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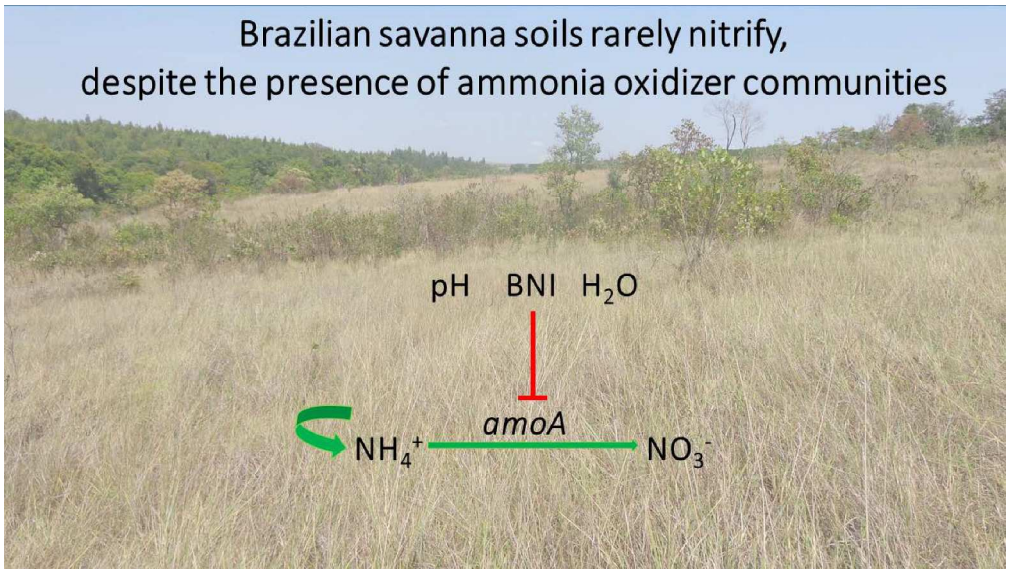

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Ammonia oxidisers in a non-nitrifying Brazilian savanna soil

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Abstract

Low nitrification rates in Brazilian savanna (Cerrado) soils haves puzzled researchers for decades. Potential mechanisms include biological inhibitors, low pH, low microbial abundance and low soil moisture content, which hinders microbial activity, including ammonia oxidation. Two approaches were used to evaluate these potential mechanisms, (i) manipulation of soil moisture and pH in microcosms containing Cerrado soil and (ii) assessment of nitrification inhibition in slurries containing mixtures of Cerrado soil and an actively nitrifying agricultural soil. Despite high ammonium concentration in Cerrado soil microcosms, little NO_3^- accumulation was observed with increasing moisture or pH, but in some Cerrado soil slurries, AOA *amoA* transcripts were detected after 14 days. In mixed soil slurries, the final NO_3^- concentration was comparative reflectedto the initial proportions of agricultural and Cerrado soils in the mixture, providing no evidence of nitrification inhibitors in Cerrado soil. AOA community denaturing gradient gel electrophoresis profiles were was similar in the mixed and nitrifying soils. These results suggest that nitrification in Cerrado soils is not constrained by water availability, ammonium availability, low pH, or biological inhibitors and alternative potential explanations for low nitrification levels are discussed. The microbial community in Cerrado soil might be adapted to N retention possibly through higher N immobilisation in organic matter rather than N loss through nitrification.

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

Introduction

Autotrophic nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a major cause of N loss in terrestrial environments. In agricultural systems, nitrification is the main pathway of N transformation, and up to 95% of total N is present as NO_3^- ~~transformed through nitrification,~~ potentially leading to ~~to nitrate (NO_3^-)~~ leaching and emission of nitric oxide (NO) and nitrous oxide (N_2O) by nitrifiers and denitrifiers (Subbarao *et al.*, 2012). Inhibitors of nitrification can decrease nitrogen losses from these systems (Subbarao *et al.*, 2006). These inhibitors target the first step in nitrification, ammonia oxidation, which is carried out by both bacterial and archaeal ammonia oxidisers. Some natural systems have lower nitrification rates and higher nitrogen fertiliser use efficiency than managed systems (Ste-Marie & Paré, 1999). For example, in soils of the tropical savanna biome in Central Brazil, also called ~~the~~ Cerrado, NO_3^- concentration is low or undetectable (Nardoto & Bustamante, 2003), the NH_4^+:NO_3^- ratio is high and the abundance of nitrifiers is low (Catão *et al.*, 2016). These ecosystems may therefore provide a model for greater and more sustainable crop productivity and decreased demand for nitrogen fertilisers.

There are several ~~several~~~~some~~ potential explanations ~~based on biological and physicochemical factors leading~~for ~~to~~ ~~for~~ low rates of nitrification in Cerrado soils, based on biological and physicochemical factors. Plants may decrease nitrification by competing for NH_4^+-N and by increasing the C:N ratio through increased carbon supply, thereby promoting immobilisation, while some plants produce nitrification inhibitors in plant litter and root exudates (Subbarao *et al.*, 2006). These inhibitors target ammonia oxidation and can benefit plants by reducing competition for ammonium (Subbarao *et al.*, 2006, Subbarao *et al.*, 2015). ~~The specific reasons for the low nitrification rates in the Cerrado biome are unclear, but~~ Both ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) are ~~both~~ present in these soils (Catão *et al.*, 2016) but ~~.~~ ~~However,~~ the relatively high ammonium concentration

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in Cerrado soil [(3 – 22 µg N g^{-1} soil, (Nardoto & Bustamante, 2003)]; 5 – 49 µg N g^{-1} soil, (Catão *et al.*, 2016)]) suggests that ammonia oxidisers are not limited by ammonia concentration and low rates of nitrification in Cerrado soils may be better explained by production of biological nitrification inhibitors.

Low nitrification rates in acidic soils have been described for many years (De Boer & Kowalchuk, 2001). Inhibition of ammonia oxidation in low pH soil was traditionally considered to be due to the low availability of ammonia, through ionisation to NH_4^+, but may be alleviated by growth in soil aggregates or on surfaces (De Boer *et al.*, 1991, Allison & Prosser, 1993), urease activity (De Boer *et al.*, 1989, Burton & Prosser, 2001), or growth of acidophilic archaeal ammonia oxidisers (Gubry-Rangin *et al.*, 2011, Lehtovirta-Morley *et al.*, 2011). Meta-analysis of net nitrification rates in a wide range of soils (Booth *et al.*, 2005) suggests that pH limitation may not be widespread, but increases increased nitrification following amendment of Cerrado soil with calcium carbonate (Rosolem *et al.*, 2003) provides evidence for pH limitation in soil.

Low water availability decreases nitrification (Placella & Firestone, 2013, Thion & Prosser, 2014) by increasing osmotic stress and reducing mobility of ammonia within the soil. In the The rainfall seasonallity dry in the Cerrado biome, and the reported increases in N_2O production and 10 fold higher NO emissions increase after following rainfall or addition of artificial rainwater (Pinto *et al.*, 2002, Pinto *et al.*, 2006) providing evidence for limitation of nitrification during dry seasons in this biome.

The specific reasons for the low nitrification rates in the Cerrado biome are unclear, but ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) are both present in these soils (Catão *et al.*, 2016). Limited nitrification is alleviated by agriculture due to fertilisation, liming, tillage or plant community change. Considering the extensive conversion of Cerrado soils to agricultural production (Marris, 2005, Catão *et al.*, 2016), it is

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~~relevant~~important to understand adaptation of natural ecosystems ~~adaptation~~ to limit N loss. The aim of this study was to test three hypotheses ~~which~~regarding ~~are~~ potential mechanisms ~~underlying~~ for the low nitrification rates: presence of biological nitrification inhibitors, low water availability and low pH. The presence of plant-derived nitrification inhibitors was tested by analysing (i) the growth of AOB and AOA in the presence of aqueous extract from Cerrado soil and (ii) the effect of Cerrado soil on ammonia oxidation by a nitrifying soil (Craibstone) in soil slurries. The effects of low water availability and low pH on nitrification were tested by manipulating Cerrado soil water content and pH in microcosms.

Materials and methods

Soil sampling

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

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which was well aerated and well drained, is classified as sandy loam with 20.8% clay and had an initial pH of 5.6 (±0.04). Physicochemical parameters from the ~~e~~Campo sujo sample were previously described (Catão *et al.*, 2016): organic matter content was 42.6 (± 2.4) g kg^{-1}, cation exchange capacity 6 (± 0.6) $cmol_c$ dm^{-3}, available phosphorus 1.8 (± 0.13) mg dm^{-3}, aluminium 1.2 (± 0.12) $cmol_c$ dm^{-3} and Fe 165.4 (± 41.0~~1~~) mg dm^{-3}. Craibstone soil, used in this study as a reference nitrifying soil, was sampled from an experimental agricultural field (Scottish Agricultural College, Craibstone, Scotland, Grid reference NJ872104) and maintained at pH 5.5 since 1961.

Cultivation of ammonia oxidisers with soil extracts

Aqueous extracts of Craibstone and Campo sujo soils were prepared by blending 20 g soil in 2 volumes of sterile distilled water for 40 s, rotating in 50-mL sterile tubes for 1 h, centrifuging (3,000×g for 15 min) and sterilising by progressive filtration through filters with 10-mm, 5-mm, 0.45-µm and 0.22-µm pore size. NH_4^+ and NO_3^- concentrations in the filtrates were below the level of detection (data not shown).

Pure cultures of AOA (*Candidatus* Nitrosocosmicus franklandus (Lehtovirta-Morley *et al.*, 2016)) and AOB (*Nitrosospira briensis* #128, *Nitrosospira tenuis* #NV-12, *Nitrosospira multiformis* - NCIMB11849, ATCC25196 and *Nitrosomonas europaea* - NCIMB11850, ATCC25978) were cultivated in inorganic growth medium in the dark without shaking. *Candidatus* Nitrosocosmicus franklandus (Lehtovirta-Morley *et al.*, 2016) was cultivated at 40°C in medium described previously (Lehtovirta-Morley *et al.*, 2011) but modified by the addition of 1 mL L^{-1} vitamin solution (Widdel & Bak, 1992), 1 mL L^{-1} selenite-tungstate solution (Widdel & Bak, 1992) and 2 mM NH_4Cl. The pH was maintained at ~7.5 by the addition of 10 mL L^{-1} 1 M HEPES buffer. The AOB were grown in Skinner

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and Walker medium (Skinner & Walker, 1961) and incubated at 30°C. Triplicate cultures were prepared in 30-mL Universal tubes by adding 5 mL of the appropriate medium, previously inoculated with exponentially growing cells (1 mL inoculum per 100 mL 2× concentrated medium), to a 5-mL volume of sterile distilled water, Craibstone or Campo sujo soil aqueous extract, or allylthiourea (100 µM final concentration), an ammonia oxidiser inhibitor. The cultures were grown without agitation, and growth was monitored for 26 days (AOA) and 13 days (AOB) by measuring nitrite accumulation (Shinn, 1941). The maximum specific growth rate was estimated as the slope of semi-logarithmic plots of nitrite concentration versus time.

Soil incubation in slurries

Soil slurries were established in 250-mL sterile Erlenmeyer flasks containing 20 g soil and 100 mL sterile distilled water, stirred at 100 rpm and maintained at 30°C in the dark. Individual flasks contained Campo sujo soil, Craibstone soil or mixtures of Campo sujo and Craibstone soils in 1:1 or 4:1 ratios. Before incubation and 1, 7, 14, and 21 days after incubation, soil slurry aliquots (8 mL) were centrifuged at 3,000×*g* for 15 min. After immediate measurement of pH in 2 mL of supernatant, the remaining supernatant (6 mL) was stored at -20°C for quantification of inorganic N (see below). The soil pellet was frozen in liquid nitrogen and stored at -80°C for ~~nucleic acid~~molecular analysis.

Soil incubation in microcosms

~~Cerrado~~ Campo sujo soil was incubated in sealed microcosms consisting of 140-mL sterile serum glass bottles containing 10 g soil. The soil had an initial water content of 24.9 ± 0.03 g H_20 100 g^{-1} dry soil, corresponding to a matric potential of -0.15 ± 0.01 MPa. Microcosms were incubated for 4 days in the dark at 30ºC (acclimation period) and then divided into two

groups. The 'dried soil' group was left to air dry, reaching a moisture content of 8.66 g H_20 100 g^{-1} dry soil (-6.34 ± 2.98 MPa matric potential). In the 'moist soil' group, the moisture content was adjusted to 37.9 ± 0.3 g H_20 100 g^{-1} dry soil by adding sterile distilled water. Soil in half of the dried soil microcosms was rewetted to 39.6 ± 1.92 g H_20 100 g^{-1} dry soil (-0.11 ± 0.02 MPa) ('Water Pulse' treatment), and the soil in the remaining dried soil microcosms was kept dry ('Dry' treatment). Finally, the pH of soil in half of the moist soil microcosms was increased to 6.34 ± 0.09 with $CaCO_3$ ('pH treatment'). The pH of soil in the remaining microcosms ('Dry', 'Water Pulse' and 'Moist' treatments) was 5.21 ± 0.02, which was slightly lower than the initial value of the sampled soil and was not adjusted. The four treatments were performed in triplicate, with or without the addition of the ammonia oxidation inhibitor acetylene (0.01% of headspace volume). The soil microcosms were incubated in the dark at 30ºC, and aerobic conditions were maintained by removing the seals for 5 - 10 minutes twice weekly. The 'Moist' and 'Water Pulse' microcosms were watered weekly to maintain moisture content. The microcosms were sampled destructively after 6 h and 1, 3, 7, 14, and 21 days, with additional sampling after 28 days for the pH treatment). For each microcosm, half of the soil was used for chemical analysis and the remainder was stored at -80ºC for molecular analysis.

Soil physicochemical analyses

Water matric potential was measured using a WP4C Dewpoint PotentiaMeter (Decagon, Pullman, UK) and pH was determined in water. Soil NH_4^+ and NO_x (NO_2^- + NO_3^-) concentrations were determined colorimetrically by flow injection analysis (FIA star 5010 Analyser, Foss Tecator AB, Höganäs, Sweden) (Allen, 1989) after extraction from 2 g wet soil in 10 mL KCl (1 M), for the microcosm soil, or directly from slurry supernatant. Because NO_2^- concentration was below the level of detection, NO_x is expressed as µg NO_3^--N g^{-1} dry

soil (ppm). Nitrification inhibition was assessed as the decrease in nitrate concentration as a percentage of that of Craibstone soil at each time point.

Molecular analysis

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

Archaeal and bacterial *amoA* genes, which encode subunit A of ammonia monooxygenase, were quantified in a MasterCycler thermal cycler (Eppendorf, Hamburg, Germany) using standard curves, as described previously (Catão *et al.*, 2016). PCR amplification was carried out using primers crenamo23f and crenamo616r for archaeal *amoA* genes (Tourna, 2008) and amoA1F and amoA2R for bacterial *amoA* genes (Rotthauwe *et al.*, 1997). Each 20-µL reaction contained 1× QuantiFast PCR Master Mix (for AOA) or QuantiTect Master Mix (for AOB) (Qiagen, Crawley, UK), 0.4 µM of each primer for AOA *amoA* or 0.6 µM of each primer for AOB *amoA*, 2 µg μL^{-1} BSA (Promega), and 2 µL DNA (or cDNA). Archaeal *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at 95ºC, followed by 40 cycles of 15 s at 94ºC and 90 s at 60ºC. Bacterial *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at 95ºC, followed by 45 cycles of 1 min at 94ºC, 1 min at 55ºC, and 1 min at 72ºC. To exclude fluorescence contamination of potential primer-dimers, SYBR Green fluorescence was measured after 5 s at 80°C or after 8 s at 83°C, for AOA and AOB, respectively. Melting curves between 65°C and 95°C were analysed for each run. AOB *amoA* transcripts were

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below the detection limit (5 copies μL^{-1}). Efficiency of amplification and r^2 for DNA were, respectively, 0.92 and 0.998 for archaeal *amoA* and 104.6 and 0.993 for bacterial *amoA*.

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

Statistical analysis

All analyses were conducted using R version (3.2.2). The effect of aqueous soil extracts on pure AOA and AOB cultures was analysed by testing differences in specific growth rate between treatments by one-way analysis of variance. Differences between nitrification rate in soil slurries were evaluated using a repeated-measures linear mixed model (package *nlme*) (Pinheiro *et al.*, 2015). Each slurry was considered a subject with random effect to analyse the effect of treatment (Campo sujo soil, Craibstone soil, or soil mixture), time, and their interaction on inorganic N concentration and *amoA* gene (and transcript) abundance. The NO_3^- concentration in the Campo sujo slurries was below the limit of detection; therefore, these samples were excluded from the analysis. Gene abundance data were log-transformed to achieve a normal distribution. When the interaction between independent variables was not significant, it was removed to analyse the effect of time or treatment independently over concentration of soil NH_4^+ and NO_x. Two-way analysis of variance, with treatment and time as independent factors, was performed to evaluate differences in mineralisation and NO_3^- in the soil microcosms.

Results

Effects of soil extracts on ammonia oxidiser cultures

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

Effects of Campo sujo soil on nitrification in Craibstone soil

Soil slurries containing Campo sujo soil, Craibstone soil, or a mixture of the two soils (at ratios of 1:1 and 4:1) were incubated for up to 21 days. In all slurries, pH increased (0.4 in the ~~grassland~~Campo sujo soil, 0.8 in Craibstone ~~samples~~soil, but only 0.1 and 0.2 for the 1:1 and 4:1 mixed samples, respectively) after the first day of incubation but did not change significantly thereafter. Net NH_4^+ ~~accumulation~~ concentration after 21 days ranged from 0.62 (±0.02) to 1.76 (±0.39) ppm (mg L^{-1} soil solution) for Craibstone soil and 0.87 (±0.02) to 2.20 (±0.02) ppm (mg L^{-1} soil solution) for Campo sujo soil (Fig. 2). Initial NH_4^+ concentration was higher in the mixed soil slurries than in controls, but the mixed slurries accumulated less NH_4^+ over the incubation period. The greatest increase in NH_4^+ concentration after 21 days was observed in Craibstone soil (2.9-fold).

NO_3^- concentration also increased in all soil slurries during incubation ($p<0.0001$, Fig. 2B), except in those containing Campo sujo only, in which NO_3^- was below the detection limit. In the mixed soil slurries, NO_3^- production was equivalent to or higher than the 50% and 20% expected for the 1:1 and 4:1 ratios of Campo sujo soil and Craibstone soil, respectively (Fig. 2C), providing no evidence for inhibition of Craibstone soil nitrification by Campo sujo soil.

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Ammonia oxidiser *amoA* gene abundance in the soil slurries did not change significantly during the incubation period, even when significant NO_3^- accumulation was observed (Fig. 3). AOA *amoA* abundance in the Campo sujo-only slurries was approximately three orders of magnitude lower than that of Craibstone-only slurries (Fig. 3A). AOA *amoA* abundance in mixed soil slurries was lower than that of Craibstone-only slurries until day 14, after which differences were not significant (p=0.132). AOB *amoA* gene abundance in the Campo sujo-only slurries was also approximately three orders of magnitude lower than that of Craibstone-only slurries, and even significantly different (p=0.024) at day 21, when Campo sujo-only AOB abundance was no longer significantly different from the those in the mixed samples (Fig. 3B).

The AOB *amoA* gene abundance was lower than AOA *amoA* gene abundance in all slurries at each time point. The AOA:AOB *amoA* gene ratio did not change significantly in the Campo sujo-only slurries but increased in the Craibstone-only and mixed soil slurries (Fig. 3C). In all slurries, AOB *amoA* transcripts were below the level of detection (5 μL^{-1}). The AOA *amoA* transcripts were detected in all slurries containing Craibstone soil throughout incubation but were detected in the Campo sujo-only slurries only at day 21 (Fig. 3D).

Before incubation, DGGE profiles of *amoA* genes amplified from Craibstone soil contained more bands (potential OTUs) than profiles of Campo sujo soil (Fig. S2) and did not change significantly after incubation for 21 days. DGGE profiles of the AOA *amoA* genes in the 1:1 mixed slurry were similar to those of Craibstone soil, possibly masking ~~the observation of~~ the less abundant *amoA* genes from the Campo sujo soil (Fig. S2).

Effects of soil pH and moisture content

The effects of pH and moisture content on nitrification in Campo sujo soil were investigated in soil microcosms. Net ~~M~~mineralisation was determined as the increase in concentration of

inorganic N (NH_4^+-N + NO_3^- -N) during incubation, assuming that other nitrogen cycle processes were not significant (Fig. S3). Mineralisation in the dry soil did not increase after wetting, in contrast to the expected 'Birch' effect (Birch, 1964) (Fig. S3). In addition, soil pH did not change significantly with time in the microcosms and remained at 5.2 for the 'Water Pulse', 'Moist' and 'Dry' treatments and at 6.3 for the 'pH' treatment, in which pH was increased artificially with $CaCO_3$. Nitrate concentration did not increase significantly in any of the treatments (Fig. S3), and no significant difference was observed between treatments (p =0.14). No significant differences were observed between samples incubated with or without acetylene, except for NO_3^--N concentrations in the 'Moist' microcosms. After incubation for 21 days, the NO_3^- -N concentration was lower in the acetylene-treated moist microcosms than in those without added acetylene (Fig. S3).

Discussion

Nitrification is frequently undetectable in undisturbed Cerrado ecosystems, although management and conversion to agricultural production increases nitrate production (Catão *et al.*, 2016). Previous studies suggest low abundance of AOA and AOB in Campo sujo soil (Catão *et al.*, 2016), which is also characterised by sparse shrubs over a continuous grass layer. The aim of this work was to determine whether the lack of nitrification and low abundance of ammonia oxidisers in this ecosystem were due to low pH, low soil moisture, NH_4^+ limitation or biological inhibition of ammonia oxidation.

Certain plants release biological nitrification inhibitors that suppress ammonia oxidation in soils (Subbarao *et al.*, 2015). For example, compounds produced by *Brachiaria* (Subbarao *et al.*, 2009) and *Sorghum* (Zakir *et al.*, 2008) inhibited a recombinant *N. europaea* strain, possibly by blocking ammonia monooxygenase and hydroxylamine oxidoreductase

(Subbarao *et al.*, 2008). Production of biological nitrification inhibitors can be promoted by exposure to high NH_4^+:NO_3^- ratios (Subbarao *et al.*, 2015), such as those found in Campo sujo soil (Catão *et al.*, 2016). However, aqueous extracts of Campo sujo soil did not inhibit growth of cultures of four AOB and one AOA, all of which were isolated from neutral to alkaline soils. Allylthiourea, used as a positive control, prevented growth of the AOB cultures but not the AOA culture, which is consistent with other studies reporting a greater tolerance of AOA to allylthiourea (Hatzenpichler & Lebedeva, 2008, Stempfhuber *et al.*, 2015). This result demonstrates the need to test potential inhibitors against both AOA and AOB, rather than *N. europaea* only.

Cultivation-based studies were ~~based~~ performed ~~only~~ usingon ~~on~~ aqueous soil extracts and a small number of cultivated strains and potential inhibition was therefore assessed more directly by mixing Campo sujo soil with Craibstone soil, a strongly nitrifying soil with similar pH, in soil slurries (Nicol *et al.*, 2008, Zhang *et al.*, 2010). The soil slurries also provided no evidence of nitrification inhibitors in Campo sujo soil. Nitrate accumulation in mixtures of Craibstone soil and Campo sujo soil was lower than that of Craibstone soil only, but this difference was less than or equal to that predicted by the lower volume of Craibstone soil in the slurry, suggesting that nitrification was not inhibited by the Campo sujo soil. Similarly, the addition of Campo sujo soil to Craibstone soil had no apparent effect on AOA and AOB *amoA* gene abundances. Archaeal *amoA* genes were more abundant than those of bacteria in the soils, and bacterial *amoA* gene expression was not detected, as reported by previous studies of Craibstone soils (Zhang *et al.*, 2010). Neither AOA nor AOB *amoA* abundance changed significantly during incubation of any of the slurries containing Craibstone soil, despite evidence of nitrate production. However, the AOA:AOB *amoA* gene ratio increased, suggesting greater growth or lower death rates of AOA, but there was no evidence for growth of AOB or AOA in the Campo sujo soil. DGGE ~~analysis~~ profiles of the

Campo sujo soil containeddetected few fewer archaeal *amoA* gene bands in the Campo sujo soil comparedthan to those of that of Craibstone soil, providing a further evidence indication of the low abundance and activity of ammonia oxidisers in Campo sujo soil. Although AOA *amoA* transcripts in Campo sujo-only slurries increased after incubation for 21 days, this increased gene expression did not appear to lead to a detectable level of nitrate. Nitrate reduction during denitrification was considered negligible due to previous experiments showing low NO emissions and undetectable N_2O in Cerrado soils (Pinto *et al.*, 2002).

In the absence of evidence for BNI, microcosm studies were performed to determine whether the low nitrification rates in Campo sujo soil were due to low pH or low soil moisture content. Gas measurements in Cerrado soil after a rainfall (natural or simulated) led to an increase in NO emissions (Pinto *et al.*, 2002), which agrees may result from with the Birch effect (Birch, 1964) of increased organic matter availability after rewetting (Fierer & Schimel, 2003). Soil pH is an important determinant of microbial diversity (Lauber *et al.*, 2009, Fierer *et al.*, 2012) and influences soil ammonia oxidiser abundance and activity (de Boer and Kowalchuk, Nicol et al. 2008), with higher transcriptional activity of Archaea than Bacteria as pH decreases (Nicol *et al.*, 2008). In our experiment, higher soil pH increased organic nitrogen mineralisation rate but did not lead to detectable nitrate production in Campo sujo soil after incubation for 28 days. Mineralisation was also lower in moist soil than in other treatments, and the increase in moisture did not lead to detectable nitrate production (see supplementary Fig. S3). Even though it can be argued that NO_3^- can be denitrified at higher soil moisture, low or undetectable NO and N_2O emission in field measurements over the seasons (Cruvinel *et al.*, 2011) and no consistent NO_3^- increase after one day or after 3 weeks of incubation, suggested limitation of nitrification by other factors.

The low nitrification and low ammonia oxidiser abundance of Campo sujo soil, in both microcosms and slurries, were not due to NH_4^+ limitation. The NH_4^+ concentration of

Campo sujo soil slurries was even higher than that of Craibstone soil slurries at the beginning of the experiment. Jack pine forest soils showed similar ~~results of~~ high ~~accumulated~~ concentrations of ammonium without detectable nitrate (Ste-Marie & Paré, 1999). None of the treatments in this study increased nitrification in Campo sujo soil and this soil did not inhibit nitrification in Craibstone soil or pure cultures of AOA or AOB. Similarly, in the jack pine forest soil, nitrification was not stimulated by increased pH or ammonium amendment but was stimulated by the addition of nitrifying soil from a forest floor (Ste-Marie & Paré, 1999). In this study, both AOA and AOB were detected in Campo sujo soil, but at low levels that are unlikely to lead to detectable nitrate production. Consequently, these soils have much greater capacity to retain N as NH_4^+ through ion exchange, with minimal NO_3^- leaching. Furthermore, NO pulses observed after rainfall are not due to nitrifier activity. However, experiments performed either for longer than 3 weeks or with rhizosphere soil might detect differences in nitrifier inhibition/stimulation and, despite the small effect of pH and H_2O on the Brazilian savanna soil nitrification rate, archaeal ammonia oxidisers started to show activity in slurries after 21 days of incubation. Taken together, our results show that low nitrification rates and ammonia oxidiser abundance in Campo sujo soil are not due to low moisture content, low pH or the presence of ammonia oxidiser inhibitors.

~~The data presented here suggest that the NO pulses observed after rainfall are not due to nitrifier activity. However, experiments performed either for longer than 3 weeks or with rhizosphere soil might detect differences in nitrifiers inhibition/stimulation. Nevertheless, and, despite the small effect of pH and H_2O on the Brazilian savanna soil nitrification rate, archaeal ammonia oxidisers started to show activity in slurries after 21 days of incubation. Most likely, nutrients stoichiometry (Mooshammer *et al.*, 2014) plays an important role on the microbial activity of those soils, as N and P co-limitation affects decomposition rates in Cerrado soils (Kozovits *et al.*, 2007, Jacobson *et al.*, 2011).~~

Although low nitrate concentrations are unlikely to be due to denitrification, nitrate assimilation and dissimilatory nitrate reduction may reduce nitrate produced by nitrifiers. However, the high ammonium concentrations, low AOA and AOB abundances, and lack of evidence for ammonia oxidiser activity and growth suggest inhibition or limitation of ammonia oxidiser growth and activity. This study was performed using soil associated with one type of vegetation sampled at the beginning of the dry season. Production of BNI may vary seasonally and with vegetation, but low nitrification rates are found in soils sampled throughout the year (Nardoto & Bustamante, 2003) and ammonium and nitrate concentrations are high and low (2.5 (±0.5) µg N-NH_4^+ g^{-1} dry soil and 0.072 (±0.01) µg N-NO_3^- g^{-1} dry soil), respectively, regardless of vegetation type (Catão *et al.*, in preparation). Inhibition of ammonia oxidiser cultures was performed with water extracts from soil and it is possible that ammonia oxidisers were inhibited by water insoluble BNI. Other features of Cerrado soils may also be important. There is evidence for co-limitation of microbial decomposition in these soils by N and P (Kozovits et al., 2007, Jacobson et al., 2011) and ammonia oxidisers may have been limited by P and other nutrients, although mixing with Craibstone soil would be expected to relieve this limitation. Cerrado soils are deficient in P (Goedert, 1983), and the values observed for the Campo sujo are similar to the upper layer of other Cerrado soils (Parron et al., 2011), where soil P sorption capacity is often related to Fe and Al contents (Goedert, 1983). The soils investigated in this study also contain relative high Fe concentrations (165.4 ± 41.01 mg dm^{-3}), which have been associated with reduction in net nitrification and AOA and AOB *amoA* gene abundance in subtropical acid soils (Jiang et al., 2015). Although these possibilities suggest future experimental studies, the reasons for low nitrification rates and low ammonia oxidiser abundances in Cerrado soil remain unclear and this study provided no evidence for ammonia limitation, pH or inhibition by water extractable inhibitors.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Ammonia oxidisers in a non-nitrifying Brazilian savanna soil

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Abstract

Low nitrification rates in Brazilian savanna (Cerrado) soils have puzzled researchers for decades. Potential mechanisms include biological inhibitors, low pH, low microbial abundance and low soil moisture content, which hinders microbial activity, including ammonia oxidation. Two approaches were used to evaluate these potential mechanisms, (i) manipulation of soil moisture and pH in microcosms containing Cerrado soil and (ii) assessment of nitrification inhibition in slurries containing mixtures of Cerrado soil and an actively nitrifying agricultural soil. Despite high ammonium concentration in Cerrado soil microcosms, little NO_3^- accumulation was observed with increasing moisture or pH, but in some Cerrado soil slurries, AOA *amoA* transcripts were detected after 14 days. In mixed soil slurries, the final NO_3^- concentration reflected the initial proportions of agricultural and Cerrado soils in the mixture, providing no evidence of nitrification inhibitors in Cerrado soil. AOA community denaturing gradient gel electrophoresis profiles were similar in the mixed and nitrifying soils. These results suggest that nitrification in Cerrado soils is not constrained by water availability, ammonium availability, low pH, or biological inhibitors and alternative potential explanations for low nitrification levels are discussed. Keywords: ammonia oxidisers, low nitrification, Brazilian savanna, inhibition, pH, soil moisture

Introduction

Autotrophic nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a major cause of N loss in terrestrial environments. In agricultural systems, nitrification is the main pathway of N transformation, and up to 95% of total N is present as NO_3^- potentially leading to leaching and emission of nitric oxide (NO) and nitrous oxide (N_2O) by nitrifiers and denitrifiers (Subbarao *et al.*, 2012). Inhibitors of nitrification can decrease nitrogen losses from these systems (Subbarao *et al.*, 2006). These inhibitors target the first step in nitrification, ammonia oxidation, which is carried out by both bacterial and archaeal ammonia oxidisers. Some natural systems have lower nitrification rates and higher nitrogen fertiliser use efficiency than managed systems (Ste-Marie & Paré, 1999). For example, in soils of the tropical savanna biome in Central Brazil, also called Cerrado, NO_3^- concentration is low or undetectable (Nardoto & Bustamante, 2003), the NH_4^+:NO_3^- ratio is high and the abundance of nitrifiers is low (Catão *et al.*, 2016). These ecosystems may therefore provide a model for greater and more sustainable crop productivity and decreased demand for nitrogen fertilisers.

There are several potential explanations for low rates of nitrification in Cerrado soils, based on biological and physicochemical factors. Plants may decrease nitrification by competing for NH_4^+-N and by increasing the C:N ratio through increased carbon supply, thereby promoting immobilisation, while some plants produce nitrification inhibitors in plant litter and root exudates (Subbarao *et al.*, 2006). These inhibitors target ammonia oxidation and can benefit plants by reducing competition for ammonium (Subbarao *et al.*, 2006, Subbarao *et al.*, 2015). Both ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) are present in these soils (Catão *et al.*, 2016) but the relatively high ammonium concentration in Cerrado soil [3 – 22 μg N g^{-1} soil, (Nardoto & Bustamante, 2003); 5 – 49 μg N g^{-1} soil (Catão *et al.*, 2016)] suggests that ammonia oxidisers are not

limited by ammonia concentration and low rates of nitrification in Cerrado soils may be better explained by production of biological nitrification inhibitors.

Low nitrification rates in acidic soils have been described for many years (De Boer & Kowalchuk, 2001). Inhibition of ammonia oxidation in low pH soil was traditionally considered to be due to the low availability of ammonia, through ionisation to NH_4^+, but may be alleviated by growth in soil aggregates or on surfaces (De Boer *et al.*, 1991, Allison & Prosser, 1993), urease activity (De Boer *et al.*, 1989, Burton & Prosser, 2001), or growth of acidophilic archaeal ammonia oxidisers (Gubry-Rangin *et al.*, 2011, Lehtovirta-Morley *et al.*, 2011). Meta-analysis of net nitrification rates in a wide range of soils (Booth *et al.*, 2005) suggests that pH limitation may not be widespread, but increased nitrification following amendment of Cerrado soil with calcium carbonate (Rosolem *et al.*, 2003) provides evidence for pH limitation in soil.

Low water availability decreases nitrification (Placella & Firestone, 2013, Thion & Prosser, 2014) by increasing osmotic stress and reducing mobility of ammonia within the soil. In the seasonally dry Cerrado biome, N_2O and NO emissions increase after rainfall or addition of artificial rainwater (Pinto *et al.*, 2002, Pinto *et al.*, 2006) providing evidence for limitation of nitrification during dry seasons.

Limited nitrification is alleviated by agriculture due to fertilisation, liming, tillage or plant community change. Considering the extensive conversion of Cerrado soils to agricultural production (Marris, 2005, Catão *et al.*, 2016), it is important to understand adaptation of natural ecosystems to limit N loss. The aim of this study was to test three hypotheses regarding potential mechanisms for the low nitrification rates: presence of biological nitrification inhibitors, low water availability and low pH. The presence of plant-derived nitrification inhibitors was tested by analysing (i) the growth of AOB and AOA in the presence of aqueous extract from Cerrado soil and (ii) the effect of Cerrado soil on ammonia

oxidation by a nitrifying soil (Craibstone) in soil slurries. The effects of low water availability and low pH on nitrification were tested by manipulating Cerrado soil water content and pH in microcosms.

Materials and methods

Soil sampling

Cerrado soil was sampled from an undisturbed shrubland (Campo sujo), with some sparse shrubs over a continuous grass layer (Eiten, 1972) , where graminoids can account for around 45% of total aboveground biomass, leading to a contribution of 46% of relative abundance of fine roots (Castro & Kauffman, 1998). Campo sujo is dominated by plants from the families Asteraceae, Leguminosae, and Poaceae (Tannus & Assis, 2004). The average monthly precipitation and temperature, measured at the nearest meteorological centre in 2014 (~30 km from the farm; Pirenopolis – GO, Station 83376, 15°50'60"S 48°57'36"W), were 143 mm (range 0 - 317 mm) and 23.4°C (range 21°C - 25.6°C), respectively. Triplicate soil samples were obtained from the upper 10 cm of soil, pooled before sieving (2-mm mesh size) and then stored at 4°C. The climate in the Cerrado biome is tropical (Köppen Aw), and samples were collected at the beginning of the dry season (May 2014). Campo sujo and Cerrado *sensu stricto* are usually found on oxisols, with low nutrient content, low pH, and high content of aluminium (Reatto *et al.*, 1998). The soil, which was well aerated and well drained, is classified as sandy loam with 20.8% clay and had an initial pH of 5.6 (±0.04). Physicochemical parameters from the Campo sujo sample were previously described (Catão *et al.*, 2016): organic matter content was 42.6 (± 2.4) g kg^{-1}, cation exchange capacity 6 (± 0.6) $cmol_c$ dm^{-3}, available phosphorus 1.8 (± 0.13) mg dm^{-3}, aluminium 1.2 (± 0.12) $cmol_c$ dm^{-3} and Fe 165.4 (± 41.0) mg dm^{-3}. Craibstone soil, used in this study as a reference nitrifying soil, was sampled from an experimental agricultural field (Scottish Agricultural

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

Cultivation of ammonia oxidisers with soil extracts

Aqueous extracts of Craibstone and Campo sujo soils were prepared by blending 20 g soil in 2 volumes of sterile distilled water for 40 s, rotating in 50-mL sterile tubes for 1 h, centrifuging (3,000×g for 15 min) and sterilising by progressive filtration through filters with 10-mm, 5-mm, 0.45-µm and 0.22-µm pore size. NH_4^+ and NO_3^- concentrations in the filtrates were below the level of detection (data not shown).

Pure cultures of AOA (*Candidatus* Nitrosocosmicus franklandus (Lehtovirta-Morley *et al.*, 2016)) and AOB (*Nitrosospira briensis* #128, *Nitrosospira tenuis* #NV-12, *Nitrosospira multiformis* - NCIMB11849, ATCC25196 and *Nitrosomonas europaea* - NCIMB11850, ATCC25978) were cultivated in inorganic growth medium in the dark without shaking. *Candidatus* Nitrosocosmicus franklandus (Lehtovirta-Morley *et al.*, 2016) was cultivated at 40°C in medium described previously (Lehtovirta-Morley *et al.*, 2011) but modified by the addition of 1 mL L^{-1} vitamin solution (Widdel & Bak, 1992), 1 mL L^{-1} selenite-tungstate solution (Widdel & Bak, 1992) and 2 mM NH_4Cl. The pH was maintained at ~7.5 by the addition of 10 mL L^{-1} 1 M HEPES buffer. The AOB were grown in Skinner and Walker medium (Skinner & Walker, 1961) and incubated at 30°C. Triplicate cultures were prepared in 30-mL Universal tubes by adding 5 mL of the appropriate medium, previously inoculated with exponentially growing cells (1 mL inoculum per 100 mL 2× concentrated medium), to a 5-mL volume of sterile distilled water, Craibstone or Campo sujo soil aqueous extract, or allylthiourea (100 µM final concentration), an ammonia oxidiser inhibitor. The cultures were grown without agitation, and growth was monitored for 26 days (AOA) and 13 days (AOB) by measuring nitrite accumulation (Shinn, 1941). The maximum

specific growth rate was estimated as the slope of semi-logarithmic plots of nitrite concentration versus time.

Soil incubation in slurries

Soil slurries were established in 250-mL sterile Erlenmeyer flasks containing 20 g soil and 100 mL sterile distilled water, stirred at 100 rpm and maintained at 30°C in the dark. Individual flasks contained Campo sujo soil, Craibstone soil or mixtures of Campo sujo and Craibstone soils in 1:1 or 4:1 ratios. Before incubation and 1, 7, 14, and 21 days after incubation, soil slurry aliquots (8 mL) were centrifuged at 3,000×*g* for 15 min. After immediate measurement of pH in 2 mL of supernatant, the remaining supernatant (6 mL) was stored at -20°C for quantification of inorganic N (see below). The soil pellet was frozen in liquid nitrogen and stored at -80°C for molecular analysis.

Soil incubation in microcosms

Campo sujo soil was incubated in sealed microcosms consisting of 140-mL sterile serum glass bottles containing 10 g soil. The soil had an initial water content of 24.9 ± 0.03 g H_20 100 g^{-1} dry soil, corresponding to a matric potential of -0.15 ± 0.01 MPa. Microcosms were incubated for 4 days in the dark at 30ºC (acclimation period) and then divided into two groups. The 'dried soil' group was left to air dry, reaching a moisture content of 8.66 g H_20 100 g^{-1} dry soil (-6.34 ± 2.98 MPa matric potential). In the 'moist soil' group, the moisture content was adjusted to 37.9 ± 0.3 g H_20 100 g^{-1} dry soil by adding sterile distilled water. Soil in half of the dried soil microcosms was rewetted to 39.6 ± 1.92 g H_20 100 g^{-1} dry soil (-0.11 ± 0.02 MPa) ('Water Pulse' treatment), and the soil in the remaining dried soil microcosms was kept dry ('Dry' treatment). Finally, the pH of soil in half of the moist soil microcosms was increased to 6.34 ± 0.09 with $CaCO_3$ ('pH treatment'). The pH of soil in the remaining

microcosms ('Dry', 'Water Pulse' and 'Moist' treatments) was 5.21 ± 0.02, which was slightly lower than the initial value of the sampled soil and was not adjusted. The four treatments were performed in triplicate, with or without the addition of the ammonia oxidation inhibitor acetylene (0.01% of headspace volume). The soil microcosms were incubated in the dark at 30ºC, and aerobic conditions were maintained by removing the seals for 5 - 10 minutes twice weekly. The 'Moist' and 'Water Pulse' microcosms were watered weekly to maintain moisture content. The microcosms were sampled destructively after 6 h and 1, 3, 7, 14, and 21 days, with additional sampling after 28 days for the pH treatment). For each microcosm, half of the soil was used for chemical analysis and the remainder was stored at -80ºC for molecular analysis.

Soil physicochemical analyses

Water matric potential was measured using a WP4C Dewpoint PotentiaMeter (Decagon, Pullman, UK) and pH was determined in water. Soil NH_4^+ and NO_x (NO_2^- + NO_3^-) concentrations were determined colorimetrically by flow injection analysis (FIA star 5010 Analyser, Foss Tecator AB, Höganäs, Sweden) (Allen, 1989) after extraction from 2 g wet soil in 10 mL KCl (1 M), for the microcosm soil, or directly from slurry supernatant. Because NO_2^- concentration was below the level of detection, NO_x is expressed as μg NO_3^--N g^{-1} dry soil (ppm). Nitrification inhibition was assessed as the decrease in nitrate concentration as a percentage of that of Craibstone soil at each time point.

Molecular analysis

Nucleic acids were extracted from 0.5 g soil as previously described (Nicol *et al.*, 2005), suspended in diethylpyrocarbonate-treated water and immediately stored at -80°C. An aliquot was treated with DNase and the RNA was reverse transcribed, as described previously

(Tourna, 2008). The nucleic acid not used for cDNA generation was considered DNA only and its concentration was estimated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Loughborough, UK).

Archaeal and bacterial *amoA* genes, which encode subunit A of ammonia monooxygenase, were quantified in a MasterCycler thermal cycler (Eppendorf, Hamburg, Germany) using standard curves, as described previously (Catão *et al.*, 2016). PCR amplification was carried out using primers crenamo23f and crenamo616r for archaeal *amoA* genes (Tourna, 2008) and amoA1F and amoA2R for bacterial *amoA* genes (Rotthauwe *et al.*, 1997). Each 20-µL reaction contained 1× QuantiFast PCR Master Mix (for AOA) or QuantiTect Master Mix (for AOB) (Qiagen, Crawley, UK), 0.4 µM of each primer for AOA *amoA* or 0.6 µM of each primer for AOB *amoA*, 2 µg μL^{-1} BSA (Promega), and 2 µL DNA (or cDNA). Archaeal *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at 95ºC, followed by 40 cycles of 15 s at 94ºC and 90 s at 60ºC. Bacterial *amoA* genes and transcripts were amplified using the following cycling conditions: 15 min at 95ºC, followed by 45 cycles of 1 min at 94ºC, 1 min at 55ºC, and 1 min at 72ºC. To exclude fluorescence contamination of potential primer-dimers, SYBR Green fluorescence was measured after 5 s at 80°C or after 8 s at 83°C, for AOA and AOB, respectively. Melting curves between 65°C and 95°C were analysed for each run. AOB *amoA* transcripts were below the detection limit (5 copies μL^{-1}). Efficiency of amplification and r^2 for DNA were, respectively, 0.92 and 0.998 for archaeal *amoA* and 104.6 and 0.993 for bacterial *amoA*.

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

Statistical analysis

All analyses were conducted using R version (3.2.2). The effect of aqueous soil extracts on pure AOA and AOB cultures was analysed by testing differences in specific growth rate between treatments by one-way analysis of variance. Differences between nitrification rate in soil slurries were evaluated using a repeated-measures linear mixed model (package *nlme*) (Pinheiro *et al.*, 2015). Each slurry was considered a subject with random effect to analyse the effect of treatment (Campo sujo soil, Craibstone soil, or soil mixture), time, and their interaction on inorganic N concentration and *amoA* gene (and transcript) abundance. The NO_3^- concentration in the Campo sujo slurries was below the limit of detection; therefore, these samples were excluded from the analysis. Gene abundance data were log-transformed to achieve a normal distribution. When the interaction between independent variables was not significant, it was removed to analyse the effect of time or treatment independently over concentration of soil NH_4^+ and NO_x. Two-way analysis of variance, with treatment and time as independent factors, was performed to evaluate differences in mineralisation and NO_3^- in the soil microcosms.

Results

Effects of soil extracts on ammonia oxidiser cultures

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

Effects of Campo sujo soil on nitrification in Craibstone soil

Soil slurries containing Campo sujo soil, Craibstone soil, or a mixture of the two soils (at ratios of 1:1 and 4:1) were incubated for up to 21 days. In all slurries, pH increased (0.4 in the Campo sujo soil, 0.8 in Craibstone soil, but only 0.1 and 0.2 for the 1:1 and 4:1 mixed samples, respectively) after the first day of incubation but did not change significantly thereafter. Net NH_4^+ concentration after 21 days ranged from 0.62 (±0.02) to 1.76 (±0.39) ppm (mg L^{-1} soil solution) for Craibstone soil and 0.87 (±0.02) to 2.20 (±0.02) ppm (mg L^{-1} soil solution) for Campo sujo soil (Fig. 2). Initial NH_4^+ concentration was higher in the mixed soil slurries than in controls, but the mixed slurries accumulated less NH_4^+ over the incubation period. The greatest increase in NH_4^+ concentration after 21 days was observed in Craibstone soil (2.9-fold).

NO_3^- concentration also increased in all soil slurries during incubation ($p<0.0001$, Fig. 2B), except in those containing Campo sujo only, in which NO_3^- was below the detection limit. In the mixed soil slurries, NO_3^- production was equivalent to or higher than the 50% and 20% expected for the 1:1 and 4:1 ratios of Campo sujo soil and Craibstone soil, respectively (Fig. 2C), providing no evidence for inhibition of Craibstone soil nitrification by Campo sujo soil.

Ammonia oxidiser *amoA* gene abundance in the soil slurries did not change significantly during the incubation period, even when significant NO_3^- accumulation was observed (Fig. 3). AOA *amoA* abundance in the Campo sujo-only slurries was approximately three orders of magnitude lower than that of Craibstone-only slurries (Fig. 3A). AOA *amoA* abundance in mixed soil slurries was lower than that of Craibstone-only slurries until day 14, after which differences were not significant ($p=0.132$). AOB *amoA* gene abundance in the Campo sujo-only slurries was also approximately three orders of magnitude lower than that of Craibstone-only slurries, and even significantly different ($p=0.024$) at day 21, when

Campo sujo-only AOB abundance was no longer significantly different from the those in the mixed samples (Fig. 3B).

The AOB *amoA* gene abundance was lower than AOA *amoA* gene abundance in all slurries at each time point. The AOA:AOB *amoA* gene ratio did not change significantly in the Campo sujo-only slurries but increased in the Craibstone-only and mixed soil slurries (Fig. 3C). In all slurries, AOB *amoA* transcripts were below the level of detection (5 μL^{-1}). The AOA *amoA* transcripts were detected in all slurries containing Craibstone soil throughout incubation but were detected in the Campo sujo-only slurries only at day 21 (Fig. 3D).

Before incubation, DGGE profiles of *amoA* genes amplified from Craibstone soil contained more bands (potential OTUs) than profiles of Campo sujo soil (Fig. S2) and did not change significantly after incubation for 21 days. DGGE profiles of the AOA *amoA* genes in the 1:1 mixed slurry were similar to those of Craibstone soil, possibly masking the less abundant *amoA* genes from the Campo sujo soil (Fig. S2).

Effects of soil pH and moisture content

The effects of pH and moisture content on nitrification in Campo sujo soil were investigated in soil microcosms. Net mineralisation was determined as the increase in concentration of inorganic N (NH_4^+-N + NO_3^- -N) during incubation, assuming that other nitrogen cycle processes were not significant (Fig. S3). Mineralisation in the dry soil did not increase after wetting, in contrast to the expected 'Birch' effect (Birch, 1964) (Fig. S3). In addition, soil pH did not change significantly with time in the microcosms and remained at 5.2 for the 'Water Pulse', 'Moist' and 'Dry' treatments and at 6.3 for the 'pH' treatment, in which pH was increased artificially with $CaCO_3$. Nitrate concentration did not increase significantly in any of the treatments (Fig. S3), and no significant difference was observed between treatments (p=0.14). No significant differences were observed between samples incubated with or

without acetylene, except for NO_3^--N concentrations in the 'Moist' microcosms. After incubation for 21 days, the NO_3^- -N concentration was lower in the acetylene-treated moist microcosms than in those without added acetylene (Fig. S3).

DiscussionNitrification is frequently undetectable in undisturbed Cerrado ecosystems, although management and conversion to agricultural production increases nitrate production (Catão *et al.*, 2016). Previous studies suggest low abundance of AOA and AOB in Campo sujo soil (Catão *et al.*, 2016), which is also characterised by sparse shrubs over a continuous grass layer. The aim of this work was to determine whether the lack of nitrification and low abundance of ammonia oxidisers in this ecosystem were due to low pH, low soil moisture, NH_4^+ limitation or biological inhibition of ammonia oxidation.

Certain plants release biological nitrification inhibitors that suppress ammonia oxidation in soils (Subbarao *et al.*, 2015). For example, compounds produced by *Brachiaria* (Subbarao *et al.*, 2009) and *Sorghum* (Zakir *et al.*, 2008) inhibited a recombinant *N. europaea* strain, possibly by blocking ammonia monooxygenase and hydroxylamine oxidoreductase (Subbarao *et al.*, 2008). Production of biological nitrification inhibitors can be promoted by exposure to high NH_4^+:NO_3^- ratios (Subbarao *et al.*, 2015), such as those found in Campo sujo soil (Catão *et al.*, 2016). However, aqueous extracts of Campo sujo soil did not inhibit growth of cultures of four AOB and one AOA, all of which were isolated from neutral to alkaline soils. Allylthiourea, used as a positive control, prevented growth of the AOB cultures but not the AOA culture, which is consistent with other studies reporting a greater tolerance of AOA to allylthiourea (Hatzenpichler & Lebedeva, 2008, Stempfhuber *et al.*, 2015). This result demonstrates the need to test potential inhibitors against both AOA and AOB, rather than *N. europaea* only.

Cultivation-based studies were performed using aqueous soil extracts and a small number of cultivated strains and potential inhibition was therefore assessed more directly by mixing Campo sujo soil with Craibstone soil, a strongly nitrifying soil with similar pH, in soil slurries (Nicol *et al.*, 2008, Zhang *et al.*, 2010). The soil slurries also provided no evidence of nitrification inhibitors in Campo sujo soil. Nitrate accumulation in mixtures of Craibstone soil and Campo sujo soil was lower than that of Craibstone soil only, but this difference was less than or equal to that predicted by the lower volume of Craibstone soil in the slurry, suggesting that nitrification was not inhibited by the Campo sujo soil. Similarly, the addition of Campo sujo soil to Craibstone soil had no apparent effect on AOA and AOB *amoA* gene abundances. Archaeal *amoA* genes were more abundant than those of bacteria in the soils, and bacterial *amoA* gene expression was not detected, as reported by previous studies of Craibstone soils (Zhang *et al.*, 2010). Neither AOA nor AOB *amoA* abundance changed significantly during incubation of any of the slurries containing Craibstone soil, despite evidence of nitrate production. However, the AOA:AOB *amoA* gene ratio increased, suggesting greater growth or lower death rates of AOA, but there was no evidence for growth of AOB or AOA in the Campo sujo soil. DGGE profiles of the Campo sujo soil contained fewer archaeal *amoA* bands than those of Craibstone soil, providing a further indication of low abundance and activity of ammonia oxidisers in Campo sujo soil. Although AOA *amoA* transcripts in Campo sujo-only slurries increased after incubation for 21 days, this increased gene expression did not appear to lead to a detectable level of nitrate. Nitrate reduction during denitrification was considered negligible due to previous experiments showing low NO emissions and undetectable N_2O in Cerrado soils (Pinto *et al.*, 2002).

In the absence of evidence for BNI, microcosm studies were performed to determine whether the low nitrification rates in Campo sujo soil were due to low pH or low soil moisture content. Gas measurements in Cerrado soil after rainfall (natural or simulated) led to

an increase in NO emissions (Pinto *et al.*, 2002), which may result from the Birch effect (Birch, 1964) of increased organic matter availability after rewetting (Fierer & Schimel, 2003). Soil pH is an important determinant of microbial diversity (Lauber *et al.*, 2009, Fierer *et al.*, 2012) and influences soil ammonia oxidiser abundance and activity (de Boer and Kowalchuk, Nicol et al. 2008), with higher transcriptional activity of Archaea than Bacteria as pH decreases (Nicol *et al.*, 2008). In our experiment, higher soil pH increased organic nitrogen mineralisation rate but did not lead to detectable nitrate production in Campo sujo soil after incubation for 28 days. Mineralisation was also lower in moist soil than in other treatments, and the increase in moisture did not lead to detectable nitrate production (see supplementary Fig. S3). Even though it can be argued that NO_3^- can be denitrified at higher soil moisture, low or undetectable NO and N_2O emission in field measurements over the seasons (Cruvinel *et al.*, 2011) and no consistent NO_3^- increase after one day or after 3 weeks of incubation, suggested limitation of nitrification by other factors.

The low nitrification and low ammonia oxidiser abundance of Campo sujo soil, in both microcosms and slurries, were not due to NH_4^+ limitation. The NH_4^+ concentration of Campo sujo soil slurries was even higher than that of Craibstone soil slurries at the beginning of the experiment. Jack pine forest soils showed similar high concentrations of ammonium without detectable nitrate (Ste-Marie & Paré, 1999). None of the treatments in this study increased nitrification in Campo sujo soil and this soil did not inhibit nitrification in Craibstone soil or pure cultures of AOA or AOB. Similarly, in the jack pine forest soil, nitrification was not stimulated by increased pH or ammonium amendment but was stimulated by the addition of nitrifying soil from a forest floor (Ste-Marie & Paré, 1999). In this study, both AOA and AOB were detected in Campo sujo soil, but at low levels that are unlikely to lead to detectable nitrate production. Consequently, these soils have much greater capacity to retain N as NH_4^+ through ion exchange, with minimal NO_3^- leaching.

Furthermore, NO pulses observed after rainfall are not due to nitrifier activity. However, experiments performed either for longer than 3 weeks or with rhizosphere soil might detect differences in nitrifier inhibition/stimulation and, despite the small effect of pH and H_2O on the Brazilian savanna soil nitrification rate, archaeal ammonia oxidisers started to show activity in slurries after 21 days of incubation. Taken together, our results show that low nitrification rates and ammonia oxidiser abundance in Campo sujo soil are not due to low moisture content, low pH or the presence of ammonia oxidiser inhibitors.

Although low nitrate concentrations are unlikely to be due to denitrification, nitrate assimilation and dissimilatory nitrate reduction may reduce nitrate produced by nitrifiers. However, the high ammonium concentrations, low AOA and AOB abundances, and lack of evidence for ammonia oxidiser activity and growth suggest inhibition or limitation of ammonia oxidiser growth and activity. This study was performed using soil associated with one type of vegetation sampled at the beginning of the dry season. Production of BNI may vary seasonally and with vegetation, but low nitrification rates are found in soils sampled throughout the year (Nardoto & Bustamante, 2003) and ammonium and nitrate concentrations are high and low (2.5 (±0.5) µg $N\text{-}NH_4^+$ g^{-1} dry soil and 0.072 (±0.01) µg $N\text{-}NO_3^-$ g^{-1} dry soil), respectively, regardless of vegetation type (Catão *et al.*, in preparation). Inhibition of ammonia oxidiser cultures was performed with water extracts from soil and it is possible that ammonia oxidisers were inhibited by water insoluble BNI. Other features of Cerrado soils may also be important. There is evidence for co-limitation of microbial decomposition in these soils by N and P (Kozovits et al., 2007, Jacobson et al., 2011) and ammonia oxidisers may have been limited by P and other nutrients, although mixing with Craibstone soil would be expected to relieve this limitation. Cerrado soils are deficient in P (Goedert, 1983), and the values observed for the Campo sujo are similar to the upper layer of other Cerrado soils

(Parron et al., 2011), where soil P sorption capacity is often related to Fe and Al contents (Goedert, 1983). The soils investigated in this study also contain relative high Fe concentrations (165.4 ± 41.01 mg dm^{-3}), which have been associated with reduction in net nitrification and AOA and AOB *amoA* gene abundance in subtropical acid soils (Jiang et al., 2015). Although these possibilities suggest future experimental studies, the reasons for low nitrification rates and low ammonia oxidiser abundances in Cerrado soil remain unclear and this study provided no evidence for ammonia limitation, pH or inhibition by water extractable inhibitors.

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Conflict of Interest

The authors have no conflict of interest to declare.

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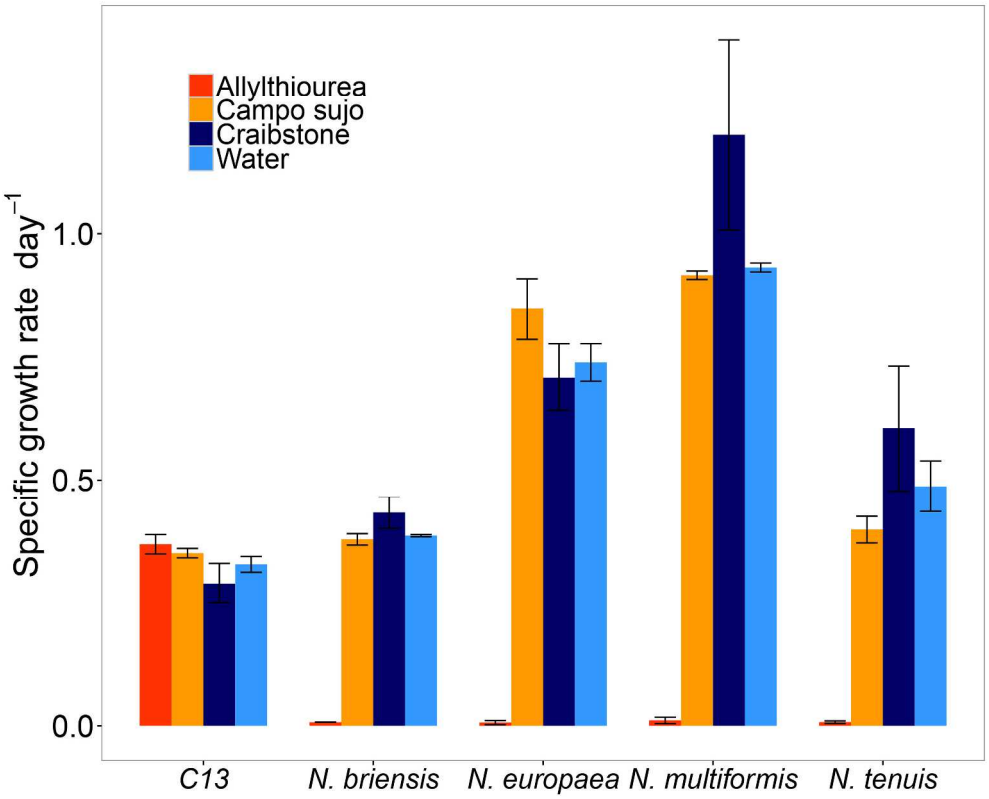

Figure 1. Effect of aqueous extracts of Campo sujo and Craibstone soils on the growth of ammonia-oxidising strains.

254x203mm (300 x 300 DPI)

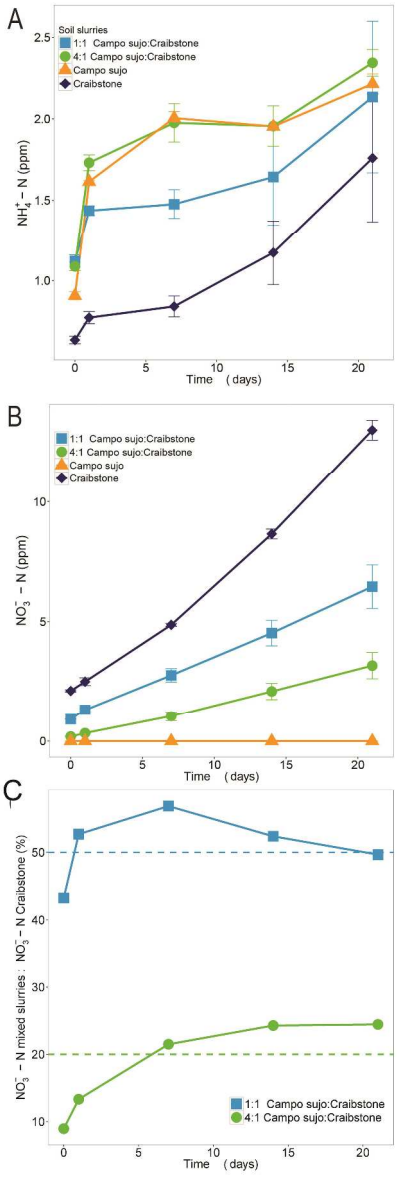

Figure 2. Changes in inorganic N concentration during incubation of in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils.

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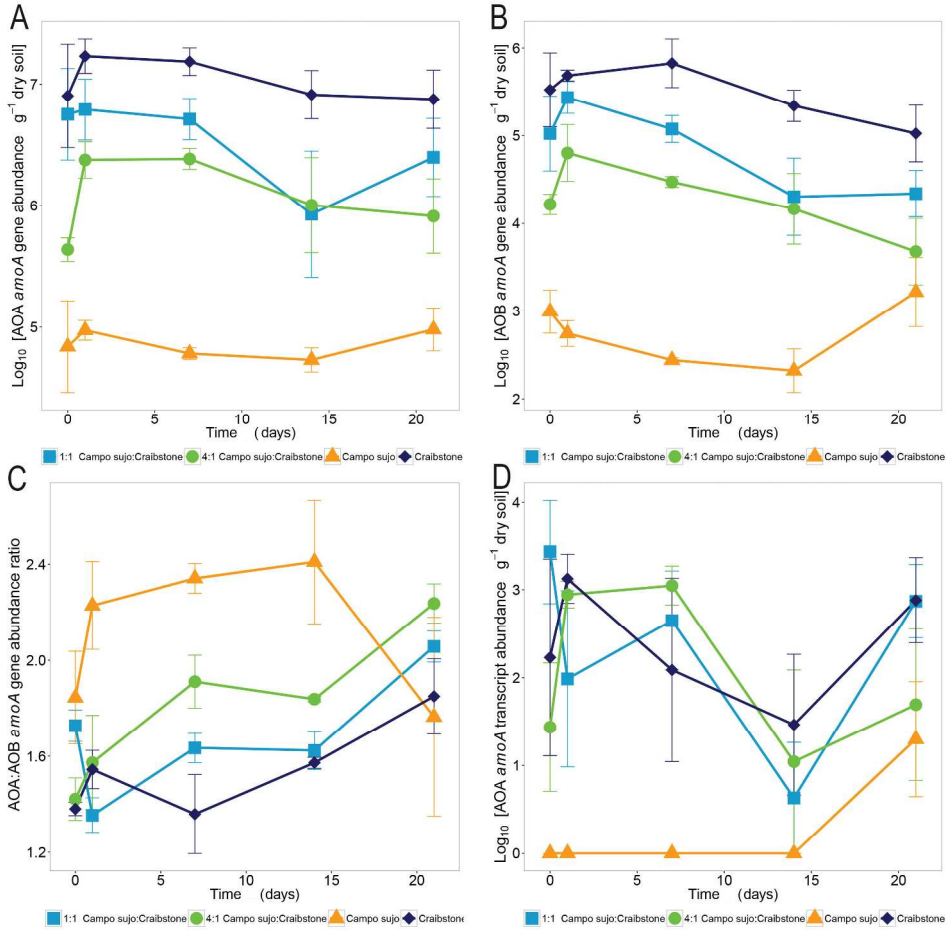

Figure 3. Changes in *amoA* gene abundance in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils.

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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 (*Nitrosospira briensis*, *Nitrosomonas europaea*, *Nitrosospira multiformis*, *Nitrosospira tenuis*) in liquid batch cultures containing aqueous extracts of Campo sujo soil or Craibstone soil, water (negative control), or 100 µM allylthiourea (positive control). Error bars represent standard deviation of the mean from triplicate cultures.

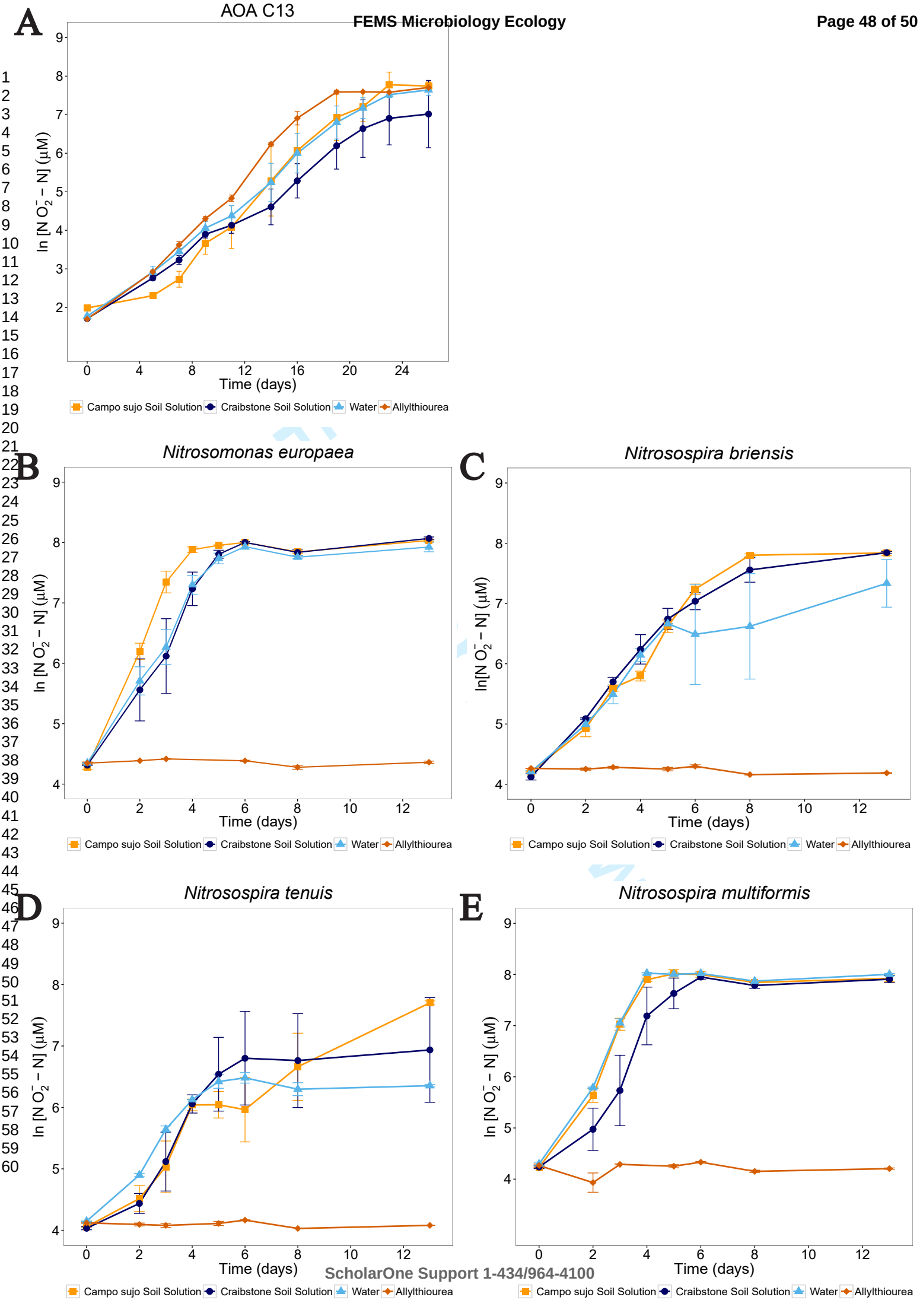
Figure 2. Changes in inorganic N concentration during incubation of ~~in~~ slurries of Craibstone soil, Campo sujo soil and mixtures of these soils. (A) NH_4^+-N concentration and (B) NO_3^- -N were determined in all soil slurries. (C) NO_3^- concentration in the mixed slurries was expressed as the percentage of 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.

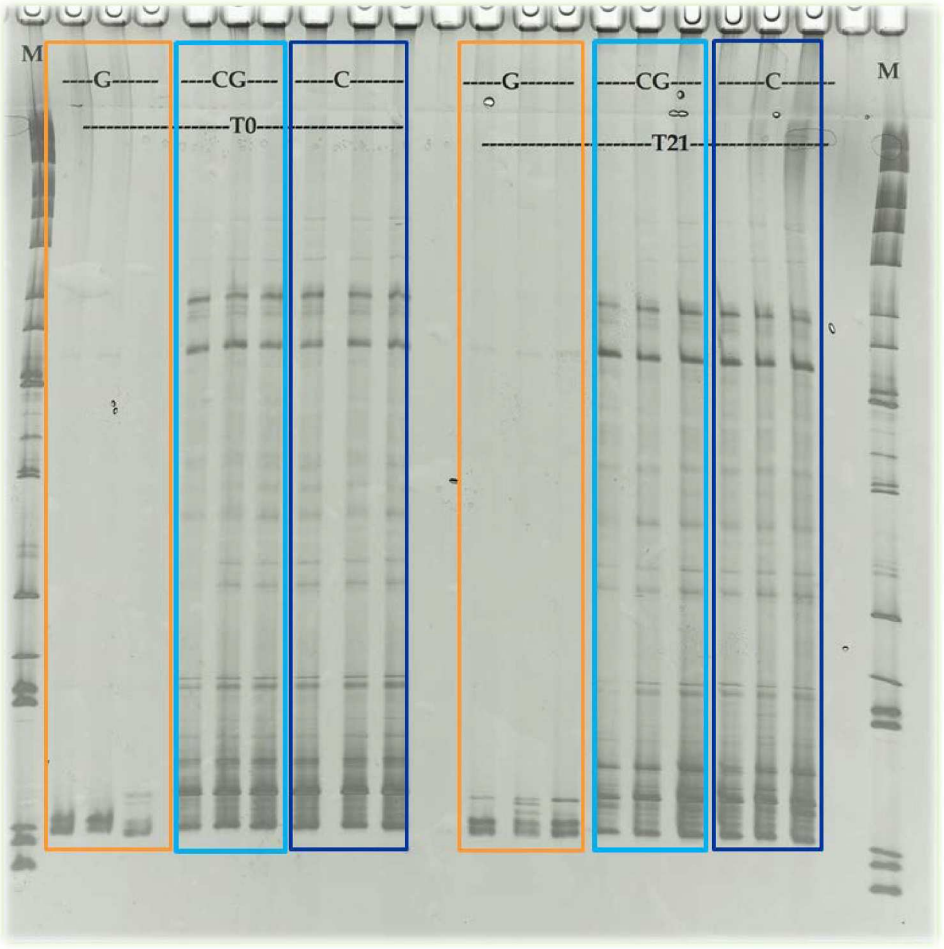
Figure 3. Changes in *amoA* gene abundance in slurries of Craibstone soil, Campo sujo soil and mixtures of these soils. (A) Ammonia-oxidising archaea (AOA) *amoA*, (B) ammonia-oxidising bacteria (AOB) *amoA*, (C) AOA:AOB *amoA* ratio and (D) AOA *amoA* transcripts were quantified during incubation of 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.

Figure S1. Effects of Campo sujo and Craibstone soils on the growth of ammonia-oxidising archaea and bacteria. Semi-logarithmic plots of nitrite concentration vs. time during liquid batch culture of (A) *Candidatus* Nitrosocosmicus franklandia (AOA C13), (B) *Nitrosomonas europaea*, (C) *Nitrosospira briensis*, (D) *Nitrosospira tenuis* and (E) *Nitrosospira multiformis* after adding aqueous extracts of Campo sujo soil or Craibstone soil, water (negative control), or 100 µM allylthiourea (positive control). Error bars represent standard deviation of the means from triplicate cultures.

Figure S2. Denaturing gradient gel electrophoresis analysis of archaeal ammonia oxidiser *amoA* genes in soil slurries. PCR-amplified archaeal *amoA* gene products from triplicate soil slurries of (G) Campo sujo soil only, (CG) Campo sujo:Craibstone mixed soil (1:1 ratio) and (C) Craibstone soil only were sampled before incubation (T0) and after incubation for 21 days (T21).

Figure S3. Effects of pH and moisture content on nitrification in Campo sujo soil. Changes in (A) (NH_4^+-N + NO_3^--N) and (B) NO_3^--N in microcosms containing Campo sujo soil after manipulation of pH and moisture content. Open symbols represent treatments with 0.01% acetylene in the headspace. Dry: air-dried soil at 8.66 g H_20 100 g^{-1} dry soil; Water: rewetted soil at 39.6 ± 1.92 g H_20 100 g^{-1} dry soil; Moist: moist soil; pH: soil treated with $CaCO_3$ (pH 6.3; pH of other treatments was 5.2).





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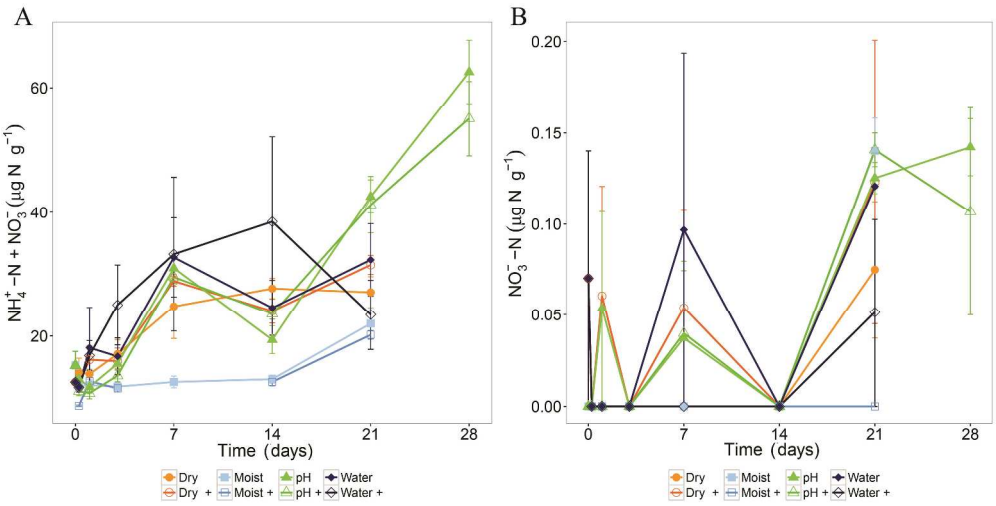

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