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>)  
45 188 concentrations were determined colorimetrically by flow injection analysis (FIA star 5010  
46 189 Analyser, Foss Tecator AB, Höganäs, Sweden) (Allen, 1989) after extraction from 2 g wet  
47 190 soil in 10 mL KCl (1 M), for the microcosm soil, or directly from slurry supernatant. Because  
48 191 NO<sub>2</sub><sup>-</sup> concentration was below the level of detection, NO<sub>x</sub> is expressed as µg NO<sub>3</sub><sup>-</sup>-N g<sup>-1</sup> dry  
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7 192 | soil (ppm). Nitrification inhibition was assessed as the decrease in nitrate concentration as a  
8 193 | percentage of that of Craibstone soil at each time point.

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12 195 ***Molecular analysis***

13  
14 196 Nucleic acids were extracted from 0.5 g soil as previously described (Nicol *et al.*, 2005),  
15 197 suspended in diethylpyrocarbonate-treated water and immediately stored at -80°C. An aliquot  
16 198 was treated with DNase and the RNA was reverse transcribed, as described previously  
17 199 (Tourna, 2008). The nucleic acid not used for cDNA generation was considered DNA only  
18 200 and its concentration was estimated using a NanoDrop 1000 Spectrophotometer (Thermo  
19 201 Scientific, Loughborough, UK).

22 202 | Archaeal and bacterial *amoA* genes, which encode subunit A of ammonia  
23 203 monooxygenase, were quantified in a MasterCycler thermal cycler (Eppendorf, Hamburg,  
24 204 Germany) using standard curves, as described previously (Catão *et al.*, 2016). PCR  
25 205 amplification was carried out using primers crenamo23f and crenamo616r for archaeal *amoA*  
26 206 genes (Tourna, 2008) and amoA1F and amoA2R for bacterial *amoA* genes (Rotthauwe *et al.*,  
27 207 1997). Each 20- $\mu$ L reaction contained 1 $\times$  QuantiFast PCR Master Mix (for AOA) or  
28 208 QuantiTect Master Mix (for AOB) (Qiagen, Crawley, UK), 0.4  $\mu$ M of each primer for AOA  
29 209 *amoA* or 0.6  $\mu$ M of each primer for AOB *amoA*, 2  $\mu$ g  $\mu$ L<sup>-1</sup> BSA (Promega), and 2  $\mu$ L DNA  
30 210 (or cDNA). Archaeal *amoA* genes and transcripts were amplified using the following cycling  
31 211 conditions: 15 min at 95°C, followed by 40 cycles of 15 s at 94°C and 90 s at 60°C. Bacterial  
32 212 *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at  
33 213 95°C, followed by 45 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. To exclude  
34 214 fluorescence contamination of potential primer-dimers, SYBR Green fluorescence was  
35 215 measured after 5 s at 80°C or after 8 s at 83°C, for AOA and AOB, respectively. Melting  
36 216 curves between 65°C and 95°C were analysed for each run. AOB *amoA* transcripts were

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7 217 below the detection limit (5 copies  $\mu\text{L}^{-1}$ ). Efficiency of amplification and  $r^2$  for DNA were,  
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9 218 respectively, 0.92 and 0.998 for archaeal *amoA* and 104.6 and 0.993 for bacterial *amoA*.

10 219 AOA community composition in soil slurries was assessed before and after incubation  
11  
12 220 for 21 days by denaturing gradient gel electrophoresis (DGGE) of archaeal *amoA* gene using  
13  
14 221 the primers described above in a linear gradient of 15% - 55% denaturant, as described  
15  
16 222 previously (Nicol *et al.*, 2005).  
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### 20 224 **Statistical analysis**

21  
22 225 All analyses were conducted using R version (3.2.2). The effect of aqueous soil extracts on  
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24 226 pure AOA and AOB cultures was analysed by testing differences in specific growth rate  
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26 227 between treatments by one-way analysis of variance. Differences between nitrification rate in  
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28 228 soil slurries were evaluated using a repeated-measures linear mixed model (package *nlme*)  
29  
30 229 (Pinheiro *et al.*, 2015). Each slurry was considered a subject with random effect to analyse  
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32 230 the effect of treatment (Campo sujo soil, Craibstone soil, or soil mixture), time, and their  
33  
34 231 interaction on inorganic N concentration and *amoA* gene (and transcript) abundance. The  
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36 232  $\text{NO}_3^-$  concentration in the Campo sujo slurries was below the limit of detection; therefore,  
37  
38 233 these samples were excluded from the analysis. Gene abundance data were log-transformed  
39  
40 234 to achieve a normal distribution. When the interaction between independent variables was not  
41  
42 235 significant, it was removed to analyse the effect of time or treatment independently over  
43  
44 236 concentration of soil  $\text{NH}_4^+$  and  $\text{NO}_x$ . Two-way analysis of variance, with treatment and time  
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46 237 as independent factors, was performed to evaluate differences in mineralisation and  $\text{NO}_3^-$  in  
47  
48 238 the soil microcosms.  
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## 51 240 **Results**

### 53 241 **Effects of soil extracts on ammonia oxidiser cultures**

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7 242 To assess the presence of nitrification inhibitors in the soil, pure cultures of four AOB and  
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9 243 one AOA were grown in liquid batch culture in medium containing aqueous soil extracts,  
10  
11 244 water (negative control) or allylthiourea (positive control). Extracts from Campo sujo and  
12  
13 245 Craibstone soils had no significant effect on the growth of any of the ammonia-oxidising  
14  
15 246 strains tested (Figs. 1 and S1). Allylthiourea completely inhibited all AOB cultures tested, but  
16  
17 247 did not inhibit the growth of the AOA *Candidatus N. franklandus* (Figs. 1 and S1).  
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19 248

#### 20 249 **Effects of Campo sujo soil on nitrification in Craibstone soil**

21  
22 250 Soil slurries containing Campo sujo soil, Craibstone soil, or a mixture of the two soils (at  
23  
24 251 ratios of 1:1 and 4:1) were incubated for up to 21 days. In all slurries, pH increased (0.4 in the  
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26 252 ~~grassland~~Campo sujo soil, 0.8 in Craibstone ~~samplesoil~~, but only 0.1 and 0.2 for the 1:1 and  
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28 253 4:1 mixed samples, respectively) after the first day of incubation but did not change  
29  
30 254 significantly thereafter. Net  $\text{NH}_4^+$  ~~accumulation-concentration~~ after 21 days ranged from 0.62  
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32 255 ( $\pm 0.02$ ) to 1.76 ( $\pm 0.39$ ) ppm ( $\text{mg L}^{-1}$  soil solution) for Craibstone soil and 0.87 ( $\pm 0.02$ ) to  
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34 256 2.20 ( $\pm 0.02$ ) ppm ( $\text{mg L}^{-1}$  soil solution) for Campo sujo soil (Fig. 2). Initial  $\text{NH}_4^+$   
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36 257 concentration was higher in the mixed soil slurries than in controls, but the mixed slurries  
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38 258 accumulated less  $\text{NH}_4^+$  over the incubation period. The greatest increase in  $\text{NH}_4^+$   
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40 259 concentration after 21 days was observed in Craibstone soil (2.9-fold).

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42 260  $\text{NO}_3^-$  concentration also increased in all soil slurries during incubation ( $p < 0.0001$ , Fig.  
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44 261 2B), except in those containing Campo sujo only, in which  $\text{NO}_3^-$  was below the detection  
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46 262 limit. In the mixed soil slurries,  $\text{NO}_3^-$  production was equivalent to or higher than the 50%  
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48 263 and 20% expected for the 1:1 and 4:1 ratios of Campo sujo soil and Craibstone soil,  
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50 264 respectively (Fig. 2C), providing no evidence for inhibition of Craibstone soil nitrification by  
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52 265 Campo sujo soil.

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7 266 Ammonia oxidiser *amoA* gene abundance in the soil slurries did not change  
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9 267 significantly during the incubation period, even when significant  $\text{NO}_3^-$  accumulation was  
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11 268 observed (Fig. 3). AOA *amoA* abundance in the Campo sujo-only slurries was approximately  
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13 269 three orders of magnitude lower than that of Craibstone-only slurries (Fig. 3A). AOA *amoA*  
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15 270 abundance in mixed soil slurries was lower than that of Craibstone-only slurries until day 14,  
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17 271 after which differences were not significant ( $p=0.132$ ). AOB *amoA* gene abundance in the  
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19 272 Campo sujo-only slurries was also approximately three orders of magnitude lower than that  
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21 273 of Craibstone-only slurries, and even significantly different ( $p=0.024$ ) at day 21, when  
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23 274 Campo sujo-only AOB abundance was no longer significantly different from the those in the  
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25 275 mixed samples (Fig. 3B).

26 276 The AOB *amoA* gene abundance was lower than AOA *amoA* gene abundance in all  
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28 277 slurries at each time point. The AOA:AOB *amoA* gene ratio did not change significantly in  
29  
30 278 the Campo sujo-only slurries but increased in the Craibstone-only and mixed soil slurries  
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32 279 (Fig. 3C). In all slurries, AOB *amoA* transcripts were below the level of detection ( $5 \mu\text{L}^{-1}$ ).  
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34 280 The AOA *amoA* transcripts were detected in all slurries containing Craibstone soil throughout  
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36 281 incubation but were detected in the Campo sujo-only slurries only at day 21 (Fig. 3D).

37 282 Before incubation, DGGE profiles of *amoA* genes amplified from Craibstone soil  
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39 283 contained more bands (potential OTUs) than profiles of Campo sujo soil (Fig. S2) and did not  
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41 284 change significantly after incubation for 21 days. DGGE profiles of the AOA *amoA* genes in  
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43 285 the 1:1 mixed slurry were similar to those of Craibstone soil, possibly masking ~~the~~  
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45 286 ~~observation of~~ the less abundant *amoA* genes from the Campo sujo soil (Fig. S2).  
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#### 48 288 ***Effects of soil pH and moisture content***

49 289 The effects of pH and moisture content on nitrification in Campo sujo soil were investigated  
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51 290 in soil microcosms. Net N mineralisation was determined as the increase in concentration of  
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7 291 inorganic N ( $\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N}$ ) during incubation, assuming that other nitrogen cycle  
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9 292 processes were not significant (Fig. S3). Mineralisation in the dry soil did not increase after  
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11 293 wetting, in contrast to the expected 'Birch' effect (Birch, 1964) (Fig. S3). In addition, soil pH  
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13 294 did not change significantly with time in the microcosms and remained at 5.2 for the 'Water  
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15 295 Pulse', 'Moist' and 'Dry' treatments and at 6.3 for the 'pH' treatment, in which pH was  
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17 296 increased artificially with  $\text{CaCO}_3$ . Nitrate concentration did not increase significantly in any  
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19 297 of the treatments (Fig. S3), and no significant difference was observed between treatments ( $p$   
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21 298  $=0.14$ ). No significant differences were observed between samples incubated with or without  
22  
23 299 acetylene, except for  $\text{NO}_3^-\text{-N}$  concentrations in the 'Moist' microcosms. After incubation for  
24  
25 300 21 days, the  $\text{NO}_3^-\text{-N}$  concentration was lower in the acetylene-treated moist microcosms than  
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27 301 in those without added acetylene (Fig. S3).  
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### 303 Discussion

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305 Nitrification is frequently undetectable in undisturbed Cerrado ecosystems, although  
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307 management and conversion to agricultural production increases nitrate production (Catão *et*  
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309 *al.*, 2016). Previous studies suggest low abundance of AOA and AOB in Campo sujo soil  
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311 (Catão *et al.*, 2016), which is also characterised by sparse shrubs over a continuous grass  
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313 layer. The aim of this work was to determine whether the lack of nitrification and low  
314  
315 abundance of ammonia oxidisers in this ecosystem were due to low pH, low soil moisture,  
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317  $\text{NH}_4^+$  limitation or biological inhibition of ammonia oxidation.

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319 Certain plants release biological nitrification inhibitors that suppress ammonia  
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321 oxidation in soils (Subbarao *et al.*, 2015). For example, compounds produced by *Brachiaria*  
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323 (Subbarao *et al.*, 2009) and *Sorghum* (Zakir *et al.*, 2008) inhibited a recombinant *N. europaea*  
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325 strain, possibly by blocking ammonia monooxygenase and hydroxylamine oxidoreductase  
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7 316 (Subbarao *et al.*, 2008). Production of biological nitrification inhibitors can be promoted by  
8 317 exposure to high  $\text{NH}_4^+:\text{NO}_3^-$  ratios (Subbarao *et al.*, 2015), such as those found in Campo  
9 318 sujo soil (Catão *et al.*, 2016). However, aqueous extracts of Campo sujo soil did not inhibit  
10 319 growth of cultures of four AOB and one AOA, all of which were isolated from [neutral to](#)  
11 320 [alkaline](#) soils. Allylthiourea, used as a positive control, prevented growth of the AOB cultures  
12 321 but not the AOA culture, which is consistent with other studies reporting a greater tolerance  
13 322 of AOA to allylthiourea (Hatzenpichler & Lebedeva, 2008, Stempfhuber *et al.*, 2015). This  
14 323 result demonstrates the need to test potential inhibitors against both AOA and AOB, rather  
15 324 than *N. europaea* only.

16 325 Cultivation-based studies were ~~based-performed only-using~~ aqueous soil extracts  
17 326 and a small number of cultivated strains and potential inhibition was therefore assessed more  
18 327 directly by mixing Campo sujo soil with Craibstone soil, a strongly nitrifying soil with  
19 328 similar pH, in soil slurries (Nicol *et al.*, 2008, Zhang *et al.*, 2010). The soil slurries also  
20 329 provided no evidence of nitrification inhibitors in Campo sujo soil. Nitrate accumulation in  
21 330 mixtures of Craibstone soil and Campo sujo soil was lower than that of Craibstone soil only,  
22 331 but this difference was less than or equal to that predicted by the lower volume of Craibstone  
23 332 soil in the slurry, suggesting that nitrification was not inhibited by the Campo sujo soil.

24 333 Similarly, the addition of Campo sujo soil to Craibstone [soil](#) had no apparent effect on AOA  
25 334 and AOB *amoA* gene abundances. Archaeal *amoA* genes were more abundant than those of  
26 335 bacteria in the soils, and bacterial *amoA* gene expression was not detected, as reported by  
27 336 previous studies of Craibstone soils (Zhang *et al.*, 2010). Neither AOA nor AOB *amoA*  
28 337 abundance changed significantly during incubation of any of the slurries containing  
29 338 Craibstone soil, despite evidence of nitrate production. However, the AOA:AOB *amoA* gene  
30 339 ratio increased, suggesting greater growth or lower death rates of AOA, but there was no  
31 340 evidence for growth of AOB or AOA in the Campo sujo soil. DGGE ~~analysis-profiles of the~~



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7 341 | ~~Campo sujo soil contained detected few fewer~~ archaeal *amoA* ~~gene bands in the Campo sujo~~  
8 342 | ~~soil compared than to those of that of~~ Craibstone soil, providing a further ~~evidence indication~~  
9 343 | of ~~the~~ low abundance and activity of ammonia oxidisers in Campo sujo soil. Although AOA  
10 344 | *amoA* transcripts in Campo sujo-only slurries increased after incubation for 21 days, this  
11 345 | increased gene expression did not appear to lead to a detectable level of nitrate. Nitrate  
12 346 | reduction during denitrification was considered negligible due to previous experiments  
13 347 | showing low NO emissions and undetectable N<sub>2</sub>O in Cerrado soils (Pinto *et al.*, 2002).

14 348 |         In the absence of evidence for BNI, microcosm studies were performed to determine  
15 349 | whether the low nitrification rates in Campo sujo soil were due to low pH or low soil  
16 350 | moisture content. Gas measurements in Cerrado soil after a rainfall (natural or simulated) led  
17 351 | to an increase in NO emissions (Pinto *et al.*, 2002), which ~~agrees may result from with~~ the  
18 352 | Birch effect (Birch, 1964) of increased organic matter availability after rewetting (Fierer &  
19 353 | Schimel, 2003). Soil pH is an important determinant of microbial diversity (Lauber *et al.*,  
20 354 | 2009, Fierer *et al.*, 2012) and influences soil ammonia oxidiser abundance and activity (de  
21 355 | Boer and Kowalchuk, Nicol *et al.* 2008), with higher transcriptional activity of Archaea than  
22 356 | Bacteria as pH decreases (Nicol *et al.*, 2008). In our experiment, higher soil pH increased  
23 357 | organic nitrogen mineralisation rate but did not lead to detectable nitrate production in  
24 358 | Campo sujo soil after incubation for 28 days. Mineralisation was also lower in moist soil than  
25 359 | in other treatments, and the increase in moisture did not lead to detectable nitrate production  
26 360 | (see supplementary Fig. S3). Even though it can be argued that NO<sub>3</sub><sup>-</sup> can be denitrified at  
27 361 | higher soil moisture, low or undetectable NO and N<sub>2</sub>O emission in field measurements over  
28 362 | the seasons (Cruvinel *et al.*, 2011) and no consistent NO<sub>3</sub><sup>-</sup> increase after one day or after 3  
29 363 | weeks of incubation, suggested limitation of nitrification by other factors.

30 364 |         The low nitrification and low ammonia oxidiser abundance of Campo sujo soil, in  
31 365 | both microcosms and slurries, were not due to NH<sub>4</sub><sup>+</sup> limitation. The NH<sub>4</sub><sup>+</sup> concentration of

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7 366 Campo sujo soil slurries was even higher than that of Craibstone soil slurries at the beginning  
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9 367 of the experiment. Jack pine forest soils showed similar ~~results of~~ high ~~accumulated~~  
10 368 concentrations of ammonium without detectable nitrate (Ste-Marie & Paré, 1999). None of  
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12 369 the treatments in this study increased nitrification in Campo sujo soil and this soil did not  
13  
14 370 inhibit nitrification in Craibstone soil or pure cultures of AOA or AOB. Similarly, in the jack  
15  
16 371 pine forest soil, nitrification was not stimulated by increased pH or ammonium amendment  
17  
18 372 but was stimulated by the addition of nitrifying soil from a forest floor (Ste-Marie & Paré,  
19  
20 373 1999). In this study, both AOA and AOB were detected in Campo sujo soil, but at low levels  
21  
22 374 that are unlikely to lead to detectable nitrate production. Consequently, these soils have much  
23  
24 375 greater capacity to retain N as  $\text{NH}_4^+$  through ion exchange, with minimal  $\text{NO}_3^-$  leaching.  
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26 376 Furthermore, NO pulses observed after rainfall are not due to nitrifier activity. However,  
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28 377 experiments performed either for longer than 3 weeks or with rhizosphere soil might detect  
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30 378 differences in nitrifier inhibition/stimulation and, despite the small effect of pH and H<sub>2</sub>O on  
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32 379 the Brazilian savanna soil nitrification rate, archaeal ammonia oxidisers started to show  
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34 380 activity in slurries after 21 days of incubation. Taken together, our results show that low  
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36 381 nitrification rates and ammonia oxidiser abundance in Campo sujo soil are not due to low  
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38 382 moisture content, low pH or the presence of ammonia oxidiser inhibitors.

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40 383 ~~The data presented here suggest that the NO pulses observed after rainfall are not due~~  
41  
42 384 ~~to nitrifier activity. However, experiments performed either for longer than 3 weeks or with~~  
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44 385 ~~rhizosphere soil might detect differences in nitrifiers inhibition/stimulation. Nevertheless,~~  
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46 386 ~~and, despite the small effect of pH and H<sub>2</sub>O on the Brazilian savanna soil nitrification rate,~~  
47  
48 387 ~~archaeal ammonia oxidisers started to show activity in slurries after 21 days of incubation.~~  
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50 388 ~~Most likely, nutrients stoichiometry (Mooshammer *et al.*, 2014) plays an important role on~~  
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52 389 ~~the microbial activity of those soils, as N and P co-limitation affects decomposition rates in~~  
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54 390 ~~Cerrado soils (Kozovits *et al.*, 2007, Jacobson *et al.*, 2011).~~

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7 391 Although low nitrate concentrations are unlikely to be due to denitrification, nitrate  
8 392 assimilation and dissimilatory nitrate reduction may reduce nitrate produced by nitrifiers.  
9 393 However, the high ammonium concentrations, low AOA and AOB abundances, and lack of  
10 394 evidence for ammonia oxidiser activity and growth suggest inhibition or limitation of  
11 395 ammonia oxidiser growth and activity. This study was performed using soil associated with  
12 396 one type of vegetation sampled at the beginning of the dry season. Production of BNI may  
13 397 vary seasonally and with vegetation, but low nitrification rates are found in soils sampled  
14 398 throughout the year (Nardoto & Bustamante, 2003) and ammonium and nitrate concentrations  
15 399 are high and low ( $2.5 (\pm 0.5) \mu\text{g N-NH}_4^+ \text{ g}^{-1}$  dry soil and  $0.072 (\pm 0.01) \mu\text{g N-NO}_3^- \text{ g}^{-1}$  dry  
16 400 soil), respectively, regardless of vegetation type (Catão *et al.*, in preparation). Inhibition of  
17 401 ammonia oxidiser cultures was performed with water extracts from soil and it is possible that  
18 402 ammonia oxidisers were inhibited by water insoluble BNI. Other features of Cerrado soils  
19 403 may also be important. There is evidence for co-limitation of microbial decomposition in  
20 404 these soils by N and P (Kozovits *et al.*, 2007, Jacobson *et al.*, 2011) and ammonia oxidisers  
21 405 may have been limited by P and other nutrients, although mixing with Craibstone soil would  
22 406 be expected to relieve this limitation. Cerrado soils are deficient in P (Goedert, 1983), and the  
23 407 values observed for the Campo sujo are similar to the upper layer of other Cerrado soils  
24 408 (Parron *et al.*, 2011), where soil P sorption capacity is often related to Fe and Al contents  
25 409 (Goedert, 1983). The soils investigated in this study also contain relative high Fe  
26 410 concentrations ( $165.4 \pm 41.01 \text{ mg dm}^{-3}$ ), which have been associated with reduction in net  
27 411 nitrification and AOA and AOB *amoA* gene abundance in subtropical acid soils (Jiang *et al.*,  
28 412 2015). Although these possibilities suggest future experimental studies, the reasons for low  
29 413 nitrification rates and low ammonia oxidiser abundances in Cerrado soil remain unclear and  
30 414 this study provided no evidence for ammonia limitation, pH or inhibition by water extractable  
31 415 inhibitors.

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31 429 **Conflict of Interest**

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33 430 The authors have no conflict of interest to declare.  
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3 1 **Ammonia oxidisers in a non-nitrifying Brazilian savanna soil**  
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7 3 Elisa C P Catão<sup>1,2</sup>, Cécile Thion<sup>2,3</sup>, R.H. Krüger<sup>1</sup> & James I Prosser<sup>2</sup>  
8

9 4 <sup>1</sup>Cellular Biology Department, Instituto Central de Ciencias Sul, Universidade de Brasilia  
10 (UnB), 700910-900 Brasilia, DF, Brazil  
11

12 5  
13 6 <sup>2</sup>Institute of Biological and Environmental Sciences, University of Aberdeen, Cruickshank  
14 Building, St. Machar Drive, Aberdeen AB24 3UU, UK  
15

16 7  
17 8 <sup>3</sup>Laboratoire Ampère, UMR CNRS 5005, École Centrale de Lyon, 34 avenue Guy de  
18 Collongue, 69130 Ecully, France  
19

20 10  
21

22 11 Corresponding author:  
23

24 12 Elisa Catão  
25

26 13 Max-Planck Institute for Biogeochemistry  
27

28 14 Department for Biogeochemical Processes  
29

30 15 Hans-Knöll Straße 10  
31

32 16 07745 Jena, Germany  
33

34 17 E-mail: [elisaccp@gmail.com](mailto:elisaccp@gmail.com)  
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36 18 Phone: +49 3641 576106  
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3 21 **Abstract**  
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5 22 Low nitrification rates in Brazilian savanna (Cerrado) soils have puzzled researchers for  
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7 23 decades. Potential mechanisms include biological inhibitors, low pH, low microbial  
8  
9 24 abundance and low soil moisture content, which hinders microbial activity, including  
10  
11 25 ammonia oxidation. Two approaches were used to evaluate these potential mechanisms, (i)  
12  
13 26 manipulation of soil moisture and pH in microcosms containing Cerrado soil and (ii)  
14  
15 27 assessment of nitrification inhibition in slurries containing mixtures of Cerrado soil and an  
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17 28 actively nitrifying agricultural soil. Despite high ammonium concentration in Cerrado soil  
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19 29 microcosms, little  $\text{NO}_3^-$  accumulation was observed with increasing moisture or pH, but in  
20  
21 30 some Cerrado soil slurries, AOA *amoA* transcripts were detected after 14 days. In mixed soil  
22  
23 31 slurries, the final  $\text{NO}_3^-$  concentration reflected the initial proportions of agricultural and  
24  
25 32 Cerrado soils in the mixture, providing no evidence of nitrification inhibitors in Cerrado soil.  
26  
27 33 AOA community denaturing gradient gel electrophoresis profiles were similar in the mixed  
28  
29 34 and nitrifying soils. These results suggest that nitrification in Cerrado soils is not constrained  
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31 35 by water availability, ammonium availability, low pH, or biological inhibitors and alternative  
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33 36 potential explanations for low nitrification levels are discussed. Keywords: ammonia  
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35 37 oxidisers, low nitrification, Brazilian savanna, inhibition, pH, soil moisture  
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## 39 Introduction

40 Autotrophic nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a major  
41 cause of N loss in terrestrial environments. In agricultural systems, nitrification is the main  
42 pathway of N transformation, and up to 95% of total N is present as  $\text{NO}_3^-$  potentially leading  
43 to leaching and emission of nitric oxide (NO) and nitrous oxide ( $\text{N}_2\text{O}$ ) by nitrifiers and  
44 denitrifiers (Subbarao *et al.*, 2012). Inhibitors of nitrification can decrease nitrogen losses  
45 from these systems (Subbarao *et al.*, 2006). These inhibitors target the first step in  
46 nitrification, ammonia oxidation, which is carried out by both bacterial and archaeal ammonia  
47 oxidisers. Some natural systems have lower nitrification rates and higher nitrogen fertiliser  
48 use efficiency than managed systems (Ste-Marie & Paré, 1999). For example, in soils of the  
49 tropical savanna biome in Central Brazil, also called Cerrado,  $\text{NO}_3^-$  concentration is low or  
50 undetectable (Nardoto & Bustamante, 2003), the  $\text{NH}_4^+:\text{NO}_3^-$  ratio is high and the abundance  
51 of nitrifiers is low (Catão *et al.*, 2016). These ecosystems may therefore provide a model for  
52 greater and more sustainable crop productivity and decreased demand for nitrogen fertilisers.

53 There are several potential explanations for low rates of nitrification in Cerrado soils,  
54 based on biological and physicochemical factors. Plants may decrease nitrification by  
55 competing for  $\text{NH}_4^+$ -N and by increasing the C:N ratio through increased carbon supply,  
56 thereby promoting immobilisation, while some plants produce nitrification inhibitors in plant  
57 litter and root exudates (Subbarao *et al.*, 2006). These inhibitors target ammonia oxidation  
58 and can benefit plants by reducing competition for ammonium (Subbarao *et al.*, 2006,  
59 Subbarao *et al.*, 2015). Both ammonia-oxidising archaea (AOA) and ammonia-oxidising  
60 bacteria (AOB) are present in these soils (Catão *et al.*, 2016) but the relatively high  
61 ammonium concentration in Cerrado soil [ $3 - 22 \mu\text{g N g}^{-1}$  soil, (Nardoto & Bustamante,  
62 2003);  $5 - 49 \mu\text{g N g}^{-1}$  soil (Catão *et al.*, 2016)] suggests that ammonia oxidisers are not

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3 63 limited by ammonia concentration and low rates of nitrification in Cerrado soils may be  
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5 64 better explained by production of biological nitrification inhibitors.  
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8 65 Low nitrification rates in acidic soils have been described for many years (De Boer &  
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10 66 Kowalchuk, 2001). Inhibition of ammonia oxidation in low pH soil was traditionally  
11  
12 67 considered to be due to the low availability of ammonia, through ionisation to  $\text{NH}_4^+$ , but may  
13  
14 68 be alleviated by growth in soil aggregates or on surfaces (De Boer *et al.*, 1991, Allison &  
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16 69 Prosser, 1993), urease activity (De Boer *et al.*, 1989, Burton & Prosser, 2001), or growth of  
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18 70 acidophilic archaeal ammonia oxidisers (Gubry-Rangin *et al.*, 2011, Lehtovirta-Morley *et al.*,  
19  
20 71 2011). Meta-analysis of net nitrification rates in a wide range of soils (Booth *et al.*, 2005)  
21  
22 72 suggests that pH limitation may not be widespread, but increased nitrification following  
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24 73 amendment of Cerrado soil with calcium carbonate (Rosolem *et al.*, 2003) provides evidence  
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26 74 for pH limitation in soil.  
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29 75 Low water availability decreases nitrification (Placella & Firestone, 2013, Thion &  
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31 76 Prosser, 2014) by increasing osmotic stress and reducing mobility of ammonia within the  
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33 77 soil. In the seasonally dry Cerrado biome,  $\text{N}_2\text{O}$  and  $\text{NO}$  emissions increase after rainfall or  
34  
35 78 addition of artificial rainwater (Pinto *et al.*, 2002, Pinto *et al.*, 2006) providing evidence for  
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37 79 limitation of nitrification during dry seasons.  
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40 80 Limited nitrification is alleviated by agriculture due to fertilisation, liming, tillage or  
41  
42 81 plant community change. Considering the extensive conversion of Cerrado soils to  
43  
44 82 agricultural production (Marris, 2005, Catão *et al.*, 2016), it is important to understand  
45  
46 83 adaptation of natural ecosystems to limit N loss. The aim of this study was to test three  
47  
48 84 hypotheses regarding potential mechanisms for the low nitrification rates: presence of  
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50 85 biological nitrification inhibitors, low water availability and low pH. The presence of plant-  
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52 86 derived nitrification inhibitors was tested by analysing (i) the growth of AOB and AOA in the  
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54 87 presence of aqueous extract from Cerrado soil and (ii) the effect of Cerrado soil on ammonia  
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3 88 oxidation by a nitrifying soil (Craibstone) in soil slurries. The effects of low water

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5 89 availability and low pH on nitrification were tested by manipulating Cerrado soil water

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7 90 content and pH in microcosms.

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10 91 **Materials and methods**

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12 92 ***Soil sampling***

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14 93 Cerrado soil was sampled from an undisturbed shrubland (Campo sujo), with some sparse

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16 94 shrubs over a continuous grass layer (Eiten, 1972), where graminoids can account for around

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18 95 45% of total aboveground biomass, leading to a contribution of 46% of relative abundance of

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20 96 fine roots (Castro & Kauffman, 1998). Campo sujo is dominated by plants from the families

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22 97 Asteraceae, Leguminosae, and Poaceae (Tannus & Assis, 2004). The average monthly

23  
24 98 precipitation and temperature, measured at the nearest meteorological centre in 2014 (~30 km

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26 99 from the farm; Pirenopolis – GO, Station 83376, 15°50'60"S 48°57'36"W), were 143 mm

27  
28 100 (range 0 - 317 mm) and 23.4°C (range 21°C - 25.6°C), respectively. Triplicate soil samples

29  
30 101 were obtained from the upper 10 cm of soil, pooled before sieving (2-mm mesh size) and

31  
32 102 then stored at 4°C. The climate in the Cerrado biome is tropical (Köppen Aw), and samples

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34 103 were collected at the beginning of the dry season (May 2014). Campo sujo and Cerrado *sensu*

35  
36 104 *stricto* are usually found on oxisols, with low nutrient content, low pH, and high content of

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38 105 aluminium (Reatto *et al.*, 1998). The soil, which was well aerated and well drained, is

39  
40 106 classified as sandy loam with 20.8% clay and had an initial pH of 5.6 ( $\pm 0.04$ ).

41  
42 107 Physicochemical parameters from the Campo sujo sample were previously described (Catão

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44 108 *et al.*, 2016): organic matter content was 42.6 ( $\pm 2.4$ ) g kg<sup>-1</sup>, cation exchange capacity 6 ( $\pm$

45  
46 109 0.6) cmol<sub>c</sub> dm<sup>-3</sup>, available phosphorus 1.8 ( $\pm 0.13$ ) mg dm<sup>-3</sup>, aluminium 1.2 ( $\pm 0.12$ ) cmol<sub>c</sub>

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48 110 dm<sup>-3</sup> and Fe 165.4 ( $\pm 41.0$ ) mg dm<sup>-3</sup>. Craibstone soil, used in this study as a reference

49  
50 111 nitrifying soil, was sampled from an experimental agricultural field (Scottish Agricultural

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3 112 College, Craibstone, Scotland, Grid reference NJ872104) and maintained at pH 5.5 since  
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5 113 1961.

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10 115 ***Cultivation of ammonia oxidisers with soil extracts***

11 116 Aqueous extracts of Craibstone and Campo sujo soils were prepared by blending 20 g soil in  
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14 117 2 volumes of sterile distilled water for 40 s, rotating in 50-mL sterile tubes for 1 h,  
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16 118 centrifuging (3,000×g for 15 min) and sterilising by progressive filtration through filters with  
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18 119 10-mm, 5-mm, 0.45-µm and 0.22-µm pore size. NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations in the filtrates  
19  
20 120 were below the level of detection (data not shown).

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23 121 Pure cultures of AOA (*Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley  
24  
25 122 *et al.*, 2016)) and AOB (*Nitrosospira briensis* #128, *Nitrosospira tenuis* #NV-12,  
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27 123 *Nitrosospira multififormis* - NCIMB11849, ATCC25196 and *Nitrosomonas europaea* -  
28  
29 124 NCIMB11850, ATCC25978) were cultivated in inorganic growth medium in the dark  
30  
31 125 without shaking. *Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley *et al.*, 2016)  
32  
33 126 was cultivated at 40°C in medium described previously (Lehtovirta-Morley *et al.*, 2011) but  
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35 127 modified by the addition of 1 mL L<sup>-1</sup> vitamin solution (Widdel & Bak, 1992), 1 mL L<sup>-1</sup>  
36  
37 128 selenite-tungstate solution (Widdel & Bak, 1992) and 2 mM NH<sub>4</sub>Cl. The pH was maintained  
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39 129 at ~7.5 by the addition of 10 mL L<sup>-1</sup> 1 M HEPES buffer. The AOB were grown in Skinner  
40  
41 130 and Walker medium (Skinner & Walker, 1961) and incubated at 30°C. Triplicate cultures  
42  
43 131 were prepared in 30-mL Universal tubes by adding 5 mL of the appropriate medium,  
44  
45 132 previously inoculated with exponentially growing cells (1 mL inoculum per 100 mL 2×  
46  
47 133 concentrated medium), to a 5-mL volume of sterile distilled water, Craibstone or Campo sujo  
48  
49 134 soil aqueous extract, or allylthiourea (100 µM final concentration), an ammonia oxidiser  
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51 135 inhibitor. The cultures were grown without agitation, and growth was monitored for 26 days  
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53 136 (AOA) and 13 days (AOB) by measuring nitrite accumulation (Shinn, 1941). The maximum  
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3 137 specific growth rate was estimated as the slope of semi-logarithmic plots of nitrite  
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5 138 concentration versus time.

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9 140 ***Soil incubation in slurries***

10 141 Soil slurries were established in 250-mL sterile Erlenmeyer flasks containing 20 g soil and  
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12 142 100 mL sterile distilled water, stirred at 100 rpm and maintained at 30°C in the dark.

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14 143 Individual flasks contained Campo sujo soil, Craibstone soil or mixtures of Campo sujo and

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16 144 Craibstone soils in 1:1 or 4:1 ratios. Before incubation and 1, 7, 14, and 21 days after

17  
18 145 incubation, soil slurry aliquots (8 mL) were centrifuged at 3,000×g for 15 min. After

19  
20 146 immediate measurement of pH in 2 mL of supernatant, the remaining supernatant (6 mL) was

21  
22 147 stored at -20°C for quantification of inorganic N (see below). The soil pellet was frozen in

23  
24 148 liquid nitrogen and stored at -80°C for molecular analysis.

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28 150 ***Soil incubation in microcosms***

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30 151 Campo sujo soil was incubated in sealed microcosms consisting of 140-mL sterile serum

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32 152 glass bottles containing 10 g soil. The soil had an initial water content of  $24.9 \pm 0.03$  g H<sub>2</sub>O

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34 153 100 g<sup>-1</sup> dry soil, corresponding to a matric potential of  $-0.15 \pm 0.01$  MPa. Microcosms were

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36 154 incubated for 4 days in the dark at 30°C (acclimation period) and then divided into two

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38 155 groups. The ‘dried soil’ group was left to air dry, reaching a moisture content of 8.66 g H<sub>2</sub>O

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40 156 100 g<sup>-1</sup> dry soil ( $-6.34 \pm 2.98$  MPa matric potential). In the ‘moist soil’ group, the moisture

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42 157 content was adjusted to  $37.9 \pm 0.3$  g H<sub>2</sub>O 100 g<sup>-1</sup> dry soil by adding sterile distilled water. Soil

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44 158 in half of the dried soil microcosms was rewetted to  $39.6 \pm 1.92$  g H<sub>2</sub>O 100 g<sup>-1</sup> dry soil ( $-0.11$

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46 159  $\pm 0.02$  MPa) (‘Water Pulse’ treatment), and the soil in the remaining dried soil microcosms

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48 160 was kept dry (‘Dry’ treatment). Finally, the pH of soil in half of the moist soil microcosms

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50 161 was increased to  $6.34 \pm 0.09$  with CaCO<sub>3</sub> (‘pH treatment’). The pH of soil in the remaining

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3 162 microcosms ('Dry', 'Water Pulse' and 'Moist' treatments) was  $5.21 \pm 0.02$ , which was  
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5 163 slightly lower than the initial value of the sampled soil and was not adjusted. The four  
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7 164 treatments were performed in triplicate, with or without the addition of the ammonia  
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9 165 oxidation inhibitor acetylene (0.01% of headspace volume). The soil microcosms were  
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11 166 incubated in the dark at 30°C, and aerobic conditions were maintained by removing the seals  
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13 167 for 5 - 10 minutes twice weekly. The 'Moist' and 'Water Pulse' microcosms were watered  
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15 168 weekly to maintain moisture content. The microcosms were sampled destructively after 6 h  
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17 169 and 1, 3, 7, 14, and 21 days, with additional sampling after 28 days for the pH treatment). For  
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19 170 each microcosm, half of the soil was used for chemical analysis and the remainder was stored  
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21 171 at -80°C for molecular analysis.  
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### 27 173 ***Soil physicochemical analyses***

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29 174 Water matric potential was measured using a WP4C Dewpoint PotentialMeter (Decagon,  
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31 175 Pullman, UK) and pH was determined in water. Soil  $\text{NH}_4^+$  and  $\text{NO}_x$  ( $\text{NO}_2^- + \text{NO}_3^-$ )  
32  
33 176 concentrations were determined colorimetrically by flow injection analysis (FIA star 5010  
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35 177 Analyser, Foss Tecator AB, Höganäs, Sweden) (Allen, 1989) after extraction from 2 g wet  
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37 178 soil in 10 mL KCl (1 M), for the microcosm soil, or directly from slurry supernatant. Because  
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39 179  $\text{NO}_2^-$  concentration was below the level of detection,  $\text{NO}_x$  is expressed as  $\mu\text{g NO}_3^- \text{-N g}^{-1}$  dry  
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41 180 soil (ppm). Nitrification inhibition was assessed as the decrease in nitrate concentration as a  
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43 181 percentage of that of Craibstone soil at each time point.  
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### 49 183 ***Molecular analysis***

50  
51 184 Nucleic acids were extracted from 0.5 g soil as previously described (Nicol *et al.*, 2005),  
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53 185 suspended in diethylpyrocarbonate-treated water and immediately stored at -80°C. An aliquot  
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55 186 was treated with DNase and the RNA was reverse transcribed, as described previously  
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3 187 (Tourna, 2008). The nucleic acid not used for cDNA generation was considered DNA only  
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5 188 and its concentration was estimated using a NanoDrop 1000 Spectrophotometer (Thermo  
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7 189 Scientific, Loughborough, UK).

9  
10 190 Archaeal and bacterial *amoA* genes, which encode subunit A of ammonia  
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12 191 monooxygenase, were quantified in a MasterCycler thermal cycler (Eppendorf, Hamburg,  
13  
14 192 Germany) using standard curves, as described previously (Catão *et al.*, 2016). PCR  
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16 193 amplification was carried out using primers crenamo23f and crenamo616r for archaeal *amoA*  
17  
18 194 genes (Tourna, 2008) and amoA1F and amoA2R for bacterial *amoA* genes (Rotthauwe *et al.*,  
19  
20 195 1997). Each 20- $\mu$ L reaction contained 1 $\times$  QuantiFast PCR Master Mix (for AOA) or  
21  
22 196 QuantiTect Master Mix (for AOB) (Qiagen, Crawley, UK), 0.4  $\mu$ M of each primer for AOA  
23  
24 197 *amoA* or 0.6  $\mu$ M of each primer for AOB *amoA*, 2  $\mu$ g  $\mu$ L<sup>-1</sup> BSA (Promega), and 2  $\mu$ L DNA  
25  
26 198 (or cDNA). Archaeal *amoA* genes and transcripts were amplified using the following cycling  
27  
28 199 conditions: 15 min at 95°C, followed by 40 cycles of 15 s at 94°C and 90 s at 60°C. Bacterial  
29  
30 200 *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at  
31  
32 201 95°C, followed by 45 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. To exclude  
33  
34 202 fluorescence contamination of potential primer-dimers, SYBR Green fluorescence was  
35  
36 203 measured after 5 s at 80°C or after 8 s at 83°C, for AOA and AOB, respectively. Melting  
37  
38 204 curves between 65°C and 95°C were analysed for each run. AOB *amoA* transcripts were  
39  
40 205 below the detection limit (5 copies  $\mu$ L<sup>-1</sup>). Efficiency of amplification and  $r^2$  for DNA were,  
41  
42 206 respectively, 0.92 and 0.998 for archaeal *amoA* and 104.6 and 0.993 for bacterial *amoA*.

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47 207 AOA community composition in soil slurries was assessed before and after incubation  
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49 208 for 21 days by denaturing gradient gel electrophoresis (DGGE) of archaeal *amoA* gene using  
50  
51 209 the primers described above in a linear gradient of 15% - 55% denaturant, as described  
52  
53 210 previously (Nicol *et al.*, 2005).

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## 212 ***Statistical analysis***

213 All analyses were conducted using R version (3.2.2). The effect of aqueous soil extracts on  
214 pure AOA and AOB cultures was analysed by testing differences in specific growth rate  
215 between treatments by one-way analysis of variance. Differences between nitrification rate in  
216 soil slurries were evaluated using a repeated-measures linear mixed model (package *nlme*)  
217 (Pinheiro *et al.*, 2015). Each slurry was considered a subject with random effect to analyse  
218 the effect of treatment (Campo sujo soil, Craibstone soil, or soil mixture), time, and their  
219 interaction on inorganic N concentration and *amoA* gene (and transcript) abundance. The  
220  $\text{NO}_3^-$  concentration in the Campo sujo slurries was below the limit of detection; therefore,  
221 these samples were excluded from the analysis. Gene abundance data were log-transformed  
222 to achieve a normal distribution. When the interaction between independent variables was not  
223 significant, it was removed to analyse the effect of time or treatment independently over  
224 concentration of soil  $\text{NH}_4^+$  and  $\text{NO}_x$ . Two-way analysis of variance, with treatment and time  
225 as independent factors, was performed to evaluate differences in mineralisation and  $\text{NO}_3^-$  in  
226 the soil microcosms.

227

## 228 **Results**

### 229 **Effects of soil extracts on ammonia oxidiser cultures**

230 To assess the presence of nitrification inhibitors in the soil, pure cultures of four AOB and  
231 one AOA were grown in liquid batch culture in medium containing aqueous soil extracts,  
232 water (negative control) or allylthiourea (positive control). Extracts from Campo sujo and  
233 Craibstone soils had no significant effect on the growth of any of the ammonia-oxidising  
234 strains tested (Figs. 1 and S1). Allylthiourea completely inhibited all AOB cultures tested, but  
235 did not inhibit the growth of the AOA *Candidatus* N. franklandus (Figs. 1 and S1).

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**237 Effects of Campo sujo soil on nitrification in Craibstone soil**

238 Soil slurries containing Campo sujo soil, Craibstone soil, or a mixture of the two soils (at  
239 ratios of 1:1 and 4:1) were incubated for up to 21 days. In all slurries, pH increased (0.4 in the  
240 Campo sujo soil, 0.8 in Craibstone soil, but only 0.1 and 0.2 for the 1:1 and 4:1 mixed  
241 samples, respectively) after the first day of incubation but did not change significantly  
242 thereafter. Net  $\text{NH}_4^+$  concentration after 21 days ranged from 0.62 ( $\pm 0.02$ ) to 1.76 ( $\pm 0.39$ )  
243 ppm ( $\text{mg L}^{-1}$  soil solution) for Craibstone soil and 0.87 ( $\pm 0.02$ ) to 2.20 ( $\pm 0.02$ ) ppm ( $\text{mg L}^{-1}$   
244 soil solution) for Campo sujo soil (Fig. 2). Initial  $\text{NH}_4^+$  concentration was higher in the mixed  
245 soil slurries than in controls, but the mixed slurries accumulated less  $\text{NH}_4^+$  over the  
246 incubation period. The greatest increase in  $\text{NH}_4^+$  concentration after 21 days was observed in  
247 Craibstone soil (2.9-fold).

248  $\text{NO}_3^-$  concentration also increased in all soil slurries during incubation ( $p < 0.0001$ , Fig.  
249 2B), except in those containing Campo sujo only, in which  $\text{NO}_3^-$  was below the detection  
250 limit. In the mixed soil slurries,  $\text{NO}_3^-$  production was equivalent to or higher than the 50%  
251 and 20% expected for the 1:1 and 4:1 ratios of Campo sujo soil and Craibstone soil,  
252 respectively (Fig. 2C), providing no evidence for inhibition of Craibstone soil nitrification by  
253 Campo sujo soil.

254 Ammonia oxidiser *amoA* gene abundance in the soil slurries did not change  
255 significantly during the incubation period, even when significant  $\text{NO}_3^-$  accumulation was  
256 observed (Fig. 3). AOA *amoA* abundance in the Campo sujo-only slurries was approximately  
257 three orders of magnitude lower than that of Craibstone-only slurries (Fig. 3A). AOA *amoA*  
258 abundance in mixed soil slurries was lower than that of Craibstone-only slurries until day 14,  
259 after which differences were not significant ( $p = 0.132$ ). AOB *amoA* gene abundance in the  
260 Campo sujo-only slurries was also approximately three orders of magnitude lower than that  
261 of Craibstone-only slurries, and even significantly different ( $p = 0.024$ ) at day 21, when

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3 262 Campo sujo-only AOB abundance was no longer significantly different from the those in the  
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5 263 mixed samples (Fig. 3B).  
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7 264 The AOB *amoA* gene abundance was lower than AOA *amoA* gene abundance in all  
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9 265 slurries at each time point. The AOA:AOB *amoA* gene ratio did not change significantly in  
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11 266 the Campo sujo-only slurries but increased in the Craibstone-only and mixed soil slurries  
12  
13 267 (Fig. 3C). In all slurries, AOB *amoA* transcripts were below the level of detection ( $5 \mu\text{L}^{-1}$ ).  
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15 268 The AOA *amoA* transcripts were detected in all slurries containing Craibstone soil throughout  
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17 269 incubation but were detected in the Campo sujo-only slurries only at day 21 (Fig. 3D).  
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20 270 Before incubation, DGGE profiles of *amoA* genes amplified from Craibstone soil  
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22 271 contained more bands (potential OTUs) than profiles of Campo sujo soil (Fig. S2) and did not  
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24 272 change significantly after incubation for 21 days. DGGE profiles of the AOA *amoA* genes in  
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26 273 the 1:1 mixed slurry were similar to those of Craibstone soil, possibly masking the less  
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28 274 abundant *amoA* genes from the Campo sujo soil (Fig. S2).  
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### 33 34 276 ***Effects of soil pH and moisture content***

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36 277 The effects of pH and moisture content on nitrification in Campo sujo soil were investigated  
37  
38 278 in soil microcosms. Net mineralisation was determined as the increase in concentration of  
39  
40 279 inorganic N ( $\text{NH}_4^+ \text{-N} + \text{NO}_3^- \text{-N}$ ) during incubation, assuming that other nitrogen cycle  
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42 280 processes were not significant (Fig. S3). Mineralisation in the dry soil did not increase after  
43  
44 281 wetting, in contrast to the expected 'Birch' effect (Birch, 1964) (Fig. S3). In addition, soil pH  
45  
46 282 did not change significantly with time in the microcosms and remained at 5.2 for the 'Water  
47  
48 283 Pulse', 'Moist' and 'Dry' treatments and at 6.3 for the 'pH' treatment, in which pH was  
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50 284 increased artificially with  $\text{CaCO}_3$ . Nitrate concentration did not increase significantly in any  
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52 285 of the treatments (Fig. S3), and no significant difference was observed between treatments  
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54 286 ( $p=0.14$ ). No significant differences were observed between samples incubated with or  
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3 287 without acetylene, except for  $\text{NO}_3^-$ -N concentrations in the 'Moist' microcosms. After  
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5 288 incubation for 21 days, the  $\text{NO}_3^-$ -N concentration was lower in the acetylene-treated moist  
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7 289 microcosms than in those without added acetylene (Fig. S3).  
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11 291 **Discussion** Nitrification is frequently undetectable in undisturbed Cerrado ecosystems,  
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14 292 although management and conversion to agricultural production increases nitrate production  
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16 293 (Catão *et al.*, 2016). Previous studies suggest low abundance of AOA and AOB in Campo  
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18 294 sujo soil (Catão *et al.*, 2016), which is also characterised by sparse shrubs over a continuous  
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20 295 grass layer. The aim of this work was to determine whether the lack of nitrification and low  
21  
22 296 abundance of ammonia oxidisers in this ecosystem were due to low pH, low soil moisture,  
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24 297  $\text{NH}_4^+$  limitation or biological inhibition of ammonia oxidation.  
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27 298 Certain plants release biological nitrification inhibitors that suppress ammonia  
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29 299 oxidation in soils (Subbarao *et al.*, 2015). For example, compounds produced by *Brachiaria*  
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31 300 (Subbarao *et al.*, 2009) and *Sorghum* (Zakir *et al.*, 2008) inhibited a recombinant *N. europaea*  
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33 301 strain, possibly by blocking ammonia monooxygenase and hydroxylamine oxidoreductase  
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35 302 (Subbarao *et al.*, 2008). Production of biological nitrification inhibitors can be promoted by  
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37 303 exposure to high  $\text{NH}_4^+:\text{NO}_3^-$  ratios (Subbarao *et al.*, 2015), such as those found in Campo  
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39 304 sujo soil (Catão *et al.*, 2016). However, aqueous extracts of Campo sujo soil did not inhibit  
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41 305 growth of cultures of four AOB and one AOA, all of which were isolated from neutral to  
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43 306 alkaline soils. Allylthiourea, used as a positive control, prevented growth of the AOB cultures  
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45 307 but not the AOA culture, which is consistent with other studies reporting a greater tolerance  
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47 308 of AOA to allylthiourea (Hatzepichler & Lebedeva, 2008, Stempfhuber *et al.*, 2015). This  
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49 309 result demonstrates the need to test potential inhibitors against both AOA and AOB, rather  
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51 310 than *N. europaea* only.  
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3 311 Cultivation-based studies were performed using aqueous soil extracts and a small  
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5 312 number of cultivated strains and potential inhibition was therefore assessed more directly by  
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7 313 mixing Campo sujo soil with Craibstone soil, a strongly nitrifying soil with similar pH, in soil  
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9 314 slurries (Nicol *et al.*, 2008, Zhang *et al.*, 2010). The soil slurries also provided no evidence of  
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11 315 nitrification inhibitors in Campo sujo soil. Nitrate accumulation in mixtures of Craibstone  
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13 316 soil and Campo sujo soil was lower than that of Craibstone soil only, but this difference was  
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15 317 less than or equal to that predicted by the lower volume of Craibstone soil in the slurry,  
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17 318 suggesting that nitrification was not inhibited by the Campo sujo soil. Similarly, the addition  
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19 319 of Campo sujo soil to Craibstone soil had no apparent effect on AOA and AOB *amoA* gene  
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21 320 abundances. Archaeal *amoA* genes were more abundant than those of bacteria in the soils,  
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23 321 and bacterial *amoA* gene expression was not detected, as reported by previous studies of  
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25 322 Craibstone soils (Zhang *et al.*, 2010). Neither AOA nor AOB *amoA* abundance changed  
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27 323 significantly during incubation of any of the slurries containing Craibstone soil, despite  
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29 324 evidence of nitrate production. However, the AOA:AOB *amoA* gene ratio increased,  
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31 325 suggesting greater growth or lower death rates of AOA, but there was no evidence for growth  
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33 326 of AOB or AOA in the Campo sujo soil. DGGE profiles of the Campo sujo soil contained  
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35 327 fewer archaeal *amoA* bands than those of Craibstone soil, providing a further indication of  
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37 328 low abundance and activity of ammonia oxidisers in Campo sujo soil. Although AOA *amoA*  
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39 329 transcripts in Campo sujo-only slurries increased after incubation for 21 days, this increased  
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41 330 gene expression did not appear to lead to a detectable level of nitrate. Nitrate reduction during  
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43 331 denitrification was considered negligible due to previous experiments showing low NO  
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45 332 emissions and undetectable N<sub>2</sub>O in Cerrado soils (Pinto *et al.*, 2002).  
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51 333 In the absence of evidence for BNI, microcosm studies were performed to determine  
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53 334 whether the low nitrification rates in Campo sujo soil were due to low pH or low soil  
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55 335 moisture content. Gas measurements in Cerrado soil after rainfall (natural or simulated) led to  
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3 336 an increase in NO emissions (Pinto *et al.*, 2002), which may result from the Birch effect  
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5 337 (Birch, 1964) of increased organic matter availability after rewetting (Fierer & Schimel,  
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7 338 2003). Soil pH is an important determinant of microbial diversity (Lauber *et al.*, 2009, Fierer  
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9 339 *et al.*, 2012) and influences soil ammonia oxidiser abundance and activity (de Boer and  
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11 340 Kowalchuk, Nicol *et al.* 2008), with higher transcriptional activity of Archaea than Bacteria  
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13 341 as pH decreases (Nicol *et al.*, 2008). In our experiment, higher soil pH increased organic  
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15 342 nitrogen mineralisation rate but did not lead to detectable nitrate production in Campo sujo  
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17 343 soil after incubation for 28 days. Mineralisation was also lower in moist soil than in other  
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19 344 treatments, and the increase in moisture did not lead to detectable nitrate production (see  
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21 345 supplementary Fig. S3). Even though it can be argued that NO<sub>3</sub><sup>-</sup> can be denitrified at higher  
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23 346 soil moisture, low or undetectable NO and N<sub>2</sub>O emission in field measurements over the  
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25 347 seasons (Cruvinel *et al.*, 2011) and no consistent NO<sub>3</sub><sup>-</sup> increase after one day or after 3 weeks  
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27 348 of incubation, suggested limitation of nitrification by other factors.

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32 349 The low nitrification and low ammonia oxidiser abundance of Campo sujo soil, in  
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34 350 both microcosms and slurries, were not due to NH<sub>4</sub><sup>+</sup> limitation. The NH<sub>4</sub><sup>+</sup> concentration of  
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36 351 Campo sujo soil slurries was even higher than that of Craibstone soil slurries at the beginning  
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38 352 of the experiment. Jack pine forest soils showed similar high concentrations of ammonium  
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40 353 without detectable nitrate (Ste-Marie & Paré, 1999). None of the treatments in this study  
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42 354 increased nitrification in Campo sujo soil and this soil did not inhibit nitrification in  
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44 355 Craibstone soil or pure cultures of AOA or AOB. Similarly, in the jack pine forest soil,  
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46 356 nitrification was not stimulated by increased pH or ammonium amendment but was  
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48 357 stimulated by the addition of nitrifying soil from a forest floor (Ste-Marie & Paré, 1999). In  
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50 358 this study, both AOA and AOB were detected in Campo sujo soil, but at low levels that are  
51  
52 359 unlikely to lead to detectable nitrate production. Consequently, these soils have much greater  
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54 360 capacity to retain N as NH<sub>4</sub><sup>+</sup> through ion exchange, with minimal NO<sub>3</sub><sup>-</sup> leaching.  
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3 361 Furthermore, NO pulses observed after rainfall are not due to nitrifier activity. However,  
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5 362 experiments performed either for longer than 3 weeks or with rhizosphere soil might detect  
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7 363 differences in nitrifier inhibition/stimulation and, despite the small effect of pH and H<sub>2</sub>O on  
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10 364 the Brazilian savanna soil nitrification rate, archaeal ammonia oxidisers started to show  
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12 365 activity in slurries after 21 days of incubation. Taken together, our results show that low  
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14 366 nitrification rates and ammonia oxidiser abundance in Campo sujo soil are not due to low  
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16 367 moisture content, low pH or the presence of ammonia oxidiser inhibitors.  
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22 369 Although low nitrate concentrations are unlikely to be due to denitrification, nitrate  
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24 370 assimilation and dissimilatory nitrate reduction may reduce nitrate produced by nitrifiers.  
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26 371 However, the high ammonium concentrations, low AOA and AOB abundances, and lack of  
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28 372 evidence for ammonia oxidiser activity and growth suggest inhibition or limitation of  
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30 373 ammonia oxidiser growth and activity. This study was performed using soil associated with  
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32 374 one type of vegetation sampled at the beginning of the dry season. Production of BNI may  
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34 375 vary seasonally and with vegetation, but low nitrification rates are found in soils sampled  
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36 376 throughout the year (Nardoto & Bustamante, 2003) and ammonium and nitrate concentrations  
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38 377 are high and low (2.5 (±0.5) µg N-NH<sub>4</sub><sup>+</sup> g<sup>-1</sup> dry soil and 0.072 (±0.01) µg N-NO<sub>3</sub><sup>-</sup> g<sup>-1</sup> dry  
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40 378 soil), respectively, regardless of vegetation type (Catão *et al.*, in preparation). Inhibition of  
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42 379 ammonia oxidiser cultures was performed with water extracts from soil and it is possible that  
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44 380 ammonia oxidisers were inhibited by water insoluble BNI. Other features of Cerrado soils  
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46 381 may also be important. There is evidence for co-limitation of microbial decomposition in  
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48 382 these soils by N and P (Kozovits *et al.*, 2007, Jacobson *et al.*, 2011) and ammonia oxidisers  
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50 383 may have been limited by P and other nutrients, although mixing with Craibstone soil would  
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52 384 be expected to relieve this limitation. Cerrado soils are deficient in P (Goedert, 1983), and the  
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54 385 values observed for the Campo sujo are similar to the upper layer of other Cerrado soils  
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3 386 (Parron et al., 2011), where soil P sorption capacity is often related to Fe and Al contents  
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5 387 (Goedert, 1983). The soils investigated in this study also contain relative high Fe  
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7 388 concentrations ( $165.4 \pm 41.01 \text{ mg dm}^{-3}$ ), which have been associated with reduction in net  
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9 389 nitrification and AOA and AOB *amoA* gene abundance in subtropical acid soils (Jiang et al.,  
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11 390 2015). Although these possibilities suggest future experimental studies, the reasons for low  
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13 391 nitrification rates and low ammonia oxidiser abundances in Cerrado soil remain unclear and  
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15 392 this study provided no evidence for ammonia limitation, pH or inhibition by water extractable  
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17 393 inhibitors.  
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### 407 **Conflict of Interest**

408 The authors have no conflict of interest to declare.  
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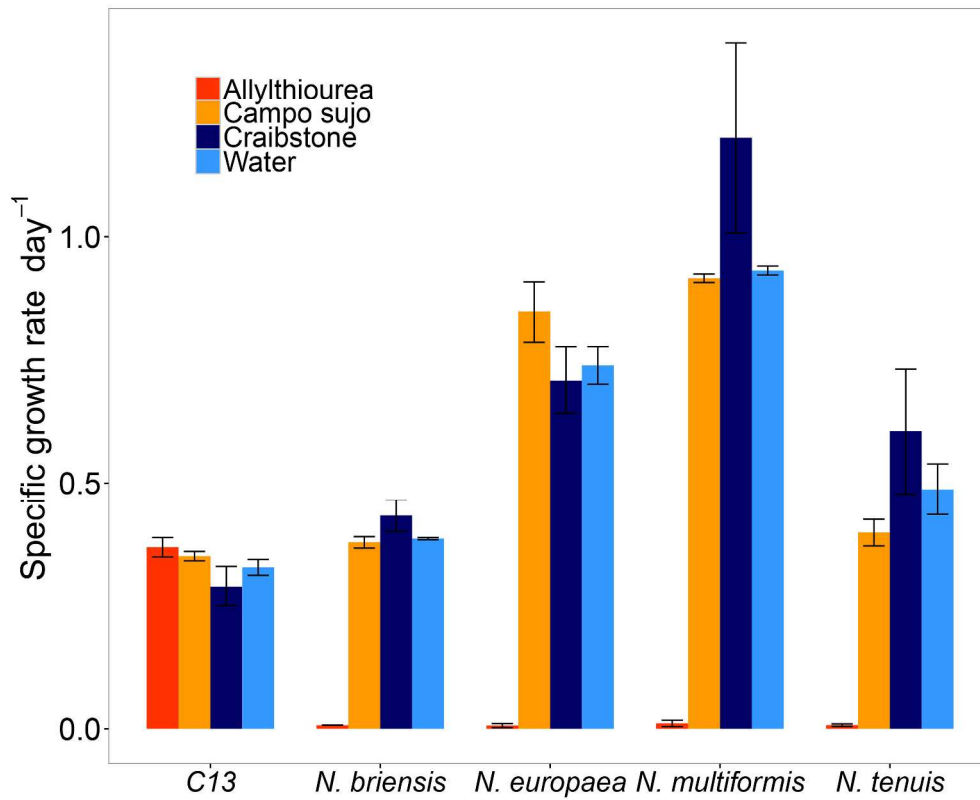


Figure 1. Effect of aqueous extracts of Campo sujo and Craibstone soils on the growth of ammonia-oxidising strains.

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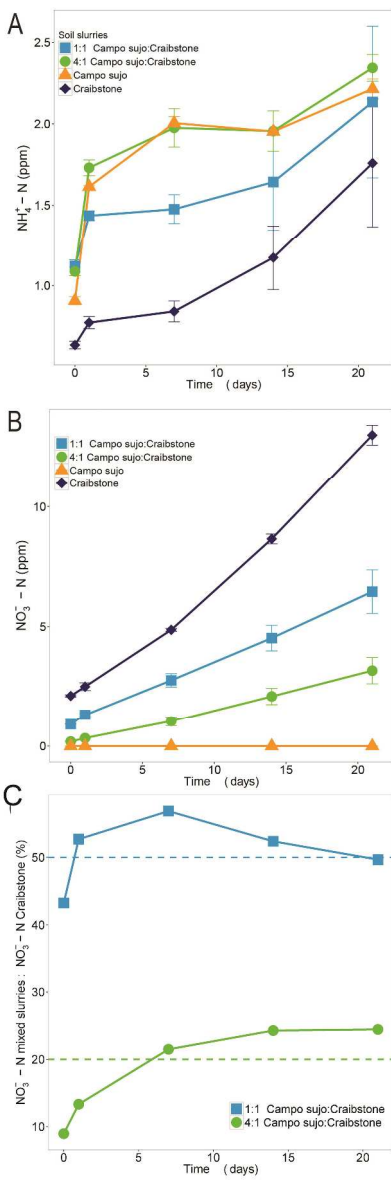


Figure 2. Changes in inorganic N concentration during incubation of in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils.

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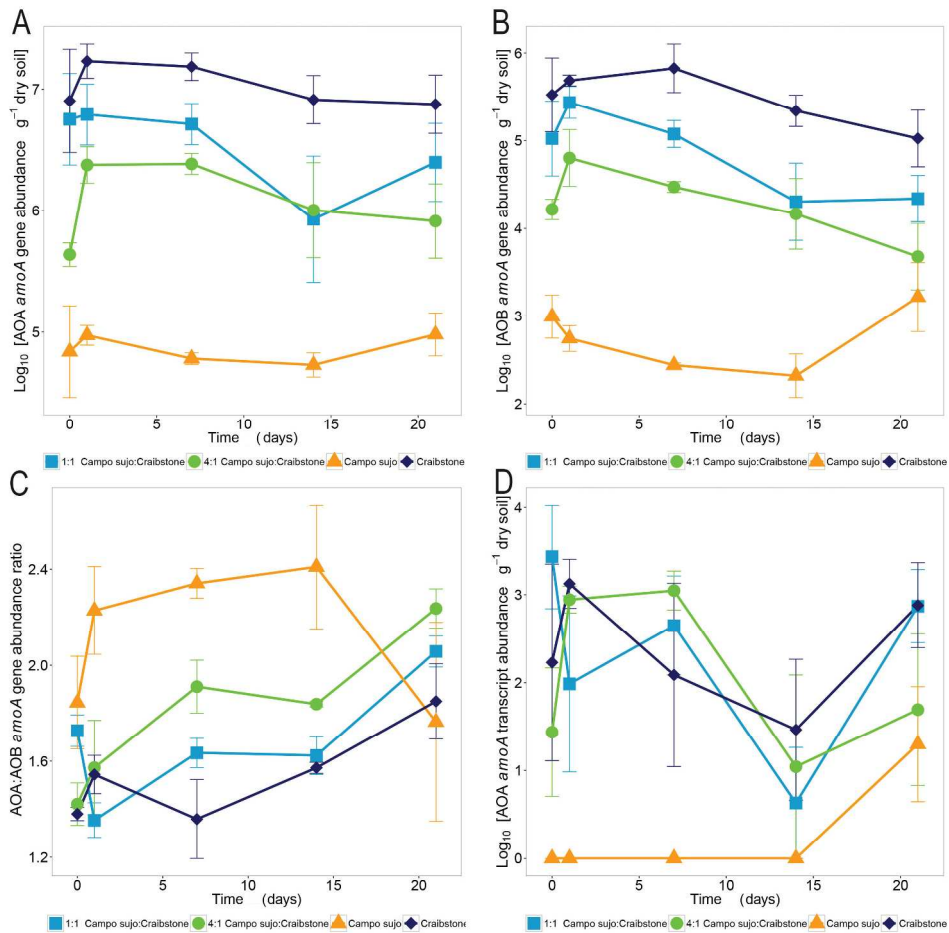


Figure 3. Changes in *amoA* gene abundance in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils.

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## Figure Legends

**Figure 1.** Effect of aqueous extracts of Campo sujo and Craibstone soils on the growth of ~~ammonia~~-~~ammonia-oxidising~~~~oxidiser~~. Maximum specific growth rate was estimated during exponential growth of the ammonia oxidising archaea (AOA) *Candidatus Nitrosocosmicus franklandia* (C13) and four soil ammonia-oxidising bacteria (*Nitrospira briensis*, *Nitrosomonas europaea*, *Nitrospira multiformis*, *Nitrospira tenuis*) in liquid batch cultures containing aqueous extracts of Campo sujo soil or Craibstone soil, water (negative control), or 100  $\mu\text{M}$  allylthiourea (positive control). Error bars represent standard deviation of the mean from triplicate cultures.

**Figure 2.** Changes in inorganic N concentration during incubation of ~~in~~-slurries of Craibstone soil, Campo sujo soil and mixtures of these soils. (A)  $\text{NH}_4^+$ -N concentration and (B)  $\text{NO}_3^-$ -N were determined in all soil slurries. (C)  $\text{NO}_3^-$  concentration in the mixed slurries was expressed as the percentage of  $\text{NO}_3^-$  in the Craibstone soil slurry. *P* values for treatment, time, and their interaction were calculated with a linear mixed model with repeated measures (*lme4* package, R version 3.2.3) for each independent variable and their interaction, and the marginal  $r^2$  associated with the fixed effects. Error bars represent standard deviation of the means from triplicate cultures. Values below the limit of detection were plotted as zero.

**Figure 3.** Changes in *amoA* gene abundance in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils. (A) Ammonia-oxidising archaea (AOA) *amoA*, (B) ammonia-oxidising bacteria (AOB) *amoA*, (C) AOA:AOB *amoA* ratio and (D) AOA *amoA* transcripts were quantified during incubation of soil slurries. *P* values for treatment, time, and their interaction were calculated with a linear mixed model with repeated measures (*lme4* package, R version 3.2.3) for each independent variable and their interaction, and the marginal  $r^2$  associated with the fixed effects. Error bars represent standard deviation of the means from triplicate cultures.

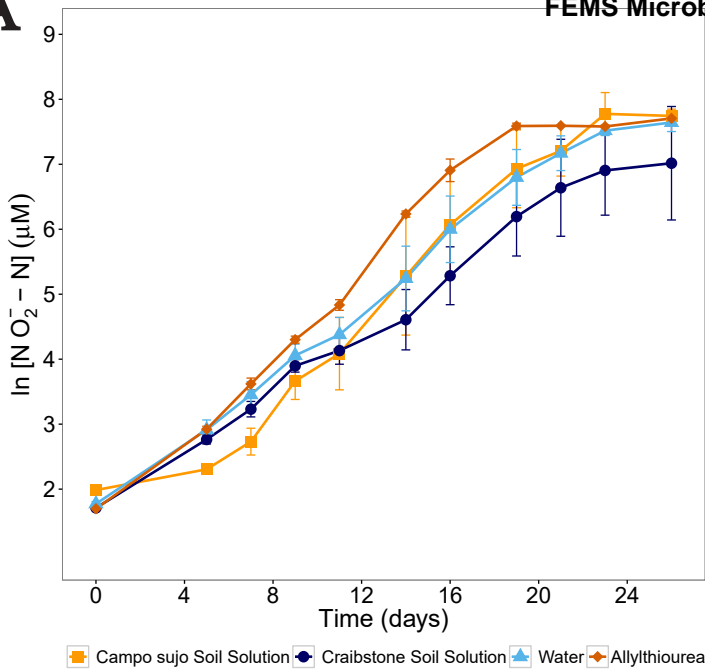
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3 **Figure S1.** Effects of Campo sujo and Craibstone soils on the growth of ammonia-  
4 oxidising archaea and bacteria. Semi-logarithmic plots of nitrite concentration vs. time  
5 during liquid batch culture of (A) *Candidatus Nitrosocosmicus franklandia* (AOA C13),  
6 (B) *Nitrosomonas europaea*, (C) *Nitrospira briensis*, (D) *Nitrospira tenuis* and (E)  
7 *Nitrospira multififormis* after adding aqueous extracts of Campo sujo soil or Craibstone  
8 soil, water (negative control), or 100  $\mu\text{M}$  allylthiourea (positive control). Error bars  
9 represent standard deviation of the means from triplicate cultures.  
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17 **Figure S2.** Denaturing gradient gel electrophoresis analysis of archaeal ammonia  
18 oxidiser *amoA* genes in soil slurries. PCR-amplified archaeal *amoA* gene products from  
19 triplicate soil slurries of (G) Campo sujo soil only, (CG) Campo sujo:Craibstone mixed  
20 soil (1:1 ratio) and (C) Craibstone soil only were sampled before incubation (T0) and  
21 after incubation for 21 days (T21).  
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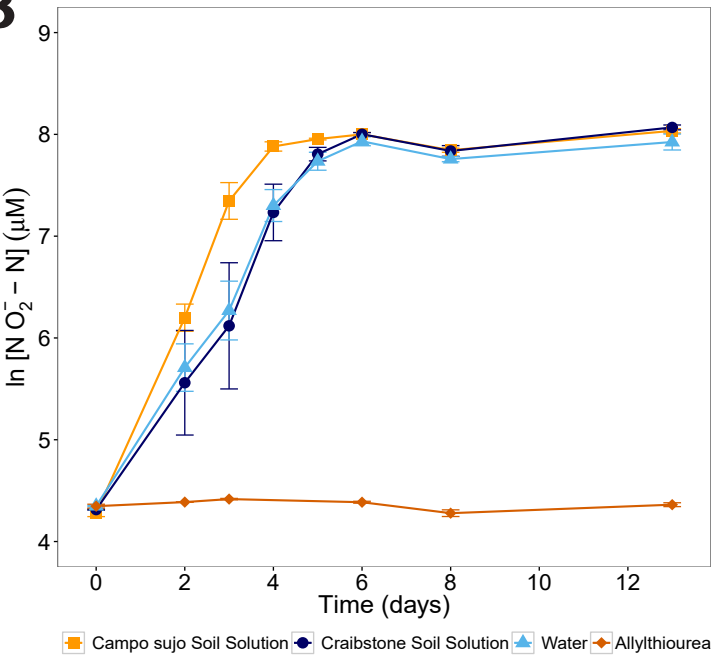
28 **Figure S3.** Effects of pH and moisture content on nitrification in Campo sujo soil.  
29 Changes in (A)  $(\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N})$  and (B)  $\text{NO}_3^-\text{-N}$  in microcosms containing Campo  
30 sujo soil after manipulation of pH and moisture content. Open symbols represent  
31 treatments with 0.01% acetylene in the headspace. Dry: air-dried soil at  $8.66 \text{ g H}_2\text{O } 100 \text{ g}^{-1}$   
32 dry soil; Water: rewetted soil at  $39.6 \pm 1.92 \text{ g H}_2\text{O } 100 \text{ g}^{-1}$  dry soil; Moist: moist soil;  
33 pH: soil treated with  $\text{CaCO}_3$  (pH 6.3; pH of other treatments was 5.2).  
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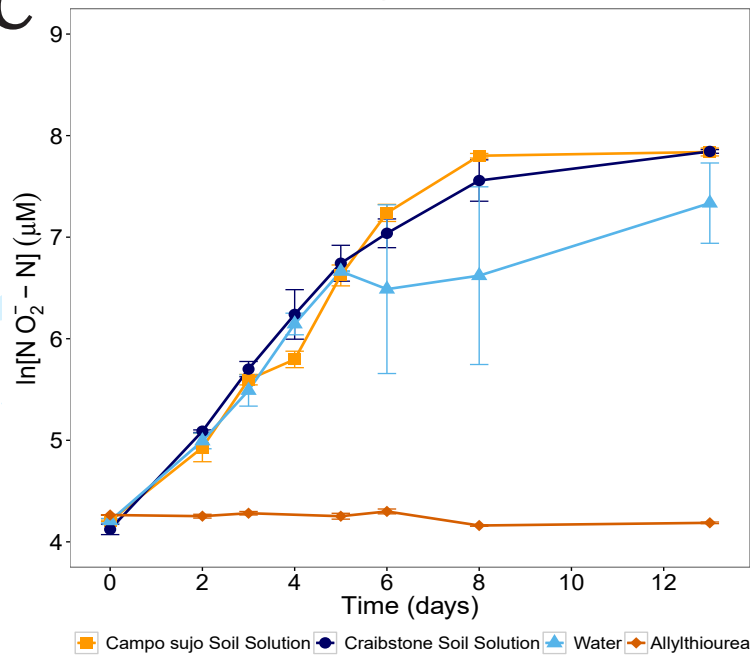
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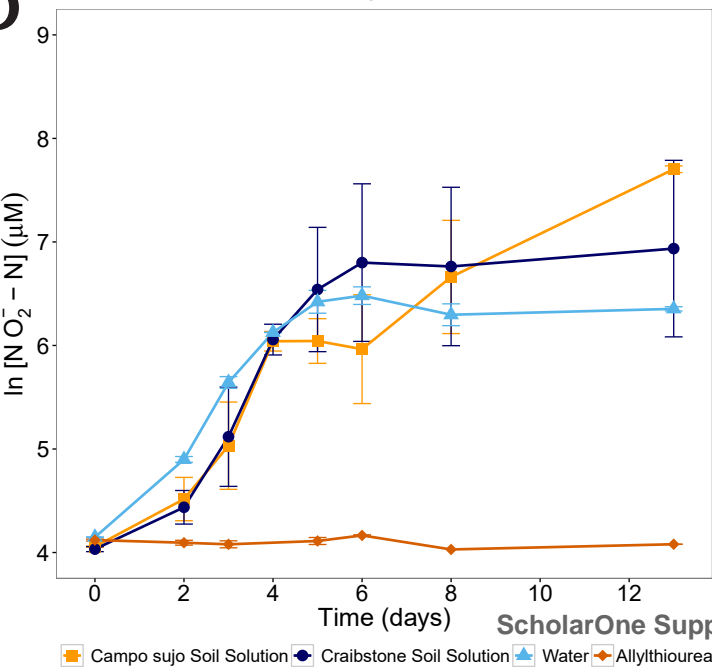
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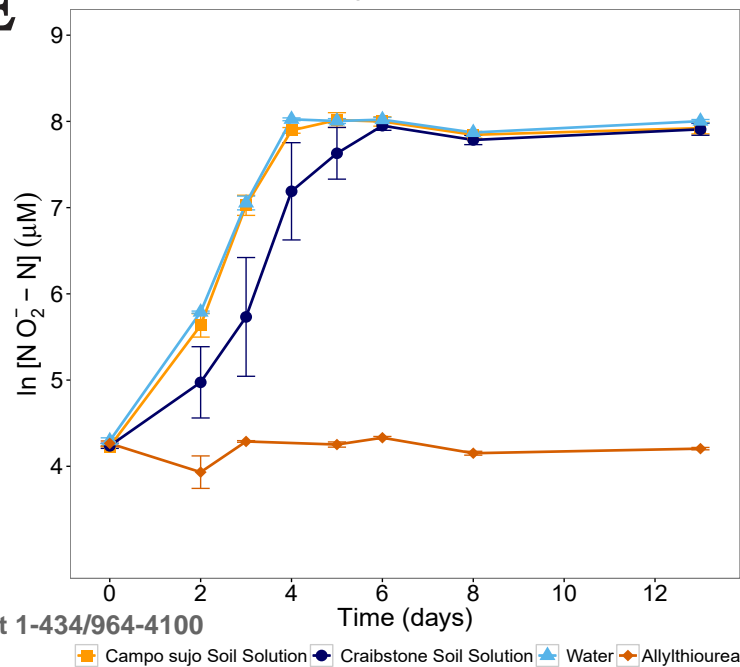
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*Nitrosospira briensis*

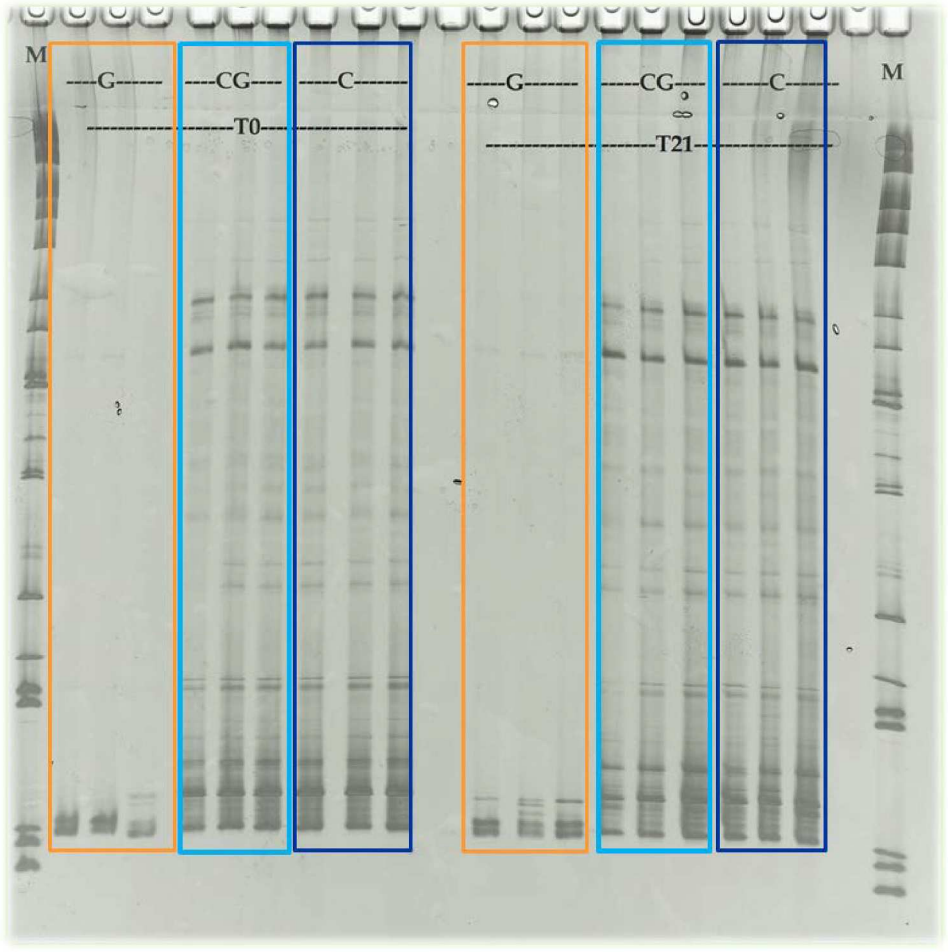
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*Nitrosospira tenuis*

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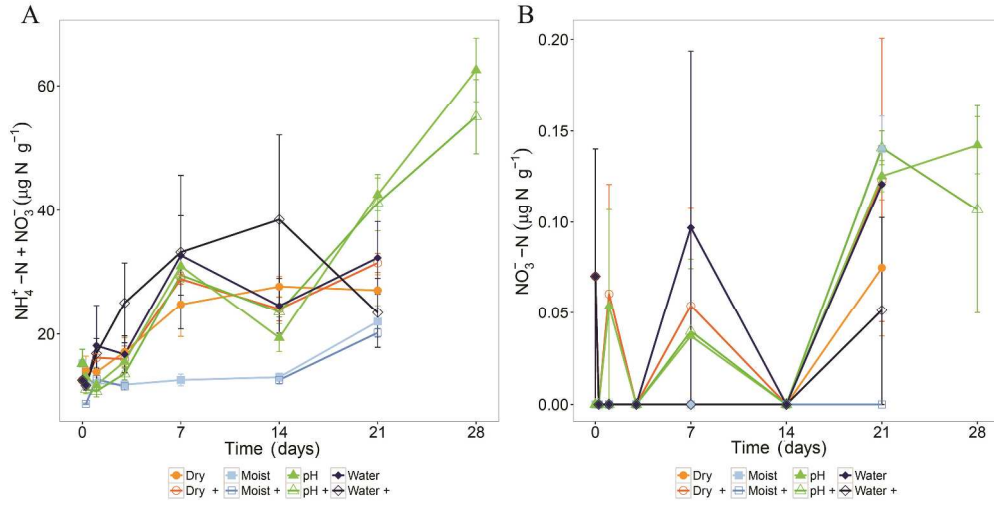
*Nitrosospira multiformis*

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