

ORIGINAL ARTICLE

Bacteria, not archaea, restore nitrification in a zinc-contaminated soil

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Biological ammonia oxidation had long been thought to be mediated solely by discrete clades of β - and γ -proteobacteria (ammonia-oxidizing bacteria; AOB). However, ammonia-oxidizing Crenarchaeota (ammonia-oxidizing archaea; AOA) have recently been identified and proposed to be the dominant agents of ammonia oxidation in soils. Nevertheless, the dynamics of AOB versus AOA, and their relative contribution to soil ammonia oxidation and ecosystem functioning on stress and environmental perturbation, remain unknown. Using a 3-year longitudinal field study and the *amoA* gene as a molecular marker, we demonstrate that AOB, but not AOA, mediate recovery of nitrification after zinc (Zn) contamination. Pristine soils showed approximately equal *amoA* gene copy numbers and transcript levels for AOB and AOA. At an intermediate Zn dose (33.7 mmol Zn per kg), ammonia oxidation was completely inhibited, and the numbers of AOB and AOA *amoA* gene copies and gene transcripts were reduced. After 2 years, ammonia oxidation in the field soils was fully restored to preexposure levels, and this restoration of function was concomitant with an increase of AOB *amoA* gene copy and gene transcript numbers. Analysis of the restored community revealed domination by a phylogenetically distinct Zn-tolerant *Nitrosospira* sp. community. In contrast, the numbers of AOA *amoA* gene copies and gene transcripts remained 3- and 10⁴-fold lower than recovered AOB values, respectively. Thus, although recent findings have emphasized a dominant role of archaea in soil-borne ammonia oxidation, we demonstrate that a phylogenetic shift within the AOB community drives recovery of nitrification from Zn contamination in this soil.

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Introduction

Nitrification is an important process in the global nitrogen (N) cycle, oxidizing ammonia to nitrate and providing substrate for reductive nitrogen processes (denitrification). The rate-limiting step in nitrification is the oxidation of ammonia to nitrite, with nitrite typically being rapidly oxidized to nitrate by nitrite-oxidizing organisms. Until recently, ammonia oxidation has been considered to be entirely mediated through activities of ammonia-oxidizing bacteria (AOB; Kowalchuk and Stephen, 2001). Cultivation-dependent (Head *et al.*, 1993), and subsequently cultivation-independent studies

(Stephen *et al.*, 1996, 1998), had suggested that members of a distinct clade of β -proteobacteria were the primary biological agents of ammonia oxidation in soil. The narrow phylogenetic breadth of this group facilitated the design of primers to monitor AOB using both phylogenetic (rRNA) and functional gene (*amoA* encoding the α -subunit of the key enzyme ammonia monooxygenase) markers (Rotthauwe *et al.*, 1997; Kowalchuk *et al.*, 2000; Kowalchuk and Stephen, 2001). Using these nucleic acid-based tools, there have been extensive environmental and ecotoxicological studies exploring the distribution, diversity and response to environmental stresses of AOB in relationship with soil nitrification (for example, Koops *et al.*, 2001; Kowalchuk and Stephen, 2001; Aoi *et al.*, 2004).

Recently, Venter *et al.* (2004) and Treusch *et al.* (2005) suggested the involvement of archaea in the global nitrification process. This genetic potential for archaeal ammonia oxidation was soon supported by the cultivation of an ammonia-oxidizing archaea isolate (AOA; Francis *et al.*, 2005; Könneke *et al.*,

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2005), and it became evident that the pool of *amoA* genes across a range of soils appeared archaea-dominated (1.5–230 times >AOB; Leininger *et al.*, 2006). Analysis of *amoA* mRNA copy levels also suggested that Crenarchaeota are the most active ammonia-oxidizing organisms in several soils (Leininger *et al.*, 2006). These recent findings have resulted in a fundamental shift in our understanding of the biology underpinning nitrification in environmental samples (Francis *et al.*, 2007). In particular, the current ethos is for an archaeal driven process, questioning the wider role of bacteria in this key N-cycle process in soil (Leininger *et al.*, 2006; Nicol and Schleper, 2006; He *et al.*, 2007).

The biological oxidation of ammonia can be influenced by numerous factors and is recognized as being a model process for ecological studies (Kowalchuk and Stephen, 2001). Although numerous studies have explored the links between environmental factors, AOB and nitrification, the response of AOA to environmental perturbations remains unknown (Nicol and Schleper, 2006; Francis *et al.*, 2007; Boyle-Yarwood *et al.*, 2008; Chen *et al.*, 2008; Shen *et al.*, 2008).

Soil nitrification is highly sensitive to elevated zinc (Zn; Smolders *et al.*, 2004), although ecotoxicological effects can be transient (Rusk *et al.*, 2004). It has been hypothesized that recovery of nitrification following Zn exposure is due to the development of Zn-tolerant AOB populations (Mertens *et al.*, 2006). However, the recent insights into the role of AOA in soil ammonia oxidation (Leininger *et al.*, 2006) place the role of AOB in nitrification restoration in doubt.

Here, we report the relative responses of AOB and AOA during the recovery of the ammonia oxidation process in an Australian agricultural soil (Spalding, South Australia) after experimental Zn contamination. To examine whether the functional recovery was due to changes in the size, activity or composition of dominant AOB and/or AOA, we measured the numbers of *amoA* gene copies and gene transcripts and assessed the *amoA* community composition for both AOB and AOA populations, and compared the data to patterns of ammonia oxidation activity.

Materials and methods

Soil treatment and nitrification

Top soil was sampled from an ongoing field trial in Spalding, South Australia, within 1 week after ZnSO₄ addition (T0) and annually for 3 years (T1–T3). The Spalding soil is classified as a chromosol (Isbell, 1996), and has a pH (0.01 M CaCl₂) of 6.3, 1.9% organic carbon and 27% clay. The background Zn concentration is 0.9 mmol Zn per kg. Zinc treatments were duplicated, and metal salts were incorporated into the soil using a rotary hoe. At each sampling, four topsoil samples (0–10 cm) were

collected per plot using a 5-cm-diameter auger, and samples were combined to yield one mixed sample. Details about the experimental design, the spiking procedure, soil sampling and soil physicochemical properties have been reported previously (Broos *et al.*, 2007b). Nine Zn treatments were imposed; a control soil ('unexposed soil') and 8 soils which received increasing Zn doses (1.1–113 mmol Zn per kg). After sampling, soils were air dried, ground, sieved to <2 mm and stored in closed containers at room temperature. Before further functional and molecular analysis, soils were remoistened with distilled water to 50% of the maximum water-holding capacity and aerobically incubated at 20 °C for 14 days in the dark to minimize the effects of storage time and of temperature and moisture content at the time of sampling.

Substrate induced nitrification (SIN) was measured in all Zn treatments (that is, the unexposed soil sample and 8 soil samples with increasing Zn doses) at T0 to T3 in duplicate per soil sample, and SIN was expressed as the percentage NH₄⁺ substrate used (%) 28 days after substrate addition (100 mg NH₄⁺-N per kg soil; Broos *et al.*, 2007b). Effects of increasing Zn concentrations on the nitrification process were related to Zn concentrations in a CaCl₂ soil extract. Total Zn concentrations in the CaCl₂ extract ranged from 0.01 to 109 mM Zn at T0 and 0.01 to 21 mM Zn at T2. The plot that received 28.3 mmol Zn per kg, corresponding to 22 mM Zn in a CaCl₂ soil extract at T0 and 6.6 mM Zn in a CaCl₂ soil extract at T2 ('Zn-exposed soil'), represented the largest Zn dose at which soil nitrification fully recovered following initial inhibition.

Zn-tolerance testing

Zn tolerance was tested in the unexposed and the Zn-exposed soil samples at T0 to T3 using the spike-on-spike test of Mertens *et al.* (2006). In short, ZnCl₂ was added to duplicate suspensions of soil samples (1:10 soil/CaCl₂ 0.01 M ratio) to final concentrations of 0, 0.77, 1.5 and 3.1 mM added Zn. Ammonium sulfate was added at 0.7 mM NH₄-N, and the pH in suspension was adjusted immediately after ammonia addition and daily during the 3-day test to pH 5.8 using 0.1 M NaOH or HCl. We preferred to adjust pH to pH 5.8 to approach the *in situ* soil pH rather than the optimal pH for nitrification (that is, pH 7–8; Jiang and Bakken, 1999). The potential nitrification rate (PNR; Smolders *et al.*, 2001) was measured, and is the nitrate production rate in the initial period (0–7 days) after ammonia addition (mg NO₃⁻-N per kg per day) during which there is unlimited substrate present. The experimental setup for SIN and PNR is essentially identical, but whereas the SIN is defined as the amount of substrate used within a 28-day incubation experiment (during which the substrate may have already been exhausted—note that SIN never exceeds 100%), PNR is defined as the rate of substrate used over a shorter

period of time. PNR is very sensitive to metal stress, and also to slight changes of other physicochemical soil characteristics (for example, pH; Broos *et al.*, 2005). Therefore, PNR rather than SIN was used as a measure of the nitrifying activity for Zn-tolerance testing in the well-defined soil suspensions (that is, constant pH and ionic strength for all soils) whereas SIN was preferred for screening the nitrification process in field soil samples over time (Broos *et al.*, 2007b). Effects of increasing Zn concentrations to the PNR were related to Zn concentrations in a CaCl₂ soil extract.

DNA extraction, PCR and denaturing gradient gel electrophoresis

DNA was extracted from all soil samples at T0 and T2 in duplicate per soil sample (Mertens *et al.*, 2006). For denaturing gradient gel electrophoresis (DGGE) community profiling, PCR targeting the AOB *amoA* gene used primers *amoA*-1F* and *amoA*-2R (Rotthauwe *et al.*, 1997; Stephen *et al.*, 1999), PCR targeting the AOA *amoA* gene used primers CrenamoA23f and CrenamoA616r (Tourna *et al.*, 2008). For amplification of AOB *amoA* genes, 1 µl of purified DNA was added to a PCR mixture containing 1.25 U of *Taq* polymerase (Qiagen, Venlo, the Netherlands), 20 pmol *amoA*-1F*, 20 pmol *amoA*-2R, 10 nmol of each dNTP, 1 × PCR buffer (Qiagen), 5 µl bovine serum albumin (1%) and PCR-grade water to a total volume of 50 µl. PCR was conducted in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) and PCR conditions were 10 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 60 s at 60 °C and 60 s at 72 °C, and a final elongation step for 10 min at 72 °C. A GC-rich clamp was attached to the 5' end of the AOB forward primer for DGGE fingerprinting. Analysis of PCR products size and integrity, and DGGE profiling of amplified bacterial *amoA* gene fragments were performed as described (Mertens *et al.*, 2006). Amplification of AOA *amoA* genes used Qiagen HotStar *Taq* PCR chemistry. DGGE separation of the AOA *amoA* genes was conducted without requirement for a GC-clamp in an Ingeny PhorU DGGE system (Tourna *et al.*, 2008). DGGE profiling was performed as previously described (Nicol *et al.*, 2005; Tourna *et al.*, 2008).

amoA sequence and phylogenetic analysis

Band excision, cloning and sequencing were performed on the unexposed and Zn-exposed soil samples at T2 as described (Mertens *et al.*, 2006). Sequences for dominant bands 'a' to 'k' (Figure 3c) have been submitted to the GenBank database under accession numbers EU515192 through to EU515202. AOB *amoA* DNA sequences 'a' to 'k' had open reading frames with translation products matching known AmoA protein sequences. The sequence data for each clone was compared with sequences on GenBank using the BlastN tool and similar and

dissimilar sequences spanning the *amoA* region of interest were recovered. Sequences were aligned in ClustalX; the alignment was manually checked. Phylogenetic analyses were conducted using MEGA version 4 (Tamura *et al.*, 2004) using the UPGMA method (Sneath and Sokal, 1973) with evolutionary distances computed using the maximum composite likelihood model (Tamura *et al.*, 2007). Phylogeny was tested using bootstrapping (Felsenstein, 1985) with 500 replicates (Supplementary Figure 2).

amoA gene copy and gene transcript quantification

Copy numbers of AOB and AOA *amoA* gene fragments were quantified in all soil samples at T2 and, additionally, in the unexposed and Zn-exposed soil samples at T0, T1 and T3 by real-time quantitative PCR (qPCR) on a Rotor-Gene 3000 Real-time PCR Cycler (Corbett Research, St Neots, UK). Real-time PCR of *amoA* gene fragments used the primers *amoA*-1F* and *amoA*-2R primers as previously described (Rotthauwe *et al.*, 1997; Stephen *et al.*, 1999), and primers Amo196F and Amo277R (Treusch *et al.*, 2005) for AOA. The protocols for quantitative amplification were based on methods described elsewhere (Okano *et al.*, 2004; Treusch *et al.*, 2005). In short, qPCR was performed in 25 µl reaction mixtures using 5 µl purified DNA as template and 12.5 µl of SYBR Green master mix (Qiagen). Primer concentrations were 2.25 pmol for primers *amoA*-1F* and *amoA*-2R, 1.5 pmol Amo196F and 3 pmol Amo277R for the AOA *amoA* amplification. Reaction mixtures were made up to a final volume of 25 µl using PCR-grade water. The protocol for amplification of AOB *amoA* fragments was 2 min at 50 °C, 15 min at 95 °C, 45 cycles of 45 s at 95 °C, 60 s at 60 °C and 45 s at 72 °C (based on Okano *et al.*, 2004), and the protocol for amplification of AOA *amoA* fragments was 15 min at 95 °C, 45 cycles of 15 s at 95 °C, 40 s at 55 °C and 45 s at 72 °C (based on Treusch *et al.*, 2005). Both protocols were followed by a final elongation step of 5 min at 72 °C. Dissociation curve analysis and agarose gel electrophoresis were performed to confirm amplification specificity. Dissociation curves for AOB and AOA *amoA* quantification were conducted by stepwise increasing the temperature with 1 °C from 60 to 95 °C or 55 to 95 °C (45 s at the lowest temperature, and 5 s for each subsequent temperature step). Dilution series of purified PCR products of plasmids (*amoA* gene of *Nitrosomonas europaea* ATCC 19718) and fosmid vectors (clone 54d9) were used as qPCR standards to allow quantification of each target number per sample.

Total soil RNA was extracted from the unexposed and Zn-exposed soil samples at T0 and T2 using the MoBio Soil RNA extraction kit and was treated with DNase (Promega, Leiden, the Netherlands). cDNA generation and quantification of AOA and AOB *amoA* transcript numbers were performed as described by Leininger *et al.* (2006).

Statistical analyses

Dose–response curves were fitted by log-logistic modeling (Doelman and Haanstra, 1989) using the Marquardt method (proc NLIN, SAS 9.1; NC, USA). Significant ($P < 0.05$) inhibitory effects on *amoA* gene copy and transcript numbers were detected using Student's *t*-tests.

Results

Zinc contamination of the arable field decreased nitrification with a characteristic log-logistic dose–response relationship (Figure 1). The EC_{50} for SIN (effective concentration with 50% reduction of SIN compared to the unexposed soil sample) was 3.6 mM Zn one week after exposure (T0; Broos *et al.*, 2007b). The EC_{50} increased significantly to 13.1 mM Zn after 2 years of exposure (T2). Zinc concentrations in the Spalding soil samples were expressed as 0.01 M $CaCl_2$ soil extracts, which mimic *in situ* Zn concentrations in porewater (Degryse *et al.*, 2003) and, hence, bioavailable Zn concentrations in soil (Mertens *et al.*, 2007). A Zn contamination level of 28.3 mmol Zn per kg soil, corresponding to 22 mM Zn at T0 and 6.6 mM Zn at T2, was the highest contamination level for which ammonia oxidation was restored within 2 years after initial inhibition (Figure 1), and this soil sample was selected for further analysis ('Zn-exposed soil').

Zinc-tolerance testing of the unexposed soil sample at T0 and T2 revealed EC_{50} values of 0.09 and 0.22 mM Zn, respectively (Figure 2). No nitrifying activity was detected for the Zn-exposed soil sample at T0. Two years after Zn addition, the PNR of the Zn-exposed soil sample was comparable to the PNR of the unexposed soil sample, and application of increasing Zn doses to the Zn-exposed soil sample at T2 did not affect the PNR (Figure 2).

The AOB and AOA *amoA* gene copy numbers in the unexposed soil samples did not differ significantly from each other, and were not significantly different between T0 and T2 (Figure 3a). AOA *amoA* gene copy numbers in the Zn-exposed soil sample were lower than AOB *amoA* gene copy numbers at both T0 and T2, with a significant difference at T2 (Figure 3a). Also, in the examination of T2 samples exposed to a range of Zn doses, severe decreases of AOA *amoA* gene copy numbers were observed at a soluble Zn concentration two orders of magnitude lower than for AOB *amoA* gene copy numbers (Supplementary Figure 1a).

At T0, the numbers of AOB and AOA *amoA* gene transcripts in the unexposed soil samples were 3.7×10^6 and 1.2×10^6 per g of soil, respectively, and similar values were found at T2 (Figure 3b). In contrast, the number of AOB and AOA *amoA* gene transcripts in the Zn-exposed soil samples was more than 3 orders of magnitude reduced at T0, compared to the values in the unexposed soil sample (Figure 3b). After two years of exposure, the number

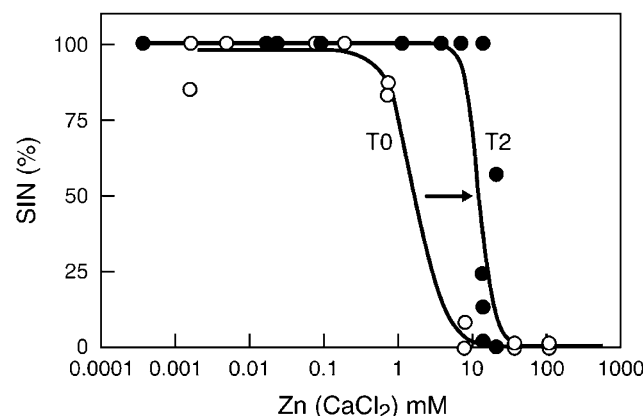


Figure 1 Substrate induced nitrification (SIN) in the Spalding field site over time. SIN, expressed as percentage substrate used (%), is plotted versus Zn concentrations in a 0.01 M $CaCl_2$ soil extract at T0 (open symbols) and T2 (closed symbols). The arrow indicates the shift in EC_{50} (Effect Concentration with 50% decrease compared to the unexposed soil sample) values between T0 and T2.

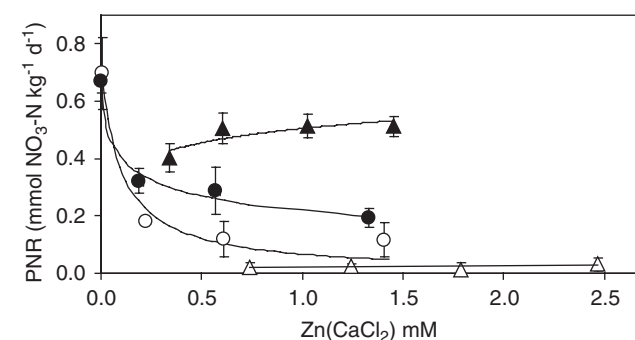


Figure 2 Zn-tolerance testing at T0 and T2. Potential nitrification rate (PNR) (\pm s.e.), expressed as mmol NO_3 -N per kg per day, of the unexposed soil sample (circle) and a Zn-exposed soil sample (triangle) at T0 (open symbols) and T2 (closed symbols), is plotted versus Zn concentrations in a 0.01 M $CaCl_2$ soil extract (\pm s.e.).

of AOB *amoA* gene transcripts recovered to the level of the unexposed soil sample at T0, but remained a bit lower than the level of the unexposed soil sample at T2. In contrast, the number of AOA *amoA* gene transcripts remained 4 orders of magnitude below that in the unexposed soil sample (Figure 3b).

Community profiling of *amoA* genes (DGGE) showed a distinct shift in the AOB community structure in the Zn-exposed soil samples at T2, yet no such shift in *amoA* genotypes was observed for the AOA community (Supplementary Figures 1b and c). In the AOB community profile of the unexposed soil, the *amoA* genotypes corresponding to bands labeled 'b', 'c', 'd' and 'e' in the DGGE fingerprint were most prevalent (Figure 3c). However, at T2, bands 'h' and 'i' were most prominent in the AOB *amoA* community profile (Figure 3c). Sequence analysis revealed that all detected AOB *amoA* bands were affiliated with several sequence clusters within

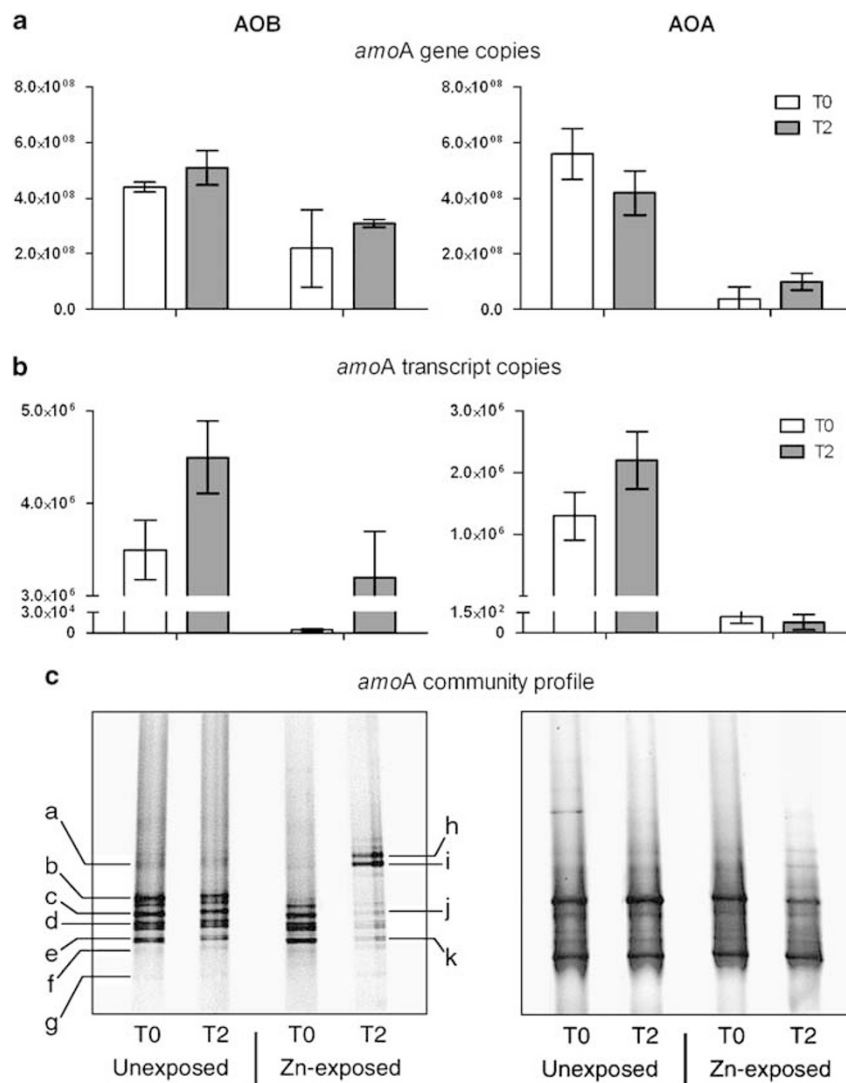


Figure 3 *amoA* gene copy numbers, *amoA* gene transcript numbers and *amoA* community profile of AOB and AOA in the unexposed and the Zn-exposed soil sample over time. Average number of *amoA* gene copies (\pm s.e.; **a**) and number of *amoA* gene transcripts (\pm s.e.; **b**) of two field replicates and the *amoA* community profile (**c**) of AOB and AOA in the unexposed soil sample and the Zn-exposed soil sample at T0 and T2. Bands marked with a letter ('a' to 'k') were cloned and sequenced and belong to the *Nitrosospira* lineage.

the *Nitrosospira* lineage (Avrahami *et al.*, 2002), and no clustering of sequences was observed with respect to the Zn tolerance of the community (Supplementary Figure 2).

Discussion

The increased EC_{50} value over time, expressed as Zn concentrations in 0.01 M $CaCl_2$ soil extracts (Figure 1), was not attributable to slow immobilization reactions of Zn in soil, but rather to adaptation of the ammonia-oxidizing community, as observed for other long-term Zn contaminated soils (Rusk *et al.*, 2004; Mertens *et al.* 2006). This was verified by testing for Zn tolerance in the Zn-exposed soil sample. In accordance to the SIN data, no nitrifying

activity was observed in the Zn-exposed soil sample at T0, whereas nitrification levels recovered to the level of the unexposed soil sample at T2. At the same time, the recovery of the nitrification process in this soil sample was accompanied with increased Zn tolerance within the nitrifying community at T2 (Figure 2).

The AOB and AOA *amoA* gene copy numbers in the unexposed soil sample were high, but comparable to those found in previous studies (Leininger *et al.*, 2006; He *et al.*, 2007). In several recent studies, the number of AOA *amoA* gene copies in soil exceeded that of AOB (for example, Adair and Schwartz, 2008; He *et al.*, 2007; Shen *et al.*, 2008), whereas numbers in the unexposed Spalding soil were roughly equal. The reduction of the AOA *amoA* gene copy numbers on contamination is very

rapid (Figure 3a; Supplementary Figure 1a). All evidence suggests the death of AOA cells on contamination, as storage and rewetting itself does not significantly affect levels of AOA, AOB or total bacteria in these soils (not shown). It should be noted that the coverage of *amoA*-based primers, especially for AOA, is not yet well established, and the assays used in this study may therefore underestimate actual *amoA* gene copy and gene transcript numbers. At T2, the nitrification activity in the Zn-exposed soil sample recovered to the value in the unexposed soil sample (Figures 1 and 2), and AOB *amoA* gene copy numbers were appreciable (Figure 3a). In contrast, the AOA *amoA* gene copy number in the Zn-exposed soil sample was fivefold lower compared to the unexposed soil sample (Figure 3a), suggesting that recovery of SIN in the Zn-exposed soil sample was exclusively attributable to the AOB community in this soil. This finding is supported by a tight association between AOB *amoA* gene copy numbers and SIN across Zn application rates at T2 (50% reduction of AOB *amoA* gene copy numbers and SIN at 8.1 mM and 13.1 mM Zn, respectively, and at 0.07 mM Zn for the AOA *amoA* gene copy numbers; Figure 1; Supplementary Figure 1a). SIN, level of Zn tolerance and AOB and AOA *amoA* gene copy numbers of the unexposed and the Zn-exposed soil samples at T1 (that is, after 1 year of exposure) were similar to those at T0 and corresponding data at T3 (that is, after 3 years of exposure) were similar to those at T2 (details not shown).

The inhibition of SIN in the Zn-exposed soil sample at T0 coincided with a sharp decrease in the AOB and AOA *amoA* gene transcript numbers, indicating that both groups were severely affected by Zn stress (Figures 1 and 3b). The restoration of the nitrification process in the Zn-exposed soil sample at T2 was associated with a recovery of the *amoA* gene transcript number within the AOB community, but not within the AOA community (Figure 3b), providing evidence for a dominant role of AOB in the restoration of the ammonium oxidation activity. At the same time, the AOB *amoA* gene community profile in the Zn-exposed soil sample had changed, yet no corresponding change could be observed within the AOA *amoA* gene community profile (Figure 3c). As sequence variation in the AOB *amoA* gene is closely linked to 16S rRNA phylogeny (Purkhold *et al.*, 2000, 2003; Aakra *et al.*, 2001), this observation suggested a shift in AOB community structure that was associated with restoration of nitrification. Interestingly, we did not observe any clustering of recovered sequences with respect to Zn tolerance of the communities (Supplementary Figure 2). Thus, it appears that the ability to acquire Zn tolerance is widespread throughout the *Nitrosospora* lineage, and specific to particular ecotypes not discerned using this functional marker gene. The selection of genotypes of Zn-tolerant AOB species

occurred from low background levels of these AOB, or from adaptation and selection of Zn-tolerant AOB over time. Our failure to detect a shift in the AOA community suggested the AOA community did not have the capacity to respond to Zn-induced stress over a 2-year time period. Similar to our results, changes of the numbers of AOB and AOA *amoA* gene copies, gene transcripts and/or *amoA* gene profiles suggested AOB dominated ammonia oxidation in an alkaline sandy loam soil under different long-term fertilization regimes (Shen *et al.*, 2008), in grassland under different grazing managements (Le Roux *et al.*, 2008) and in long-term acidified soil samples (Nicol *et al.*, 2008). In contrast, data on the numbers of AOB and AOA *amoA* gene transcripts in soils incubated at different temperatures (Tournia *et al.*, 2008) and in nitrogen fertilized paddy rhizosphere soils (Chen *et al.*, 2008) suggest an AOA-dominated ammonia-oxidation process. It must be recognized that the nitrification process, *amoA* gene community profiles, *amoA* gene copy numbers and *amoA* gene transcript numbers were analyzed in dried, stored and reconditioned soils. Detected target numbers may therefore not be an accurate portrayal of *in situ* conditions. However, we have observed that soil drying and storage, which mimic the conditions present for extended periods in these fields (Broos *et al.*, 2007a), had no detectable effects on determined process rates, as well as on bacterial numbers (data not shown). Furthermore, our molecular analyses were geared toward determining the populations responsible for activities in the reconditioned soil samples, not necessarily *in situ* field conditions at the time of sampling.

Thus, contrary to the general view of archaea being more tolerant to chronic stress conditions than bacteria (Schleper *et al.*, 2005; Valentine, 2007), we demonstrated that AOA were more sensitive to Zn contamination than AOB in this soil. It is unlikely that the effects on the AOA community were caused by other physicochemical or biological soil processes besides Zn, as the numbers of AOA and AOB *amoA* gene copies and gene transcripts and AOA and AOB *amoA* gene profiles of the unexposed soil samples were similar across all replicates and sampling times (that is, T0 to T3). Similar consistent results were observed by Wheatley *et al.* (2003) in three uncontaminated fields. Interestingly, significant numbers of AOA *amoA* genes still remained in the Zn-exposed soil samples at T2, even though virtually no expression was observed. Thus, the prospect remains that some AOA populations may be able to survive such stress, in dormant states or as spores (Onyenwoke *et al.*, 2004). Alternatively, these organisms may obtain energy through processes other than ammonia oxidation. Further research will be necessary to determine if AOA have long-term adaptive capabilities in response to heavy metal-induced stress.

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