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Short communication

Activity of the ammonia oxidising bacteria is responsible for zinc tolerance development of the ammonia oxidising community in soil: A stable isotope probing study

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ABSTRACT

Both bacterial and archaeal ammonia oxidizers (AOB and AOA) are abundant in most soils, but their relative contributions to soil nitrification under different conditions are still debated. This study investigates the influence of long-term zinc (Zn) stress on the ammonia oxidizer community and determines the role of AOB and AOA in Zn tolerant nitrification. This was assessed by artificially spiking a grassland soil with 1300 and 2400 mg Zn kg^{-1} (final concentration). Both treatments increased Zn tolerance after incubation for 12 months and increased the AOB/AOA *amoA* gene abundance ratio from 0.1 to 30. Soil samples were subsequently subjected to stable isotope probing (SIP) of DNA by incubation under $^{13}CO_2$ atmosphere. $^{13}CO_2$ was assimilated by AOB in the uncontaminated soil. In contrast, this assimilation was not observed for the AOA. Similarly, AOB and not AOA assimilated $^{13}CO_2$ after Zn exposure for 12 months. These results show that recovery of nitrification and development of Zn tolerance after long-term Zn exposure in this soil is due to the initial activity of AOB, rather than AOA.

Ammonia oxidation, the first and rate-limiting step in nitrification, was thought to be performed by a relatively small group of ammonia oxidising bacteria (AOB) (Stephen et al., 1996). However, a number of studies have demonstrated that archaeal *amoA* genes are widely distributed in the environment and are sometimes more dominant than bacterial *amoA* genes (e.g. Francis et al., 2005; Leininger et al., 2006). However, the actual role of ammonia oxidising archaea (AOA) in soil nitrification is still debated (Prosser and Nicol, 2008) and a number of factors have been suggested to influence the distribution and activities of AOA and AOB in soil, in particular the pH, the concentration of soil ammonium and the soil organic matter content (organic versus mineral ammonium fertilizer) (Prosser and Nicol, 2012).

We previously showed that the AOB/AOA gene abundance ratio increased with increasing zinc (Zn) stress and Zn tolerance. Mertens et al. (2009) showed that the AOB/AOA transcript number ratio also increased in a different Zn contaminated soil compared to the uncontaminated control soil. Therefore, we have speculated that Zn tolerance development after long term Zn exposure was due to the activity of the AOB community rather than the AOA community. This hypothesis is tested in this study using stable isotope probing of DNA (DNA-SIP). In contrast to abundance analysis, SIP is not a quantitative technique, but it tracks active AOB and AOA populations that actually consumed CO_2. Incubation of soil in the presence of $^{13}CO_2$ followed by molecular analysis of ^{12}C- and ^{13}C- labelled DNA allows to assess active and autotrophic ammonia oxidisers (Radajewski et al., 2000). This technique has been used previously to assess activity of soil ammonia oxidizers (e.g. Jia and Conrad, 2009; Zhang et al., 2010; Xia et al., 2011). Combined with qPCR quantification of AOB and AOA *amo*A gene abundance it is possible to detect whether AOB and/or AOA actively consumed CO_2 (Zhang et al., 2010).

We have previously induced development of Zn tolerance of the ammonia oxidising community by long term, outdoor incubation (1 year) of an artificially Zn-spiked soil (Ruyters et al., 2010). From this previous experiment the uncontaminated soil and two artificially Zn contaminated soils in which the ammonia oxidising community had developed Zn tolerance (1300 mg Zn kg^{-1} and 2400 mg Zn kg^{-1}) were selected for SIP analysis. These soils were stored air-dried at 4 °C for 2 years and therefore activity and

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community analyses were performed again (Supplementary Information Table 1 and Supplementary Information Fig. 1). Storage had no major effects on the ammonia oxidising community if you compare these results with those in Ruyters et al. (2010) (details not shown). SIP was performed as described by Zhang et al. (2010). Briefly, NH_4^+–N-amended (200 mg N kg^{-1}), pre-incubated soils were incubated under a $^{12}CO_2$ or $^{13}CO_2$ atmosphere (each in triplicate) for 28 days at 30 °C and soil was sampled after incubation for 0 and 28 days and DNA was extracted (Griffiths et al., 2000). One μg DNA was added at the top of a tube containing a CsCl solution with a buoyant density of 1.3999. Isopycnic centrifugation (186,000 *g*, 24 h) created a buoyant density gradient in the tube. The solution was then fractionated into different fractions of 240 μl and as such ^{13}C-labelled DNA (heavier, bottom part of the tube) was separated from ^{12}C-labelled DNA. After fractionation of the CsCl gradient, the abundance of *amoA* genes was measured in each fraction by qPCR (Zhang et al., 2010), using primers amoA-1F and amoA-2R to target AOB (Rotthauwe et al., 1997) and primers Crenamo23F and Crenamo616R to target AOA (Tourna et al., 2008). Relative abundances are plotted versus the buoyant density of the CsCl solution which was measured in each fraction with a digital refractometer (Fig. 1). Analysis of the relative abundances in the $^{12}CO_2$ microcosms showed that the peak of unlabelled AOA *amo*A gene abundance occured at lower buoyant densities compared to the peak of unlabelled AOB *amo*A gene abundance (Fig. 1), which might result from the lower GC content in genomic archaeal DNA.

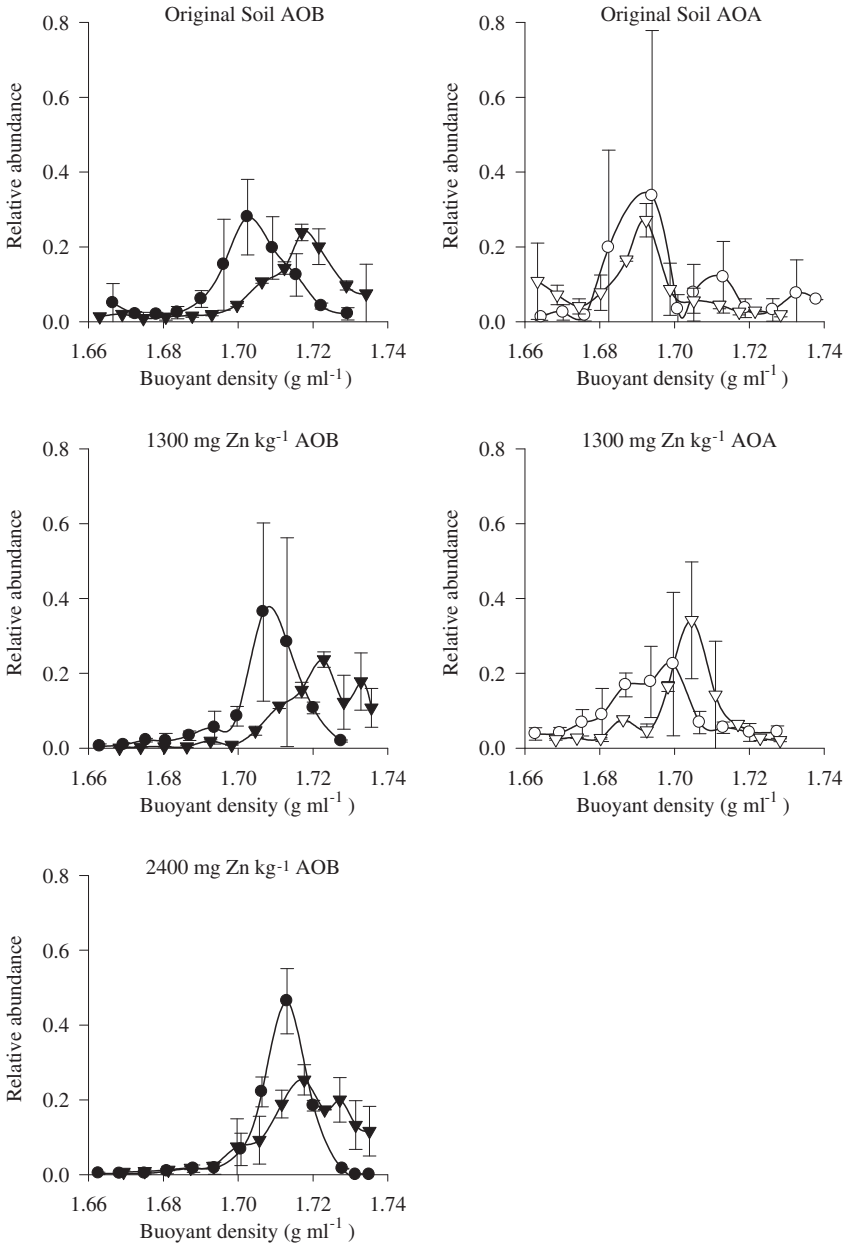

Fig. 1. Distribution of AOB (left panels) and AOA (right panels) *amoA* gene abundance in a CsCl density gradient of DNA extracted from an uncontaminated and two Zn contaminated soils (1300 mg Zn kg^{-1}; 2400 mg Zn kg^{-1}) incubated under $^{12}CO_2$ (circles) and $^{13}CO_2$ (triangles) atmosphere. Standard deviations are given as error bars ($n = 3$). Black filled symbols represent relative AOB *amoA* gene abundances, white symbols represent relative AOA *amoA* gene abundances. A shift towards heavier fractions (^{13}C labelled DNA) was observed for AOB *amoA* gene abundance in the $^{13}CO_2$ incubated microcosms compared to the $^{12}CO_2$ microcosms in all three soils, but was not observed for the AOA *amoA* gene abundance. Archaeal *amoA* genes were below the detection limit for qPCR in CsCl fractions from the soil containing 2400 mg Zn kg^{-1} due to low AOA *amoA* gene abundance in the original DNA extract of that soil (Supplementary Information Table 1) and therefore a graph was not obtained.