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Short communication

Effect of nitrification inhibitors on the growth and activity of *Nitrosotalea devanaterra* in culture and soil

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ABSTRACT

The soil archaeon *Nitrosotalea devanaterra* is a representative of an abundant ammonia oxidising archaeal lineage. The effects of three nitrification inhibitors (allylthiourea (ATU), dicyandiamide (DCD) and nitrapyrin) on growth in both soil and liquid culture were compared. DCD and nitrapyrin inhibited nitrification at similar concentrations to bacterial ammonia oxidisers. Although DCD completely inhibited nitrification, some growth occurred in liquid cultures. Surprisingly, *Nitrosotalea devanaterra* was less susceptible to inhibition by ATU, which stimulated, rather than inhibited, nitrification in soil microcosms. Inhibition thresholds and responses may reflect differences in ammonia oxidation mechanisms between archaea and bacteria.

Nitrification is an essential part of the global nitrogen cycle and accounts for considerable loss of nitrogen from agricultural ecosystems through leaching of nitrate and as gaseous forms of nitrogen, estimated as approximately 70% of annual 100 Tg N fertiliser input (Raun and Johnson, 1999). The nitrogen-use efficiency of soils has vital economic and ecological importance and application of nitrification inhibitors is used to mitigate nitrogen loss from the ecosystem (Dinnes et al., 2002; Subbarao et al., 2006). Dicyandiamide (DCD) and nitrapyrin (2-chloro-6-(trichloromethyl) pyridine) are the most frequently used commercial nitrification inhibitors in agriculture (Subbarao et al., 2006) and allylthiourea (ATU) has been widely employed in nitrification research (Bedard and Knowles, 1989). All three inhibitors exert their effects indirectly; while nitrapyrin and ATU are believed to act by chelating copper, the mode of action of DCD is poorly characterised (Subbarao et al., 2006). DCD has been previously suggested to either prevent ammonia uptake or utilisation, or act as a copper chelator (Zacherl and Amberger, 1990; Subbarao et al., 2006). Ammonia oxidation is the first and rate-limiting step of nitrification, and is largely carried out by two distinct groups of autotrophic microorganisms in soil, ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA). Although ammonia oxidising archaea are numerically, and in some soils functionally dominant over their bacterial counterparts (Leininger et al., 2006; Nicol et al., 2008; Gubry-Rangin et al., 2010; Stopnišek et al., 2010), investigation of nitrification inhibitors has focused almost exclusively on ammonia oxidising bacteria. A recently cultivated acidophilic ammonia oxidising archaeon, *Nitrosotalea devanaterra*, belongs to an abundant lineage found in many terrestrial ammonia oxidising communities (Gubry-Rangin et al., 2011; Lehtovirta-Morley et al., 2011) and could provide a useful model for comparing growth responses in laboratory culture and native soil environments.

The effects of three nitrification inhibitors, DCD, ATU and nitrapyrin, were investigated by monitoring growth (*amo*A gene abundance) and activity (process rates) of *N. devanaterra* in liquid batch culture and in soil microcosms. Culture experiments were performed under conditions described previously (Lehtovirta-Morley et al., 2011) and nitrite and ammonia concentrations were determined colorimetrically (Shinn, 1941; Kandeler and Gerber, 1988; Allen, 1989). Nitrapyrin was used at the following concentrations: 1, 10 and 50 μM in 0.1% dimethylsulfoxide (DMSO) (due to its low solubility in water), DCD at 1, 5 and 10 mM and ATU at 10, 50 and 100 μM in dH_2O. Triplicate soil microcosms were assembled in 120-ml serum vial bottles for destructive sampling and contained 8 g (30% w/w (wet weight)) sieved sandy loam soil (pH 4.5) from the Scottish Agricultural College on Craibstone Estate, NE Scotland. Microcosms were incubated at 30 °C degrees in the dark and aerated at regular intervals. Concentrations of inhibitors were

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adjusted based on the threshold inhibitory concentrations observed in liquid culture; nitrapyrin to 10 and 50 μmol kg^{-1} soil in 0.05% (v/w) DMSO, dicyandiamide to 5 and 10 mmol kg^{-1} soil and allylthiourea to 100, 500 and 1000 μmol kg^{-1} soil. Soil ammonia, nitrite and nitrate concentrations were determined as described previously (Nicol et al., 2008). DNA extractions and qPCR targeting *amo*A genes specific to the archaeal lineage containing *N. devanaterra* were performed as described previously (Tourna et al., 2010; Lehtovirta-Morley et al., 2011). DGGE analysis was carried out as previously described (Nicol et al., 2008). Statistical analyses using one-way ANOVA and the Student's *t*-test were performed in SigmaPlot version 11 (Systat Software).

Ammonia oxidation by *N. devanaterra* was completely inhibited (defined as lack of significant growth after introduction of the inhibitor) in liquid culture in the presence of 5 mM and 10 mM DCD (*t*-test, $p = 0.051$ and $p = 0.51$), while 1 mM DCD led to 33% (±1.7%, standard error) inhibition based on the final nitrite yield (*t*-test, $p < 0.001$) (Fig. 1a). ATU led to partial inhibition, giving significant inhibition of 85% (±0.7%), 63% (±4.3%) and 29% (±0.4%) compared to control cultures in the presence of 100 μM, 50 μM and 10 μM ATU, respectively (ANOVA, $p < 0.001$) (Fig. 1b). Nitrapyrin resulted in almost complete inhibition of ammonia oxidation: 92% (±1.8%) and 100% inhibition at concentrations of 10 and 50 μM, respectively, and 15% (±5.6%) inhibition at 1 μM (Fig. 1c). In general, inhibition of growth (assessed as a lack of increase in *amo*A gene abundance) was similar to inhibition of nitrite production. In contrast to nitrapyrin, which inhibited both ammonia oxidation and growth (Fig. 1f), growth occurred in all treatments after addition of ATU and DCD, even after nitrite production ceased. After addition of 5 mM and 10 mM DCD, *amo*A gene abundance increased ~3-fold despite the lack of nitrite accumulation (*t*-test, $p < 0.001$ and $p < 0.001$, respectively) (Fig. 1d), although these growth yields were lower than in the control with no inhibitor (ANOVA, $p = 0.033$). In the presence of 50 and 100 μM ATU, archaeal yield, measured by the increase in *amo*A gene abundance, was 34% (±5.3%) (*t*-test, $p < 0.001$) and 32% (±4.4%) (*t*-test, $p < 0.001$) of the control yield after 21 days, respectively (equivalent to ~3.5-fold increase) (Fig. 1e), although nitrite concentration increased slightly up to day 14, indicating possible growth.

To determine whether inhibition of *N. devanaterra* in liquid culture reflected inhibition in the environment, effects of inhibitors were investigated in microcosms containing soil in which

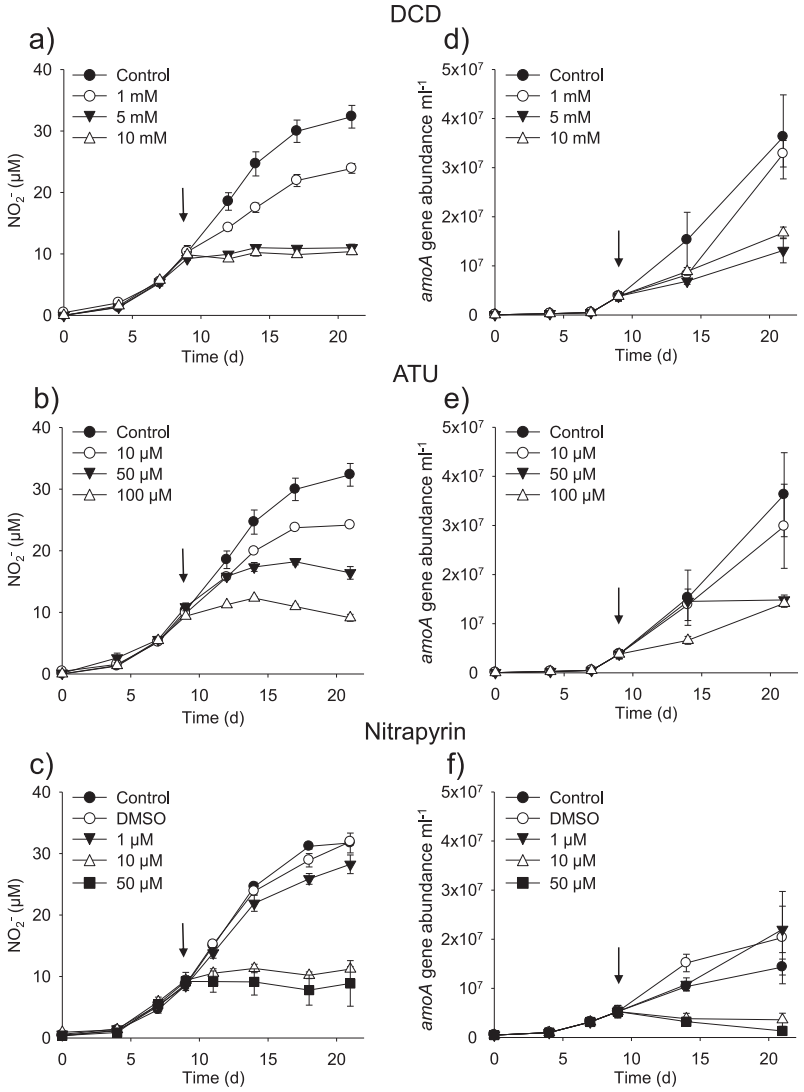

Fig. 1. The effect of nitrification inhibitors on nitrite accumulation (a, b, c) and growth assessed by qPCR of the *amo*A gene (d, e, f) of *N. devanaterra* in liquid batch culture at pH 4.5. (a, d) DCD; (b, e) ATU; (c, f) nitrapyrin. Error bars represent standard errors of biological triplicates. Arrows indicate the time at which inhibitor was added.

nitrification is driven by thaumarchaea, rather than bacteria (Gubry-Rangin et al., 2010), and where *N. devanaterra* is a dominant member of the archaeal community (Gubry-Rangin et al., 2011). Inhibition of ammonia oxidation by DCD in soil microcosms closely resembled that in liquid culture. Inhibition was complete at 5 μmol g^{-1} soil, with 25% (±2.6%) inhibition at 1 μmol g^{-1} DCD (Fig. 2a). In contrast, ATU was less inhibitory in soil microcosms, with no detectable inhibition at 100 nmol g^{-1} soil, and significant stimulation of nitrification at higher concentrations (500 and 1000 nmol g^{-1} soil, 24% (±1.7%) and 26% (±2.6%) increases, respectively) (Fig. 2b, ANOVA, $p < 0.001$). *amo*A gene abundance mirrored the trends observed for nitrification in both DCD and ATU treatments (Fig. 2d,e). However, there was a decrease in *amo*A gene abundance associated with 5 μmol g^{-1} soil DCD (Fig. 2d) as opposed to an increase observed in liquid culture, which could reflect greater sensitivity to this inhibitor in soil. Although DMSO had an adverse effect on nitrification in the control soil microcosms, inhibition in the presence of nitrapyrin was significantly greater than in the DMSO control (Fig. 2c, ln-transformed data, ANOVA, $p = 0.003$). *amo*A gene abundance in nitrapyrin-treated microcosms was lower than in the control with DMSO, thus following the trends of nitrification (Fig. 2f). While nitrification proceeded at a low rate in the control microcosms containing DMSO, *amo*A gene abundance declined, which may indicate activity by other ammonia oxidisers.

The qPCR data were consistent with DGGE data, which indicated that a band representing *N. devanaterra*-like phylotypes increased in relative intensity in the absence of inhibitors (Fig. 3a,b) and decreased in the presence of DMSO (and nitrapyrin) and DCD at 5 μmol g^{-1} soil (Fig. 3a,c). In addition, three archaeal phylotypes other than *N. devanaterra* were affected by the nitrification inhibitors in soil microcosms and were inhibited by DMSO, nitrapyrin and DCD.

Differential inhibition of bacterial and archaeal ammonia oxidisers by ATU has been suggested previously; while AOB are inhibited at 8–80 μM (Ginestet et al., 1998), AOA appear less sensitive in both liquid culture and the soil environment (Hatzenpichler et al., 2008; Taylor et al., 2010; Jung et al., 2011). In contrast with previous studies, ATU resulted in significant inhibition of *N. devanaterra* in liquid culture. However, ATU at equivalent concentrations did not inhibit thaumarchaeal growth in soil microcosms, as also reported by Taylor et al. (2010), possibly due to

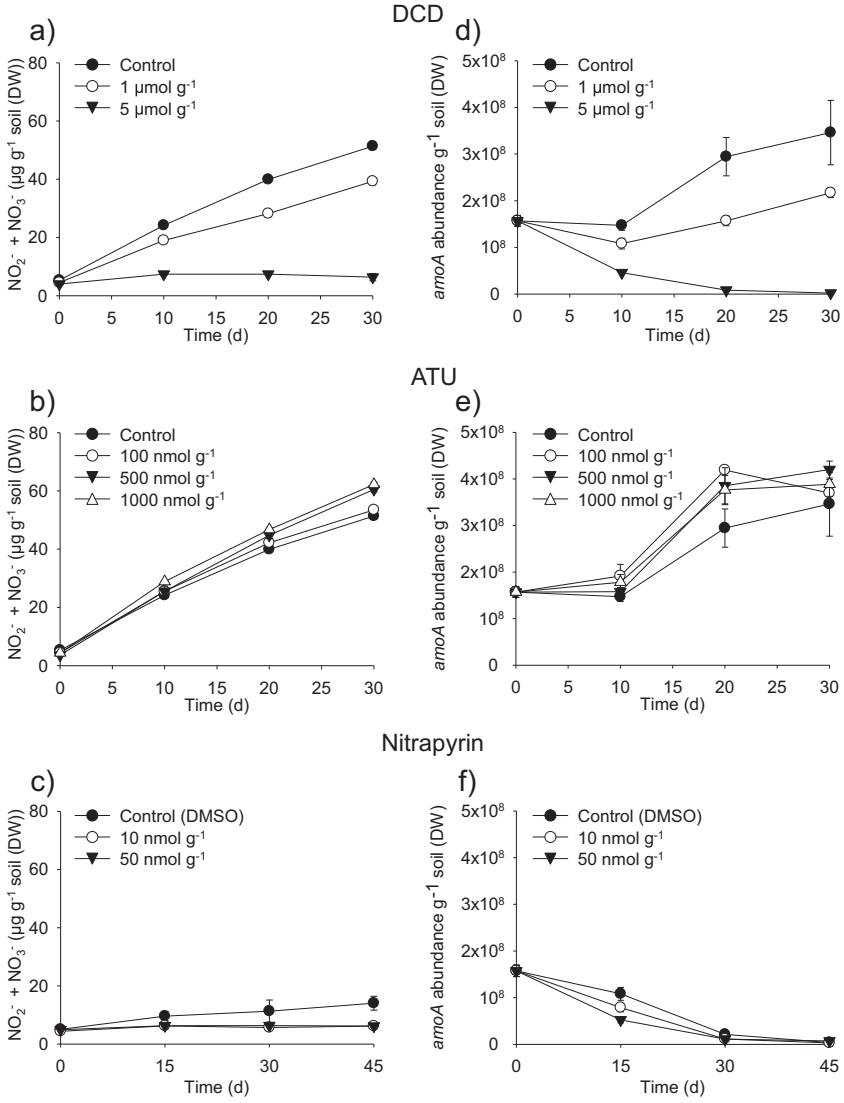

Fig. 2. The effect of nitrification inhibitors and DMSO on $NO_2^- + NO_3^-$ accumulation (a, b, c) and growth (d, e, f) by *N. devanaterra*-associated phylotypes in soil microcosms (pH 4.5). (a, d) DCD; (b, e) ATU; (c, f) nitrapyrin. Error bars represent standard errors of biological triplicates.

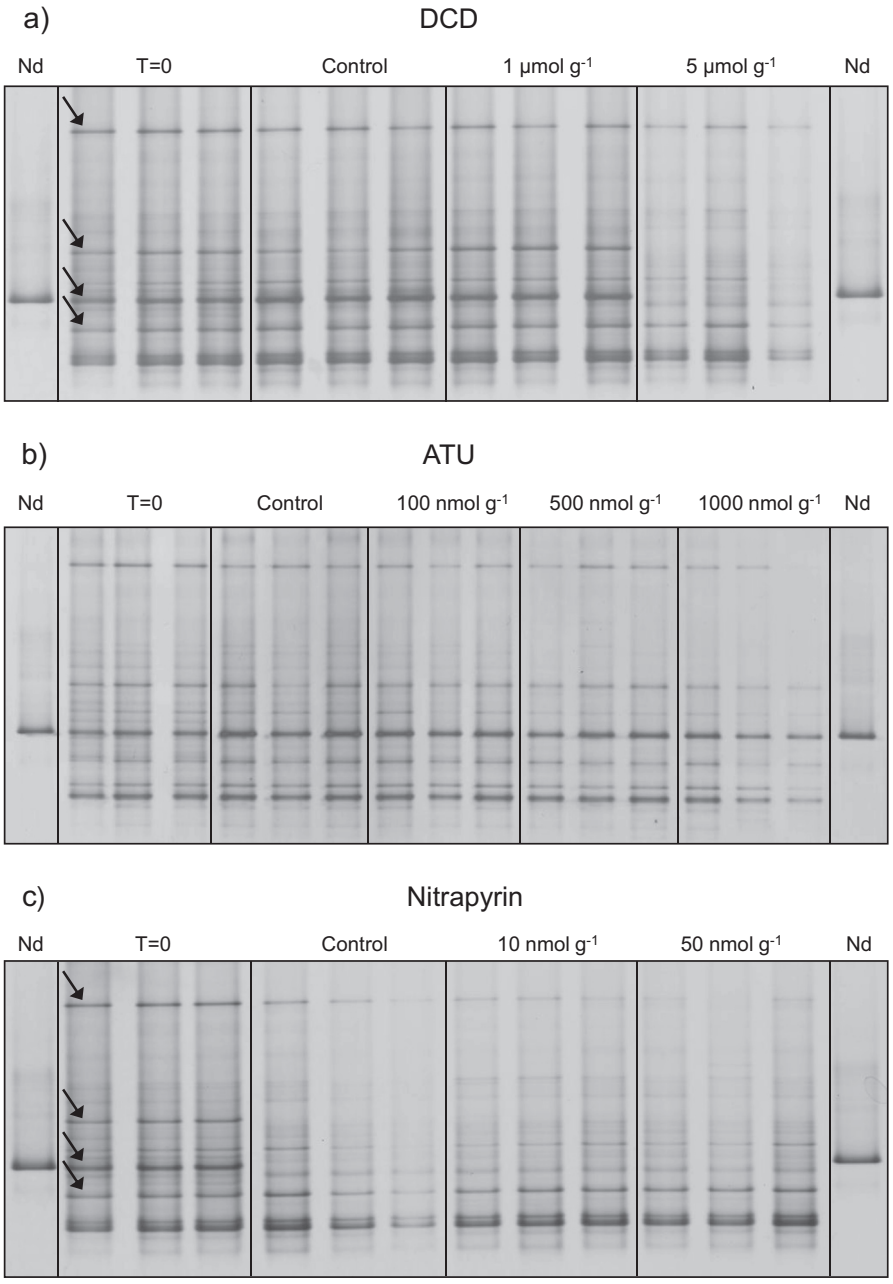

Fig. 3. Effect of nitrification inhibitors on archaeal ammonia oxidiser community structure examined by denaturing gradient gel electrophoresis (DGGE) of amplified archaeal *amo*A genes. (a) DCD, (b) ATU, (c) nitrapyrin. Nd = *Nitrosotalea devanaterra amo*A gene amplicon. Arrows indicate bands representing phylotypes affected by inhibitors with a reproducible change in relative band intensity in some treatments compared to the start of the experiment.

spatial distribution and protection by soil structure, lower diffusion of the inhibitor through the soil matrix or degradation. Stimulation of soil nitrification by high concentrations of ATU may result from degradation and subsequent release of ammonia. Although both nitrapyrin and ATU are considered Cu-chelators, their effects on *N. devanaterra* were contrasting. AOA are believed to depend heavily on copper because, unlike AOB, they lack cytochromes and have an alternative electron transfer mechanism consisting of copper-containing proteins (Walker et al., 2010). The unexpected observation that DCD and ATU inhibited nitrite accumulation, while *amo*A gene abundance increased slightly in liquid culture experiments, may indicate an alternative energy source to ammonia oxidation, or ability to store energy before addition of the inhibitor (Kadouri et al., 2005; Ingalls et al., 2006; Tourna et al., 2011).

Slightly different inhibition thresholds of archaeal ammonia oxidisers may affect agricultural practise and archaea can contribute to nitrogen fertiliser loss even in conditions where their bacterial counterparts would be inhibited. In particular, the effect of ATU can be compromised. In contrast, the effect of DCD could be reduced as it failed to stop archaeal growth in liquid culture, potentially allowing nitrification to proceed rapidly if inhibition is removed, e.g. through degradation, making repeated application necessary. When inhibition thresholds between further thaumarchaeal strains can be assessed, it will be interesting to determine whether the effects of nitrification inhibitors are consistent against different soil archaeal communities.

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