SOIL MICROBIOLOGY

Response of Ammonia Oxidizing Bacteria and Archaea to Acute Zinc Stress and Different Moisture Regimes in Soil

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Abstract Ammonia oxidation has been intensively studied for its sensitivity to environmental shifts and stresses. However, acute stress effects on the occurrence and composition of ammonia oxidizing bacteria (AOB) and archaea (AOA) based on expression of related molecular markers in complex soil environments have been to an extent overlooked, particularly concerning transient but commonly occurring environmental changes like soil moisture shifts. The present study investigates the responses of AOB and AOA to moisture shifts and high Zn soil content. *AmoA* gene copies and transcripts of AOB and AOA along with potential nitrification activity were measured in a soil microcosm approach for investigating the referred environmental shifts. Moisture change from 87 to 50 % of the water holding capacity caused a ~99 % reduction of AOB but not of AOA *amoA* transcripts that did not change significantly. Increasing applied zinc concentrations resulted in a reduction of potential nitrification rates and negatively affected studied gene expressions of both AOB and AOA, with AOB being more responsive. Both 16 S rRNA and *amoA* transcripts of AOB had an inverse relation to the applied zinc, indicating a gradual loss in total cell activity. Our results suggest the existence of pronounced differences between AOB and AOA concerning ammonia oxidation activity.

Introduction

Nitrification, a central pathway of the global nitrogen cycle [1] and defined as the turnover of ammonia to nitrate, was used in ecotoxicology studies and proposed for risk assessment, due to its sensitivity to various stresses and environmental shifts [1–9; ISO 15685:2004]. Ammonia oxidation (rate-limiting step of nitrification) has been long considered to be solely performed in soil by bacterial ammonia oxidizers (AOB) of the betaproteobacterial class [10–12]. For tracking the presence and activity of AOB in soil, the *amoA* gene (encoding for the alpha subunit of ammonia monooxygenase—AMO) has been used as molecular marker [13] together with betaproteobacterial ammonia oxidizer-specific 16 S rRNA gene targeting primers [14]. Recently, isolation of crenarchaeal strains encompassing in their genomes a homolog to the AOB *amoA* and being able to use ammonia for energy acquisition [15–17] changed the general perception of ammonia oxidation. Studies revealed the wide distribution of archaeal ammonia oxidizers like the ones belonging to mesophilic *Crenarchea* (ammonia oxidizing archaea—AOA), which was recently proposed to comprise the new distinct phylum of *Thaumarchaeota*, in soil environments [18–25]. Despite being cosmopolitan, differences observed between AOA and the well-studied AOB raised

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debates about the potential niche separation between the two groups [23, 26–30].

Some of the observed differences were derived from studies of AOB and AOA responses and adaptation to trace element toxicity stresses occurring in soil environments [31–33]. However, little information exists about the in situ differences of AOB and AOA during initial stages of these stresses and also commonly occurring stresses like moisture.

This study aimed at gaining insights concerning fundamental differences between the two microbial groups as an outcome of their initial responses to perturbations. In particular, the acute effects on ammonia oxidation partition between AOB and AOA regarding the two types of stresses were investigated. These were the shift of moisture (commonly occurring in natural environments) and one severe stress relevant to risk assessment studies—zinc stress. Total nitrification, amoA gene and transcript differences for both microbial groups, and also the 16 S rRNA gene(s) presence and expression for AOB were assessed for soil microcosms subjected to moisture shifts (commonly occurring environmental change) and increasing zinc concentrations (severe stress).

Materials and Methods

Experimental Setup

Topsoil (0–15 cm depth) was collected from a maize field located close to Alsenio (PC, Italy) at the end of October 2009. Total soil carbon and nitrogen and also soil texture were measured according to standard soil methods [34, 35], while background trace element concentrations were determined via the *aqua regia* digestion method as described in Coppolecchia et al. [36]. Measured soil properties are shown in Tables 1 and 2.

Moisture shift effects on total nitrification activity and amoA and amoA transcripts per ammonia oxidizer microbial group were assessed for two water contents. Soil microcosms in triplicates (each parted by 800 g of soil maintained in 2 l glass containers with 85 mm openings) were water-saturated and incubated at room temperature in the dark. Microcosms were sampled 1 day (water content equal to

Table 1 Basic properties of the soil used for generating the microcosms

Total C (%)	Total N (%)	pH	WHC (%)	Particle size distribution (%)		
				Clay	Silt	Sand
0.81	0.28	8.2	34	24	42	33

Table 2 Trace element background concentration of the soil used for generating the microcosms

Trace element	Pb	Ni	Cu	Cd	Cr	Co	Zn
mg kg^{-1}	18.2	38.6	19.6	0.1	67.9	13.7	84.9

87 % of the water holding capacity—WHC) and 4 days (water content 50 % WHC) post leaching, and samples were stored at −20 °C until analysis as described further on.

AOB and AOA acute responses to zinc were examined by treating soil microcosms with increasing concentrations and assessing potential nitrification, amoA gene and transcript content, and also amoA variant changes, for soil samples obtained after overnight incubation. Briefly, soil microcosms of 200 g each were spiked with ZnCl$_2$ to nominal Zn concentrations ranging from 0 to 5,000 mg kg^{-1} (hereafter treatments will be referred to according to nominal zinc content). Potential Cl$^-$ effects were reduced by a single soil leaching with 60 ml of ddH$_2$O (double distilled water). The leaching process eliminated pH decrease caused by ZnCl$_2$ spiking for [ZnCl$_2$] applications below 2,000 mg kg^{-1} according to the measurement carried out prior to ZnCl$_2$ spiking, after ZnCl$_2$ spiking, and after ddH$_2$O leaching. Post-leaching pH decrease above 0.5 U was observed only for higher zinc concentrations where measured potential nitrification activity and gene transcripts were close to or below detection limits as presented further on. The soil microcosms were concomitantly incubated in open air overnight at room temperature and sampled the following day, and samples were stored at −20 °C for downstream analyses.

CaCl$_2$ 0.01 M Extractable Zn (Labile Fraction)

Post-leaching Zn-extractable fractions of soil samples were determined via the CaCl$_2$ (0.01 M) extraction method. Briefly, 10 g of soil was soaked in 100 ml of CaCl$_2$ 0.01 M and incubated at room temperature in a rotary shaker at 30 rpm for 2 h. Extracts were obtained after separation by centrifuging at 3,000 g for 10 min and were acidified with HNO$_3$. The acidified extracts were stored at 4 °C until inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis. The wavelength used for Zn determination with ICP-OES was based on the related results of Bettinelli et al. [37].

Potential Nitrification Assay

Potential nitrification was assessed according to the Kandeler method [38], with the colorimetric determination of the nitrite released after 5 h of incubation with (NH$_4$)$_2$SO$_4$ as a substrate and the addition NaClO$_3$ for inhibiting nitrate reduction and nitrite oxidation. Five grams of moist soil was incubated with 20 ml of (NH$_4$)$_2$SO$_4$ 1 mM and 0.1 ml NaClO$_3$ 1.5 M in

closed Erlenmeyer flasks for 5 h at 37 °C with shaking at 100 rpm. Five milliliters of KCl 2 M was added in all flasks and briefly mixed, and the contents were filtered through a 0.2-μm filter. Five milliliters of filtrates was mixed with 3 ml of NH_4Cl 0.19 M pH 8.5 solution and 2 ml of color reagent (2 g of sulfanilamide, 0.1 g of N-(1-naphtyl)-ethylenediamine hydrochloride diluted in 180 ml distilled water, and 20 ml of 85 % phosphoric acid solution) and was incubated at room temperature for 15 min. Two types of controls were used for validation of the potential nitrification measurement method. These were the additional no incubation controls, used to assess false positive effects of soil solutes and reagents apart from the coloring agent as described further on, and the no filtrate controls, used to assess false positives caused by the color reagent. As no incubation controls, flasks containing soil, $(NH_4)_2SO_4$, and $NaClO_3$ were directly stored at −20 °C until the filtration step, and concomitantly, the filtrates were incubated with the color reagent for 15 min at room temperature. The no filtrates control was performed by incubating 5 ml ddH_2O with 2 ml of color reagent for 15 min at room temperature. Finally, filtrates from samples and controls were compared to the no filtrate controls according to 520 nm light wavelength absorbance.

Nucleic Acid Isolation, Quality Control, and Quantification

For the nucleic acid isolation, the RNA–DNA co-extraction protocol of the MoBio PowerSoil™ RNA kit (Carlsbad, CA, USA) when combined with the RNA PowerSoil™ DNA elution accessory kit was followed, with a slight modification. The modification referred to the addition of EDTA pH 8.0 to a final concentration of 0.5 M right after the SDS containing reagent and prior vortexing for cell lysis enhancement.

RNA was further purified using the RNeasy ® Mini Kit (Qiagen, Valencia, CA, USA) coupled with the on column DNase digestion using the RNase free DNase set (Qiagen). DNA purity analysis was performed spectrophotometrically (260/280 nm and 260/230 nm ratios), while DNA and RNA extracts were also analyzed on 0.8 and 1 % agarose gels. DNA and RNA were quantified using the Quant-iT™ (HS dsDNA Assay and RNA Assay kits, respectively; Invitrogen, Paisley, UK) in combination with the Qubit™ fluorometer (Invitrogen).

General PCR Conditions and RNA Reverse Transcription

The betaproteobacterial and crenarchaeal *amoA* and also the betaproteobacterial 16 S rRNA coding gene were studied along with their transcripts. Primer sets used for the respective gene amplifications were the amoA 1 F/2R T [13], the CrenamoA 23f/616r [39], and the CTO 189fABC/654r [14]. PCR amplification was carried out in 50 μl reaction mixtures using the AmpliTaq® DNA polymerase with buffer I kit (Applied Biosystems, Foster City, CA, USA). Twenty nanograms of template was added in each mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.4 mM dNTPs, 0.5 μM of each primer, and ddH_2O. Conditions used were 94 °C for 5 min, 35 cycles of 94 °C for 30 s denaturation, 54 °C for 30 s annealing, 72 °C for 30 s extension, and a final extension step of 72 °C for 10 min for the amoA 1 F/2R T and the CTO 189fABC/654r primer sets, while for the CrenamoA 23f/616r primer set, an extension step of 1 min instead of 30 s was used.

Reverse transcription of RNA to cDNA was performed using the iScript™ cDNA Synthesis kit from BioRad Laboratories (Hercules, CA, USA) according to the manufacturer's instructions. Verification of the purity of RNA extracts from DNA carryover prior to reverse transcription was carried out with real-time quantitative PCR (see section below) using no reverse transcription controls for all selected primer sets.

Real-Time Quantitative PCR (qPCR) and Standard Curve Generation

For the qPCR assays, the iQ™ SYBR® Green Supermix (BioRad Laboratories) was used in 50 μl reactions. Absolute quantification was carried out in all cases based on standard curves as described further on. Transcript and gene copies were quantified using 10 ng (for cDNA) and 20 ng (for DNA) templates in a mixture containing 25 μl of Supermix and 0.5 μM of each primer and ddH_2O. The PCR program followed was as referred in the "General PCR Conditions and RNA Reverse Transcription" section for each primer set with differences for the initial enzyme activation stage time (95 °C for 3 min instead of 5 min) and the cycles performed (50 instead of 35). A melting curve was performed right after each reaction set was finished for the evaluation of the specificity of the amplifications.

Standard curves were generated according to the following procedure: for each primer set, PCR products of soil DNA extracts were quantified as described in the "Nucleic Acid Isolation, Quality Control, and Quantification" section. The amplicon numbers per product were estimated according to the average molecular weight of amplicons. For assessing the variability of existing amplicon molecular weights, representative bands of the gradient range from denaturing gradient gel electrophoresis (DGGE) gels (performed as described in the next section) were excised and sequenced by Primm (Milan, Italy). Ten best BLAST matches in the non-redundant database of NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/) were used for obtaining the full amplicon average molecular weight information according to nucleic acid composition. Serial dilutions corresponding to the range of 10 to 10^8

amplicons per product were performed for generating the standard curves in duplicates and also assessing the detection limits and R^2 values. Obtained R^2 values were above 0.98 for all performed reactions.

DGGE Analysis

PCR products obtained with the primer sets amoA 1 F/ amoA 2R T with forward primer containing the GC clamp in the 5′ end previously published by Muyzer et al. [40] and CrenamoA23f/616r without a GC clamp [39] were used for DGGE analysis. PCR conditions were the same as described above.

DGGE was carried out using a DCode Universal Mutation Detection System (BioRad Laboratories) according to the manufacturer's instructions. Denaturant contents of polyacrylamide gels used were 15–55 % for the CrenamoA 23f/616r primer set and 50–65 % of denaturant for the AmoA1F GC/ 2R T primer set as described previously [14, 31, 39]. Gels were SYBR green-stained and image analysis was carried out with the Cross Checker software [41] for generating genotype presence/absence (binary) matrices.

Statistical Tests and Analyses

Range weighted richness (Rr) values were estimated according to the provided formula of Marzorati et al. [42]: Rr= $(S^2 \times Dg)$ (S is the observed band richness per DGGE gel lane and Dg the gradient difference between the first and last band). Hierarchical clustering analysis was performed on the binary matrices generated by the DGGE gel images using the unweighted pair group comparison method with arithmetic means (UPGMA) algorithm and the Jaccard distance estimation. Principal coordinates analysis (PCoA) was also performed for the Jaccard algorithm transformed binary matrices. Vector analysis [43] after plotting of both treatments

and the DGGE genotypes according to PCoA was used to assess potential genotype–treatment correlations. One-way analysis of variance of means (ANOVA) and Tukey's honestly significant differences (HSD) test were performed for the potential nitrification, the real-time PCR, and the richness (S) and Rr data. Spearman's rank (r_s) and Pearson product-moment (r) correlation coefficients were used for assessing the level of ranked and linear correlation, respectively, between AOB amoA and 16 S rRNA gene expressions. Analysis of similarity (ANOSIM) test using 1,000 permutations and the Jaccard distance transformation were performed to identify correlations between banding pattern shifts of the DGGE gels and treatments or measured variables, respectively. The R software [44] with the Hmisc (http://biostat.mc.vanderbilt.edu/trac/Hmisc) and the Biodiversity R [45] packages were used for all statistical analyses.

Results

Effects of Moisture Shift on Nitrification, amoA Genes, and Transcripts

A significant effect of moisture values dropping from 87 to 50 % of the WHC was observed only for AOB amoA transcripts having a decrease of approximately 99 % (Fig. 1). The potential nitrification, AOB and AOA gene copy number, and also amoA AOA transcript copy number remained similar.

Zinc Effects on Potential Nitrification and AOA and AOB Copy and Transcript Numbers

Potential nitrification results indicate a Zn concentration dose-dependent reduction of the enzymatic activity (Fig. 2a). Zn concentration of 125 mg kg^{-1} nominal did not induce significant reduction of the measured potential

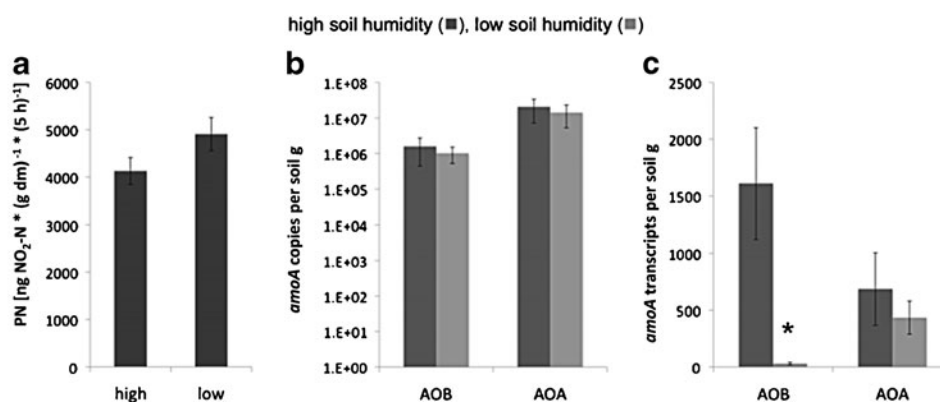high soil humidity (■), low soil humidity (■)

Figure 1 Soil moisture drop (from high =87 % to low =50 % of the WHC) effects on measured potential nitrification rates expressed on the basis of dry soil matter (**a**) and also amoA gene (**b**) and transcript (**c**) abundance of AOB and AOA. Significant differences due to change in moisture were observed only for the betaproteobacterial ammonia oxidizer transcript numbers according to performed ANOVA (indicated with the *asterisk* for $\alpha < 0.05$)
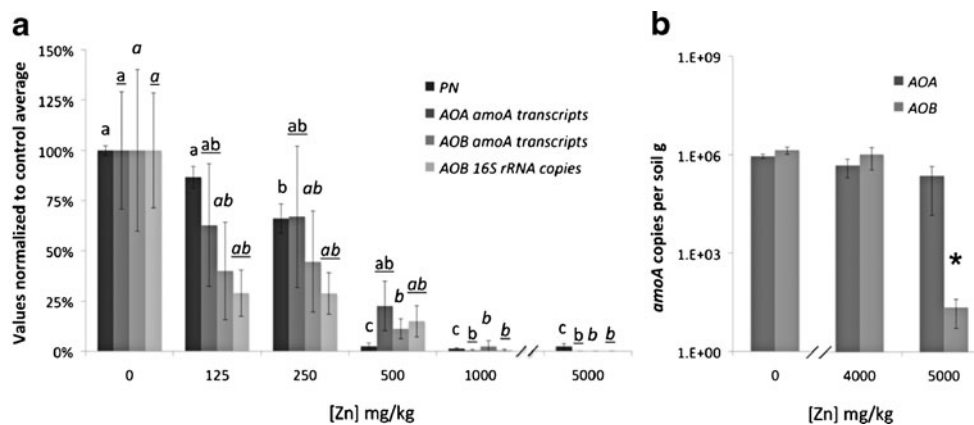
Figure 2 Potential nitrification (PN) results, AOA *amoA* transcripts, AOB *amoA* transcripts, and AOB 16 S rRNA copies normalized to the average control (nominal [Zn]=0 mg kg^{-1}) values for Zn treatments ranging from 0 to 5,000 mg kg^{-1} (**a**); *amoA* copy numbers for AOA and AOB for the [Zn] range mentioned for **a** (**b**). *Letters of various formats* in **a** indicate pairwise comparisons among different treatment groups according to Tukey's HSD pairwise comparisons performed for ANOVA per measurement type: *normal letters* are used in the treatment group comparisons for PN rate measurements, *italics* for AOA *amoA* transcript counts, *underlined* for AOB *amoA* transcript counts, and *underlined italics* for AOB 16 S rRNA counts. The average control values (used for expressing values as relative) for each measured variable were: PN rate of 446,378 ng N per g of dry soil weight and per 5 h, 3,211 AOB *amoA* transcripts per soil g, 315 AOA *amoA* transcripts per soil g, 631,745,245 AOB 16 S rRNA copies per soil g

nitrification. Statistically significant reduction was observed for 250 mg kg^{-1} of applied [Zn], while 500 mg kg^{-1} or higher of applied [Zn] almost eliminated potential nitrification. Transcripts of all studied genes had similar reduction as in the case of the potential nitrification values. Control treatment ([Zn]=0 mg kg^{-1}) average *amoA* transcript numbers were one order of magnitude lower for AOA (315 transcrips per soil gram) compared to the AOB (3,211 transcripts per soil gram) having clear differences as also indicated by the soil moisture shift test. Screened bacterial transcripts were indicated to be more responsive than the archaeal ones to the increased [Zn] applications. AOB had significantly lower average relative *amoA* transcript copy values at 500 mg kg^{-1} of applied [Zn] compared to 250 mg kg^{-1} of applied [Zn], while in AOA, this difference was not statistically significant. A strong correlation was indicated for AOB *amoA* and 16 S rRNA gene transcripts according to the obtained Spearman (r_s=0.9, P=0) and Pearson (r=0.76, P=0.0037) correlation values. Gene abundance showed no significant differences, except for the treatment that received 5,000 mg kg^{-1} and only for AOB *amoA* (Fig. 2b).

Effect of Zn Spiking on *amoA* Richness and Structure

AmoA gene and transcript composition shifts of the environmental samples in response to nominal Zn concentrations between 0 and 500 mg kg^{-1} were assessed with DGGE. DGGE *amoA* gene fingerprints were consistent throughout the referred range of [Zn] applications for both betaproteobacterial and crenarchaeal ammonia oxidizers (data not shown) and therefore are not further discussed.

Differences between AOB and AOA were observed with regards to fingerprints obtained from *amoA* transcripts. Hierarchical clustering results showed formation of three major [Zn] application-related clusters for AOB, but no such correlation was indicated for AOA (panels b and a of Fig. 3, respectively). Clusters formed for AOB *amoA* separated the samples into three treatment-related groups: (1) the control group, (2) the 125 and 250 mg kg^{-1} of applied [Zn] group, and (3) the 500 mg kg^{-1} applied [Zn] group. Range weighted richness of *amoA* expression decreased between the control and 500 mg kg^{-1} [Zn] for both examined groups (Fig. 3c). However, this response was significant for 125 mg kg^{-1} [Zn] and above for AOB, while such clear difference was observed between 0 and 500 mg kg^{-1} for AOA. Observed richness (S) was similar to the Rr values (data not shown).

PCoA analysis of the DGGE *amoA* transcript patterns showed sample topology corresponding to the inferred groupings by the hierarchical clustering results for both AOB (Fig. 3d) and AOA (data not shown). The referred AOB fingerprints were further analyzed using vector analysis in the performed PCoA in order to assess the presence of genotypes more related with any of the inferred sample groups. Vector analysis indicated five distinct groups of genotypes in relation to the applied treatments (an example of extrapolated correlation is shown for g12 in Fig. 3d). These were the following treatment groups: (1) the 0 mg kg^{-1} [Zn] (g2, g5, g6, g7, g8); (2) the 0, 125, and 250 mg kg^{-1} [Zn] (g9, g11—with slightly higher correlation to 0 mg kg^{-1} of [Zn]); (3) the 125 and 250 mg kg^{-1} (g1, g10); (4) the 0 and 500 mg kg^{-1} [Zn] (g3, g4); and (5) the 500 mg kg^{-1} [Zn] (g12).
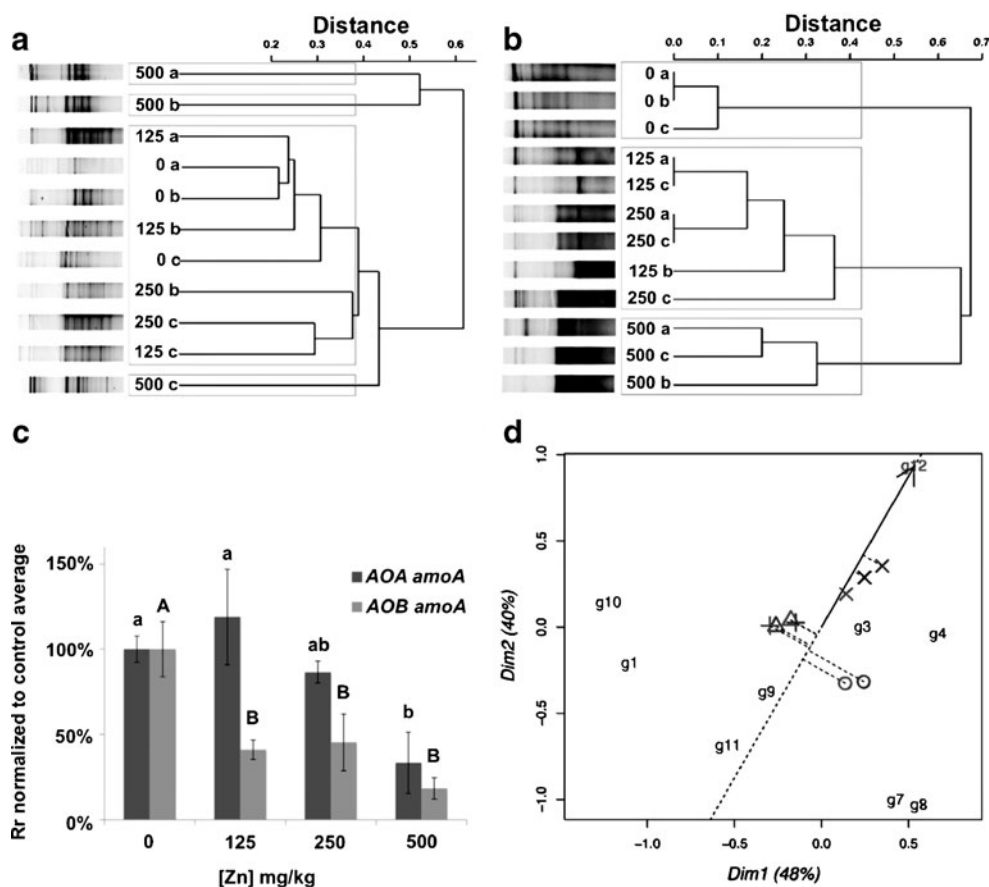
Figure 3 DGGE-based transcriptional analysis results for AOA and AOB *amoA* of the [Zn] treatment range of 0–500 mg kg^{-1}. AOA and AOB hierarchical clustering results using UPGMA and the Jaccard coefficient along with the banding patterns are shown in **a** and **b,** respectively. Average Rr values for each treatment, normalized to the average [Zn]=0 mg kg^{-1} treatment values (64.516 for AOA and 2.615 for AOB) are shown in **c** (*letters— normal* for AOA and *italics* for AOB—indicate statistically significant differences between treatments according to ANOVA and

Tukey's HSD for $\alpha < 0.05$). **d** PCoA results are provided as biplot of the various DGGE identified genotypes (g1–g12) and samples (denoted by the *different symbols* according to [Zn] treatment groups: *open circles* for 0 mg kg^{-1}; *open triangles* for 125 mg kg^{-1}; *crosses* for 250 mg kg^{-1}; *times symbol* for 500 mg kg^{-1}). The correlation of genotype 12 (g12 or gel band number 12) with the various treatments is indicated by vector analysis (projected distances to the g12 vector are indicative of the correlation of the genotype with corresponding samples)

ANOSIM results showed two clearly different responses between bacterial and archaeal *amoA* transcriptional profiles (Fig. 4). Bacterial *amoA* transcriptional patterns had higher

variability between applied [Zn] sample groups than within (R values), while the respective archaeal did not (significance of differences based on 1,000 permutations).

Figure 4 ANOSIM results of the treatment groups for bacterial (*left*) and crenarchaeal (*right*) *amoA* banding patterns obtained by DGGE analysis of transcripts. *Notched boxplots* indicate the dissimilarity rank distributions for between and within treatments presented in plots
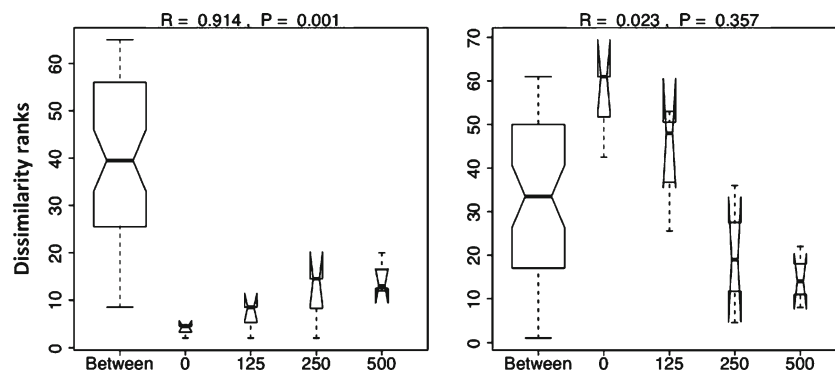
Discussion

In the present study, we studied the response differences between two prokaryotic groups known to carry the *amoA* homologue and be accounted for most ammonia oxidation, the AOB and AOA, using a commonly occurring environmental stress and one severe stress.

Soil Moisture

Significant moisture reductions in the soil environment were previously shown to affect nitrification activity due to reduction of substrate availability and triggering of physiological changes (e.g., downregulation of basic metabolism and upregulation of stress-related gene expression) in order to tolerate the induced osmotic changes [46]. The outcomes of this study did not indicate that the applied moisture shift was severe enough to cause loss of enzymatic activity as shown according to the potential nitrification results (Fig. 1a). With the presence of the *amoA* gene maintained, *amoA* transcript number reduction for AOA and AOB was in concordance to the soil moisture shifts, thus indicating that even relatively mild environmental shifts are capable of inducing downregulation of basic metabolism gene expression and possibly causing the entry of microbial cells to an alarming stage.

Significant differences were observed only for AOB *amoA* transcripts during the moisture stress test. Such differences might be due to the wider taxonomic affiliations of the two microbial groups (higher extracellular osmolality tolerated by archaea compared to bacteria as depicted by Martin et al. [47]). Furthermore, they are indicative of the potential difference of the importance *of amoA* gene for basic metabolic activity among the two microbial groups, as indicated elsewhere [27, 48–50]. This is further supported by the identified differences in *amoA* transcript abundance, where average AOB *amoA* transcript copies were up to twofold higher than the AOA *amoA* transcript copies (a difference similar to the one observed also in the control treatment of the severe stress experiment performed in this study), while *amoA* gene copy levels were similar between the microbial groups.

Zinc Dose Response

Potential Nitrification Transcript and Gene Copy Numbers

Microcosm water-leaching reduced the effects of spiking on pH, previously shown to affect enzymatic activity, *amoA* presence, and expression in soil [11, 30]. Moreover, significant reduction of the CaCl$_2$-extractable Zn after leaching when compared to the total (background plus applied) prior leaching was observed only in Zn concentrations above

1,000 mg kg^{-1}, way above the [Zn] point where gene expression and potential nitrification reached almost zero values.

Potential nitrification data indicate a decrease of the enzymatic activity along with increasing zinc concentration, with most of it being abolished at applied Zn concentrations of 500 mg kg^{-1} and above (Fig. 1a). This is consistent with previous observations of enzyme inhibition due to competition of zinc with copper for placement in the AMO active site [4].

Relative average values of transcripts showed a more immediate response compared to potential nitrification (Fig. 2a), implying a higher resilience of the enzymatic functionality and presence as opposed to measured transcripts. Transcript numbers readily dropped for both the bacterial functional and the 16 S rRNA marker genes at above 0 mg kg^{-1} of applied [Zn], thus indicating total cell activity loss for AOB. This result is consistent with the results of previous studies indicating a downregulation of the carbon fixation RubisCo marker in *Nitrosococcus mobilis* and *Nitrosomonas europaea* under [Zn] ranging from 65.8 to 658 mg l^{-1} of liquid cultures [4, 6], hence suggesting that several basic metabolic functions and therefore total cell activity of ammonia oxidizers are affected by zinc stress.

The constitutive expression of unicellular organism housekeeping genes like the 16 S rRNA gene has been debated in the past, particularly concerning natural environments [51, 52]. In the present study, bacterial *amoA* transcript counts were correlated with measured AOB 16 S rRNA (Fig. 2a and obtained $r_s = 0.9$, $r = 0.76$ with respective P values of 0 and 0.0036) copies in the examined soils, while they were simultaneously downregulated as shown by *amoA* gene level stability (Fig. 2b), thus indicating that phenotypic *amoA* expression stability when the 16 S rRNA gene expression is used for normalizing the measured *amoA* transcript copies might be observed but not necessarily true. Therefore, both AOB 16 S rRNA gene and *amoA* expression are good total cell activity markers, while 16 S rRNA transcripts have reduced value for use as a normalization means in quantitative assays for soil environments like the one presented here.

Concerning DNA qPCR templates, [Zn] effects were observed only for AOB at 5,000 mg kg^{-1} according to qPCR results (Fig. 2b). Such reduction is consistent with extracellular DNA degradation, previously found to occur in some cases within 12 h, revealing cell death [53].

AmoA Transcript Patterns and Correlations with Applied [Zn]

Rr and richness (*S*) values of *amoA* transcripts followed similar to the *amoA* transcript qPCR trends for both AOA and AOB with the AOA slightly deviating with an increase for 125 [Zn] and AOB having a significant drop for

125 mg kg^{-1} [Zn] and above. Such reduction could indicate a high selectivity of the applied stress. Nevertheless, DGGE patterns showed [Zn]-dependent transcriptional profiles for AOB, while this was not the case for AOA where results showed more random patterns according to clustering analysis (Fig. 3a), while strong support to this outcome was provided by ANOSIM *R* values (Fig. 4). Therefore, higher importance of AOB was indicated concerning potential nitrification rates in the examined agricultural soil. However, the soils examined here were soils with low organic matter content, receiving nitrogen in mineral forms, previously shown to favor AOB [54, 55]. Similar responses were observed also in the study of Mertens et al. [31] where no major differences of crenarchaeal *amoA* DGGE patterns obtained from DNA templates could be connected to the applied Zn doses as opposed to the bacterial ones.

Distinct AOB genotypes were observed according to PCoA analysis of *amoA* transcript fingerprints for the various applied [Zn] (Fig. 3d), while less respective specificity could be extrapolated for AOA. Lack of specific sensitivity of the *amoA* homologue transcription in AOA to [Zn] along with the relatively lower *amoA* expression compared to AOB may imply lower importance of this gene for the physiology of AOA found in the examined soils. This outcome is further supported by the correlation identified between AOB *amoA* transcript pattern shifts with respective *amoA* and 16 S rRNA gene transcripts. These results are consistent with the results of Xia et al. [56] where AOB mainly were found to be responsible for ammonia oxidation in the tested agricultural soil, as indicated by *amoA* sequence analysis combined with stable isotope probing for assessing carbon fixation by ammonia oxidizers. Out of the screened AOA, only a small portion was shown to perform carbon fixation and therefore demonstrated an autotrophic lifestyle.

Concluding Remarks

Recent discoveries related to nitrification have changed the perception of several related concepts in this research area and generated several questions. One step towards shedding light in nitrification partition among AOB and AOA in agricultural soils was investigated in the present study by looking into the effects caused by one common mild stress and one severe stress.

AOB were shown to be more affected by the moisture shift compared to AOA concerning transcription indicating a stress response. Moisture shift-associated microenvironment changes are indicative of physiological differences between AOB and AOA. Acute responses to Zn indicated that *amoA* expression was higher for AOB than AOA when not completely suppressed by zinc. On the other hand,

bacterial ammonia oxidizers appear to be more sensitive to the applied stress than the respective archaeal guild. The latter was demonstrated in the highest applied Zn dose in a DNA level and also according to the *amoA* average relative expression values per treatment that were overall lower for AOB. Rapid reduction of the 16 S rRNA for bacterial ammonia oxidizers in a similar fashion as with the *amoA* transcripts demonstrated a most probable viability loss rather than a recoverable state. Particular bacterial genotypes showed a strong correlation to certain applied Zn doses while poor connection was observed for archaeal ammonia oxidizers.

Collectively, differences observed in the transcriptional responses between AOB and AOA *amoA* reflect potential differences in the importance of the encoded protein for basic metabolism between the referred groups. Therefore, our results further support previously stated opinions and study outcomes about potential niche differentiation [48, 57].

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