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Recovery of soil ammonia oxidation after long term zinc exposure is not related to the richness of the bacterial nitrifying community

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Abstract

A soil sterilization-reinoculation approach was used to manipulate soil microbial diversity and to assess the effect of the diversity of the Ammonia Oxidizing Bacteria (AOB) on the recovery of the nitrifying community to metal stress (zinc). Gamma irradiated soil was inoculated with 13 different combinations of up to 22 different soils collected worldwide to create varying degrees of AOB diversity. Two months after inoculation, AOB *amoA* DGGE based diversity (weighted richness) varied more than 10 fold among the 13 treatments, the largest value observed where the number of inocula had been largest. Subsequently, the 13 treatments were either or not amended with ZnCl_2 . Initially, Zn amendment completely inhibited nitrification. After six months Zn exposure, recovery of the potential nitrification activity in the Zn amended soils ranged from <10% to >100% of the potential nitrification activity in the corresponding non-amended soils. This recovery was neither related to DGGE based indices of AOB diversity nor to the AOB abundance assessed two months after inoculation ($p>0.05$). However, recovery was significantly related ($r=0.75$) to the potential nitrification rate before Zn amendment and only weakly to the number of soil inocula used in the treatments ($r=0.46$). The lack of clear effects of AOB diversity on recovery may be related to an inherently sufficient diversity and functional redundancy of AOB communities in soil. Our data indicate that potential microbial activity can be a significant factor in recovery.

Key words: Recovery/Microbial diversity/DGGE/Nitrifying community/zinc

Introduction

Microbial biodiversity only weakly affects generic soil microbial functions such as respiration and N-mineralisation, likely because of functional redundancy [12]. Biodiversity may however have a more important role in the functional stability (resistance and resilience) of the community following disturbances or stresses. Indeed, it has been proposed that the resistance of the soil microbial community, i.e. the ability to withstand a disturbance or stress, can increase with increasing biodiversity because of a higher likelihood that populations tolerant to the disturbance or stress are present [15]. Moreover, the resilience of a microbial community, i.e., its functional recovery rate after a disturbance or stress, may increase with increasing biodiversity, since more populations are available to adapt to the changing environment. However, there is little evidence that supports these hypotheses in soil microbial ecosystems. Girvan et al. [6] showed no difference in the recovery of carbon mineralization after copper (Cu) amendment as a stress factor between two soils with intrinsic differences in microbial diversity. Griffiths et al. [7] reported that the addition of Cu initially reduced the decomposition rate of plant residues to a larger extent in soils that have been fumigated to decrease the microbial biodiversity compared to the non-fumigated soil. In contrast, adaptation after two months Cu exposure was as fast in the fumigated soil as in the non-fumigated soils [9]. Similar trends were observed in a study on the effect of a transient heat stress on nitrification [37].

To experimentally achieve different soil microbial biodiversities, these authors either applied fumigation [7] or dilution of soil samples and reinoculation in sterile soil [8; 37]. Both methods have their limitations. While dilution of the soil results into a gradual elimination of low abundant populations, the relative proportions of the dominant populations, presumed to

contribute the most to the community function, will remain constant. Fumigation decreases the diversity by selectively eliminating fumigation-sensitive populations. An alternative method to manipulate soil microbial diversity, designated as sterilization-reinoculation methods, applies inoculation of a sterile soil with different bacterial species [21] or with soil aliquots containing different communities [10]. Rapid recolonization of sterile soil (within a few days) using the latter method has been shown [16]. This method has been used previously to ensure constant abiotic soil properties in experiments that investigated the toxicity of heavy metals to microbial processes [2; 4; 30] as metal toxicity depends on soil properties such as pH or effective cation exchange capacity (eCEC). For instance, metal toxicity decreases with increasing eCEC due to decreasing metal bioavailability [34]. Hence, abiotic factors might obscure biological parameters and should be excluded in order to assess the effect of metal stress on the microbial community.

Several studies have demonstrated that the initial negative effect of increased Zn concentrations on nitrification disappears on the long term (> one year) due to selection of Zn tolerant species as indicated by a changing nitrifying community structure with increasing Zn concentrations [30; 20; 31; 38]. It was speculated that this is the result of the proliferation of tolerant species already present in the community at the expense of Zn sensitive species which become less dominant or disappear [1]. Soil contains both ammonia oxidising bacteria (AOB) and archaea (AOA) [14]. Although the role of AOA in ammonia oxidation in soil is still debated [26], recovery of nitrification to Zn stress seems to be mainly due to the activity of AOB, rather than AOA [19; 31]. However, until now no information exists about the role of the AOB diversity in this recovery. The objective of this study is to relate the functional recovery of the potential nitrification after Zn stress to the AOB diversity. AOB diversity was experimentally varied using the sterilization-inoculation method. Sterilized soil was inoculated with different soils containing

different AOB communities or soil mixtures thereof. After two months incubation, Zn was amended and recovery of the soil bacterial nitrifying community was determined after six months Zn exposure by comparing the potential nitrification rate (PNR) in Zn amended soils to this of the control soil which did not receive Zn. The AOB diversity was quantified using indices calculated from AOB *amoA* DGGE profiling and by the number of soils used as inoculum which is a technique-independent index of diversity.

Materials and methods

Soil treatments

Ter Munck (TM) soil (Belgium) [33] was used as the matrix soil for creating 14 soil treatments with different microbial diversity, including one non-inoculated control. This was achieved by inoculation of sterile TM soil with aliquots from other soils (see below). Sterile TM soil was obtained by γ -irradiation (Co-60) at 25 kGy [36]. Sterility of the soil was confirmed by the absence of CFU on LB agar medium plated with a soil extract (1:5 Soil:Liquid ratio) after 10 days of incubation at 30 °C. Soil pH (CaCl₂ 0.01M) remained unchanged after irradiation (pH 6.2). The irradiated soil had 11% clay, 70% silt, 1% organic C and the effective Cation Exchange Capacity (eCEC) was 12.2 cmol_c kg⁻¹ [33]. The soil was stored in a closed container at room temperature for two weeks and then divided into 14 portions of 500 g. At inoculation and during the course of the experiment, the soil was not handled in sterile conditions and only covered with parafilm to ensure aerobic conditions.

Twenty two top soils used for inoculation were collected worldwide (six soils from Belgium, three from Australia, three from Spain, one from Italy, Greece, Denmark, Sweden, Hungary, Canada, Vietnam, USA, Kenya and Nigeria) and were stored air-dry. After rewetting to 70% of

field capacity, these soils showed different AOB *amoA* DGGE profiles and detectable potential nitrification activity. The pH of the soils ranged from 4.0 to 7.7. The individual soils or mixtures thereof were inoculated into sterile TM soil at 5 wt% in total (25 g soil in 475 g sterile TM soil). The soils for inoculation were selected based on contrasting AOB diversities as suggested by their AOB *amoA* DGGE profiles. Moreover, dedicated mixtures of the individual soils were included assuming that diversity increases with an increasing number of inoculum soils. Eight treatments were inoculated with one of eight different soils as listed in Table 1 (single soil inocula treatments) and four treatments were inoculated with mixtures of these soils (mixture 1-4; Table 1). Mixture 5 contained the same eight and 14 other soils (four soils from Belgium, three from Australia, two from Spain, one from Italy, Canada, Vietnam, USA and Nigeria) (Table 1). In case of inoculation with mixtures, the 25 g inoculum consisted of equal fractions (dry weight based) of each soil except for the Teso and Souli soils that had a lower AOB *amoA* abundance than the other soils (6.4×10^5 and 6.4×10^6 , respectively). Therefore, Teso and Souli soils were added at 5-fold larger doses than the other soils in the mixtures. A 14th treatment was not inoculated. After inoculation, all treatments were amended with 20 mg $\text{NH}_4^+\text{-N kg}^{-1}$ (as $(\text{NH}_4)_2\text{SO}_4$) to stimulate nitrification and the establishment of a nitrifying microbial community. Treatments were moistened to 20% moisture content, well homogenized and incubated in 1L plastic pots (height 14 cm, diameter 11 cm) as one replicate for two months at 25 °C while covered with parafilm, before Zn amendment.

Table 1: Soils and soil mixtures used to inoculate the sterile Ter Munck soil to vary the microbial diversity. Eight treatments were inoculated with one of the eight soils listed. Four other treatments (Mixture 1-4) were inoculated with a mixtures of these eight soils as indicated. Mixture 5 consisted of all these soils and 14 other soils (not shown).

Soil name	Country	Mixture 1	Mixture 2	Mixture 3	Mixture 4
Teso ¹	Kenya		X	X	X
Guadalajara	Spain	X			
Zeveren	Belgium		X	X	
Souli ¹	Greece			X	X
Borris	Denmark			X	X
Nagyhörösök	Hungary	X		X	
Kasterlee	Belgium	X		X	
Kövlinge	Sweden		X	X	

¹ The weight proportion of these soils in the mixtures was 5 times higher than that of the other soils to equalize the proportion of the AOB abundance of each soil in the mixture

Recovery experiment

The recovery experiment started two months after inoculation. Prior to Zn amendment, soil DNA was extracted in duplicate samples from all treatments to assess the *initial* AOB diversity and AOB abundance (as described below). In addition, the *initial* Potential Nitrification Rate (PNR) was determined in duplicate samples per treatment (two technical replicates) and the *initial* Zn tolerance was determined on one soil sample per treatment. Subsequently, each soil treatment was divided into four portions (125 g) of which two were amended with 1500 mg Zn kg⁻¹ (as ZnCl₂) and two control portions received no Zn. This resulted in 56 soils (14 treatments each divided into two non-Zn amended soils and two Zn amended soils). The soils were incubated for an extra 6 months in perforated 200 ml plastic pots (height 10 cm, diameter 5 cm) covered with

parafilm in darkness at 25 °C. After one week incubation, soils were leached with artificial rain water to remove excess of salts due to Zn application as described by Oorts et al. [25]. Non-Zn amended soils were also leached. The soil pH was subsequently measured. Soil pH decreases due to Zn^{2+} salt amendment and nitrification and, conversely, pH affects Zn bioavailability and nitrification activity. The pH of the Zn amended soils decreased by 1 pH unit three weeks after Zn application and the soils were limed with $0.41 \text{ g CaO kg}^{-1}$ to restore soil pH to 6.2 as in non-Zn amended soils. The soil pH was not determined during the remaining incubation period. Simultaneously with liming, $150 \text{ mg NH}_4^+\text{-N kg}^{-1}$ was applied to Zn amended and control soil samples to accelerate recovery of the potential nitrification [31]. Soils were leached once more at two months after Zn amendment with artificial rain water and CaCl_2 (0.01M) to remove produced $\text{NO}_3^-\text{-N}$ which can inhibit nitrification, followed by application of $150 \text{ mg NH}_4^+\text{-N kg}^{-1}$. A third leaching was conducted two weeks before determining recovery, without a subsequent $\text{NH}_4^+\text{-N}$ application. After six months Zn exposure (eight months incubation in total), samples were taken from all soils to measure the PNR and calculate the recovery.

Potential nitrification rate (PNR)

Soil nitrification was quantified as the Potential Nitrification Rate (PNR) [33] and performed in suspension at a pH of 6.2. Briefly, soil moisture content of the soil samples was adjusted to 20% 7 days prior to the start of the assay. Then, soil samples were suspended in 0.01M CaCl_2 with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.2) (2 g soil in 10 ml liquid) and NH_4Cl solution (15 mg ml^{-1}) was added at an added concentration of $200 \text{ mg NH}_4^+\text{-N kg}^{-1}$ dry soil. Samples of 3 ml were taken immediately after $\text{NH}_4^+\text{-N}$ addition and after seven days head-over-end (28 rpm) incubation at 20 °C. Potassium chloride 4 N was added to a final concentration of 1

N, samples were shaken head-over-end for two hours (28 rpm), centrifuged (3000 g, 10 min) and the NO_3^- -N concentration was measured colorimetrically in the supernatant (SA 40, Skalar, The Netherlands). The PNR was calculated as the amount of nitrate produced per kg dry soil over time (mg NO_3^- -N $\text{kg}^{-1} \text{ d}^{-1}$). The PNR at two months was determined on two soil samples for each inoculated treatment, the PNR at 8 months was determined on one replicate of each of the four pots.

Zn tolerance assay

Zinc tolerance was measured by means of the spike-on-spike test as described by Mertens et al. [20]. Briefly, ZnCl_2 (50 mg ml^{-1}) was added to a 2.5 g soil sample suspended in CaCl_2 0.01 M (1:10 solid:liquid ratio) with 25 mM MES buffer (pH 6.2) at added concentrations of 0 (two samples), 15, 30, 75, 150, 300 and 500 mg Zn l^{-1} . Ammonium chloride solution (15 mg ml^{-1}) was added at a concentration of 100 mg NH_4^+ -N kg^{-1} . The pH in suspension was adjusted immediately after N addition and every other day during the seven day incubation period to $\text{pH } 6.2 \pm 0.1$ by adding 0.1 M NaOH or HCl if required. The samples were incubated at 20 °C and continuously shaken head-over-end (28 rpm). Nitrate concentrations were measured as described in the PNR assay at the start of the experiment and after seven days, and the potential nitrification rate was calculated as the amount of nitrate produced per kg dry soil over time (mg NO_3^- -N $\text{kg}^{-1} \text{ d}^{-1}$). Soluble Zn in the extract was measured in the supernatant after centrifugation of the soil suspension (3000 g, 15 min) using ICP-OES (Perkin Elmer Optima 3300 DV, Norwalk, CT, USA). The potential nitrification rate in suspension was related to measured equilibrium Zn concentrations in the CaCl_2 soil extract. Zinc tolerance was expressed as the dissolved Zn concentration at which the potential nitrification rate was 50% reduced compared to a control

suspension (no Zn added) (EC50) which was interpolated from the dose-response curve fitted by log-logistic modeling using the Marquardt method (proc NLIN, SAS 9.2, NC, USA) [3]. Zinc tolerance was determined on one soil sample of each inoculated treatment after two months incubation.

amoA AOB abundance and diversity

Soil DNA was extracted (0.5 g) and purified using the MO BIO Powersoil DNA Isolation Kit according to the manufacturer's protocol (MO BIO laboratories, California, USA). Phylogeny of the AOB *amoA* gene, responsible for the first step in nitrification, has previously been shown to be congruent with 16S rRNA phylogeny [27] suggesting that the *amoA* gene is a suitable marker to determine the AOB community structure and diversity [13]. Copy numbers of AOB *amoA* were quantified in duplicate in each DNA extract by means of real-time PCR using a Corbett Rotor-Gene 3000 real-time PCR cycler (Qiagen, Venlo, The Netherlands) as described previously [31] using primers *amoA*-1F and *amoA*-2R [29]. Dilution series of purified PCR products from the cloned *amoA* gene of *Nitrosomonas europaea* ATCC 19718 (AOB) were used as qPCR standards. The efficiency of the reaction was 0.84. Copy numbers of AOB *amoA* were transformed to the numbers of AOB assuming that on average 2.5 *amoA* copies were present per AOB cell [23]. PCR amplification of AOB *amoA* using primers GC-*amoA*-1F* and *amoA*-2R [29; 35] was performed at an annealing temperature of 60 °C as described [19]. Denaturing Gradient Gel Electrophoresis (DGGE; Ingeny, Goes, The Netherlands) profiling of AOB *amoA* was performed on 6% polyacrylamide gels with a linear gradient of 40-60%, run for 16h at 60 °C and 100V as described [20].

Data analysis

The initial PNR, AOB diversity and AOB abundance, i.e. values after two months incubation prior to Zn amendment, is reported as average values of two replicates per treatment. Duncan grouping ($p < 0.05$) was performed to detect significant differences among treatments. Differences in initial Zn tolerance among treatments were determined by analyzing the 95% confidence limits of the EC50 value and treatments were manually grouped in statistical different groups if confidence intervals were not overlapping. Each treatment was separated in two Zn amended and non-Zn amended soils and the PNR was measured on these duplicates six months after Zn amendment. Recovery was quantified as the ratio of the PNR in the Zn amended soils to the average of the corresponding PNR in the non-Zn amended soils after six months Zn exposure according to:

$$\text{recovery (\%)} = \frac{\text{average PNR}_{\text{Zn 6 months}}}{\text{average PNR}_{\text{controls 6 months}}} \times 100 \quad (\text{Equation 1})$$

The standard deviation of recovery was calculated with the standard deviation of the nominator and denominator assuming no covariance. Significant recovery was assumed when the PNR in the non-Zn amended soils did not significantly differ from the PNR in the Zn amended soils (t-test, $p > 0.05$). Stepwise multiple regression analysis was performed to analyze recovery in relation to AOB diversity, abundance, activity and Zn tolerance of the treatments after two months incubation and prior to Zn amendment (proc REG, SAS 9.2, NC, USA; significant limits to enter a variable set at $p = 0.15$). Pearson correlation coefficients among variables were calculated (proc CORREL, SAS 9.2, NC, USA). For both analyses, average values per treatment were used for the initial AOB diversity, initial AOB abundance, initial PNR and initial Zn tolerance whereas the duplicate recovery values were used individually. The DGGE profiles were

analyzed using Gelcompar 4.6 (Applied Maths, Belgium). To determine the AOB diversity all bands in each DGGE profile were manually assigned. The AOB diversity based on *amoA* DGGE profiling was expressed as the weighted richness as proposed by Mertens et al. [18]. The weighted richness is a dimensionless value representing the number of bands corrected for the gradient span on the DGGE gel and is a measure for the number of different phylotypes present and the genetic variability within these phylotypes [17]. The number of bands in the DGGE profile, the Shannon-Wiener index based on *amoA* DGGE profiling were used as additional indices for diversity to show whether relations are due to the use of a particular diversity index. The number of soils used in the different inocula - i.e. a *nominal* proxy of diversity - is a technique-independent diversity index, since a larger AOB diversity is expected with increasing number of soils used in the inocula. However, it does not account for possible shifts in diversity upon subsequent growth of the communities after inoculation. Band matching classes grouping similar bands across the DGGE profiles were automatically defined in Gelcompar using default parameters and manually checked. Band matching data (band presence and intensity) obtained from the DGGE analysis were used to graphically compare the AOB community in the single soil inocula treatments with the AOB community in the treatments inoculated with the mixtures using two-dimensional nMDS ordination based on the Bray-Curtis similarity measure (default parameters, PC-ORD v5) [28].

Results

Establishment and diversity of the AOB community after two months incubation and prior to Zn amendment

No potential nitrification activity (PNR) was detected after two months incubation prior to Zn amendment in the non-inoculated treatment (the soil that was sterilized at the start) and only a few, weak bands were observed in the AOB *amoA* DGGE profile (Figure 1). The PNR after two months incubation in the inoculated treatments ranged from 1.5-4.5 mg NO₃⁻-N kg⁻¹ d⁻¹ in treatments inoculated with soil mixtures 1 to 5 and from 0.5-3.5 mg NO₃⁻-N kg⁻¹ d⁻¹ in single soil inocula treatments (Table 2). The AOB abundance was of the same order of magnitude for all inoculated treatments (6.6×10⁷ AOB g⁻¹ soil on average; Table 2) and comparable with values previously reported in soils [14; 19; 22; 24; 31]. Zinc tolerance before Zn exposure was low for all inoculated treatments, but significantly differed among treatments ranging from 0.4 mg Zn l⁻¹ in the treatment inoculated with Teso (most sensitive) to 7 mg Zn l⁻¹ in the treatment inoculated with the Zeveren or Nagyhöröcsök soil (Table 2). Expected AOB weighted richness, calculated based on the AOB *amoA* DGGE profiles of the soils that served as inocula (DGGE profiles not shown) were compared to the measured AOB weighted richness based on the AOB *amoA* DGGE profiling of the inoculated treatments two months after inoculation and prior to Zn amendment (Table 2).

Table 2: Relevant microbial properties of re-inoculated soils two months after inoculation (before Zn exposure) (diversity, abundance, PNR and zinc tolerance) and recovery after 6 months Zn exposure. The AOB diversity is the weighted richness based on the DGGE bands. The Shannon Wiener index, number of bands and the number of soil inocula are shown in the Supplementary information (Table S-1). The potential nitrification rate (PNR) refers to the non-Zn amended soils. Recovery is calculated as given in equation 1. Significantly different means ($p < 0.05$) are denoted with different letters and standard deviations are given in brackets ($n = 2$).

Treatment (inocula)	Expected ¹ AOB Diversity (Richness)	Measured AOB Diversity (Richness)	AOB abundance ² (log AOB g ⁻¹ soil)	Zn tolerance (mg Zn l ⁻¹)	PNR (2 months) ² (mg NO ₃ ⁻ -N kg ⁻¹ d ⁻¹)	PNR (8 months) ² (mg NO ₃ ⁻ -N kg ⁻¹ d ⁻¹)	Recovery ² (%)
Mixture 1	83	33 ^{CDE} (4.5)	8.0 ^{ABC} (0.4)	4.3 ^A	2.8 ^C (0.04)	4.6 ^F (1.3)	77 (80)
Mixture 2	72	94 ^{AB} (16.5)	7.7 ^{AB} (0.3)	1.0 ^D	1.6 ^E (0.004)	6.9 ^{BCD} (1.2)	8 (9)
Mixture 3	196	71 ^{BC} nd	7.7 ^{ABC} (0.2)	2.0 ^{CD}	2.6 ^{CD} (0.5)	2.2 ^{EF} (0.7)	21 (27)
Mixture 4	97	98 ^{AB} (3.5)	7.4 ^{BCD} (0.1)	1.2 ^D	1.6 ^E (0.1)	6.4 ^{BCD} (0.4)	3 (3)
Mixture 5	>>	114 ^A (51.3)	7.8 ^{ABC} (0.3)	4.6 ^A	4.5 ^A (0.2)	1.9 ^F (0.9)	138 (122)
Kövlinge	5	6 ^E (0.6)	6.4 ^E (0.7)	2.3 ^{BC}	0.1 ^F (0.04)	7.7 ^{CB} (0.5)	1 (1)
Nagyhörcsök	17	7 ^E (0.6)	8.3 ^A (0.6)	6.5 ^A	2.3 ^D (0.01)	11.1 ^A (0.04)	89 (21)
Kasterlee	46	10 ^E (0.1)	7.6 ^{BC} (0.5)	5.9 ^A	3.4 ^B (0.3)	2.6 ^{EF} (0.003)	124 (57)
Borris	46	20 ^{ED} (0.1)	7.4 ^{DC} (0.7)	3.9 ^B	1.4 ^E (0.1)	7.5 ^{BCD} (0.9)	3 (3)
Souli	15	57 ^{BCD} (9.7)	8.0 ^{AB} (0.2)	5.7 ^A	0.6 ^F (0.1)	4.8 ^{ED} (3.1)	1 (1)
Zeveren	31	67 ^{BC} (1.8)	7.9 ^{ABC} (0.1)	6.6 ^A	1.3 ^E (0.1)	2.0 ^F (1.8)	45 (51)
Guadalajara	20	70 ^{BC} (8.2)	7.9 ^{AB} (0.4)	3.3 ^{BC}	0.5 ^F (0.1)	8.5 ^B (1.1)	6 (4)
Teso	36	81 ^{AB} (19.3)	7.0 ^D (0.5)	0.4 ^{CD}	1.3 ^E (0.1)	5.4 ^{CD} (1.0)	5 (3)

¹ The expected AOB diversity is derived from the *amoA* DGGE profiles of the soils before inoculation (see text); ² Standard deviation is given in brackets ($n = 2$); >> Richness of mixture 5 could not be estimated, but is assumed to be very high, since it is comprised of 22 soils; nd: not determinable

The values of the additional diversity measures (number of bands and Shannon-Wiener index) are reported in Supplementary Information Table 1. As expected, the AOB weighted richness and number of bands was low in the treatments inoculated with the Kövlinge and Nagyhörsök soils. In contrast, the measured weighted richness was well below the expected weighted richness in the treatments inoculated with the Borris and Kasterlee soils. For all the single soil inocula treatments, except for the treatment inoculated with the Kasterlee soil, the measured number of bands was higher than the expected number of bands (Supplementary Information Table 1). In the treatments inoculated with mixtures, the expected AOB weighted richness was calculated as the sum of the AOB weighted richness measured in the soils used in the mixture before inoculation. For the treatments inoculated with mixtures the measured AOB weighted richness was similar (mixture 4), larger (mixture 2) or lower (mixture 1, 3 and 5) than what was expected, whereas the number of bands after inoculation was lower than expected for all treatments (Supplementary Information Table 1). The measured AOB weighted richness and number of bands of the treatments inoculated with mixture 2, 4 and 5 was larger than those of the treatments inoculated with single soils (Table 2). The AOB *amoA* DGGE profiles of the treatments inoculated with the soil mixtures showed resemblances with those of single soil inocula treatments of the soils that were used in these mixtures (Figure 1). Figure 1 in Supplementary Information shows the two-dimensional nMDS ordination graphs based on Bray-Curtis similarity for the treatments inoculated with the mixtures compared to the single soil inocula treatments inoculated with the soils used in the mixture. In total, both axes explained at least 60% of the variation among all mixtures. The established community in the treatments inoculated with the mixtures was structurally a mixture of the single soil inocula treatments except for the treatment inoculated with mixture 2 where the Teso soil dominated the AOB community. Teso soil was

inoculated 5 times more than other soils to equalize the inoculated abundance of the AOB in the mixtures. Note that mixture 5 consisted of more than the eight soils displayed, the remaining 14 soils were not used as single inocula and, therefore, not included in the graph. In conclusion, communities with varying AOB diversity established among treatments and overall, the AOB diversity was increased by inoculating mixtures of several soils.

Figure 1: AOB *amoA* DGGE profiles of the inoculated treatments after two months incubation in the absence of Zn.

Recovery in inoculated treatments after six months Zn exposure

One week after Zn contamination, potential nitrification activity was undetected in all Zn amended soils. The PNR in the non-Zn amended soils were similar to the PNR in the corresponding treatments after 2 months incubation before Zn addition. After eight months incubation, potential nitrification activity restored (PNR = 11 mg NO₃⁻-N kg⁻¹ d⁻¹) in the non-Zn amended soils of the *non-inoculated treatment* and its AOB *amoA* DGGE showed two weak bands suggesting that some AOB phylotypes established, probably due to non-sterile handling (data not shown). No recovery of the PNR was recorded in the Zn amended soils of the non-inoculated treatment. No significant recovery of the potential nitrification was observed in the Zn amended soils of seven of the *inoculated treatments* (recovery <10%; Table 2). In contrast, full recovery was observed in the Zn amended soils of the treatment inoculated with mixture 5 (138% recovery), the Nagyhörcsök (89%) and the Kasterlee (124%) soil (Table 2). Partial recovery ranging from 21%-77% was observed in the Zn amended soils of the treatments inoculated with mixtures 1, 3 and with the Zevenen soil. Recovery was not significantly correlated with the initial AOB weighted richness (Table 3 and Figure 2). Other AOB diversity indices were correlated to the AOB weighted richness (Table 3). Recovery was significantly, and positively, correlated to the number of soil inocula, the initial Zn tolerance and, most strongly, to the initial PNR (Table 3 and Figure 2). Multiple regression showed that, among the variables displayed in Table 3, only the initial PNR and initial Zn tolerance were significantly correlated to recovery (partial R² = 0.56, p < 0.15 and partial R² = 0.10, p < 0.15, respectively). Similarly, when grouping the treatments inoculated by either mixtures or single soils or with similar initial PNR values, no direct effect of the AOB diversity on recovery was observed (Table 4).

Figure 2: Recovery (equation 1) of the potential nitrification after long term Zn exposure in relation to the initial diversity of the corresponding inoculated treatments (□ weighted richness, left graph), and to the initial potential nitrification activity (○ PNR, right graph). Initial diversity and initial PNR are average values of two technical replicates, two replicate values of recovery are given. Linear regression was used to evaluate the relation (weighted richness: $R^2 = 0.005$, $p=0.63$; PNR: $R^2 = 0.56$, $p<0.001$). Gray shaded symbols: treatments inoculated with soil mixtures; open symbols: single soil inocula treatments.

Table 3: Pearson correlation (r-values) among microbial properties before Zn stress (all properties except recovery) and recovery after Zn stress.

	Initial	Zn	AOB	AOB	# AOB	AOB Shannon	#	
	PNR	tolerance	abundance	richness	bands	Wiener	inocula	Recovery
Initial PNR	1.00							
Zn tolerance	0.26	1.00						
AOB abundance	0.20	0.47 ¹	1.00					
AOB richness	0.18	-0.44 ¹	0.11	1.00				
# AOB bands	0.26	-0.45 ¹	0.03	0.93 ¹	1.00			
AOB Sh-Wi	0.20	-0.38	0.14	0.89 ¹	0.92 ¹	1.00		
# inocula	0.72 ¹	0.00	-0.07	0.55 ¹	0.59 ¹	0.42 ¹	1.00	
Recovery	0.75 ¹	0.50 ¹	0.20	-0.10	-0.05	-0.08	0.46 ¹	1.00

¹ Significant correlations ($p < 0.05$)

Table 4: Stepwise multiple linear regression to explain recovery with microbial properties before Zn stress (units in Table 2) for three groups of data: all treatments, mixtures and single soil inocula treatments. Grouping the measurements by similar initial PNR values did not reveal any factor explaining recovery ($p > 0.15$). Average values were used for the initial PNR, AOB abundance, AOB richness (technical replicates, $n=2$), treatment replicates ($n = 2$) for recovery were included. Zinc tolerance was not determined in duplicate.

All	partial					Mixtures	partial				
	Intercept	Slope	R ²	p			Intercept	Slope	R ²	p	
Step 1						Step 1					
PNR	-23.77	34.45	0.56	< 0.0001		PNR	-68.85	44.73	0.56	0.01	
Step 2											
Zn											
tolerance	-49.56	9.03	0.10	0.01							
PNR		30.47	0.56	< 0.0001							

Single	Soil	partial			
Inocula		Intercept	Slope	R ²	p
Step 1					
PNR		-21.56	41.06	0.73	<0.0001

Discussion

The contribution of the different single soil inocula to the AOB community that developed in the treatments inoculated with mixtures was clearly visible (Figures 2 and Supplementary Information Figure 1), but especially for the Teso soil in mixture 2, suggesting that AOB phylotypes of Teso were positively selected. However, the dominance of the AOB community of Teso in this treatment might also be attributed to the relative larger proportion of the soil in the mixtures to equalize the initial AOB abundance inoculation. Marschner and Rumberger [16] showed that the similarity of the reinoculated soil community with the original soil community increased with increasing inoculum proportion. In general, all soils used in the mixtures contributed to the established community two months after inoculation as displayed in Supplementary Information Figure 1. The obtained dissimilarity shows the creation of a new community structure and hence a modified AOB diversity due to mixing soils. Among all 13 treatments the AOB weighted richness varied between 6 and 114 and the number of bands between 15 and 34 showing the creation of treatments with a varying AOB diversity. In conclusion, we created 13 different soil microbial communities with contrasting AOB diversity, without changing abiotic soil properties, such as eCEC and pH by inoculating a sterilized soil with aliquots of soils and soil mixtures. In addition, potential nitrification was detected in all treatments after two months incubation. This potential nitrification (initial PNR) was different among treatments and was not correlated with the AOB abundance. Apparently, a similar AOB abundance among treatments does not ensure a similar activity, suggesting that not all populations are active or that nitrification activity is different among different phylotypes.

The applied Zn stress after two months incubation was sufficient to completely inhibit nitrification after one week Zn exposure in all Zn amended soils of all treatments. As such, the AOB diversity did not affect the initial resistance to the applied Zn stress, which is in contrast to a previous study where a positive relation between diversity and resistance of the plant decomposition rate directly after copper addition was found [7]. This lack of an effect of AOB diversity on the resistance might be ascribed to the larger Zn dose used in this study (1500 mg Zn kg⁻¹), which might not be suitable for resistance testing. This dose was chosen based on a previous study in another soil, which showed that adaptation of potential nitrification occurred concomitant with changes of the nitrifying community at this concentration [31]. Lower concentrations might fail to induce changes of the community structure.

Potential nitrification recovered in some of the Zn amended soils after six months Zn exposure. It is speculated that a soil with a more diverse microbial community can adapt faster, because of a larger likelihood that populations tolerant to the disturbance or stress are present and that more populations are available to phenotypically or genotypically adapt to the changing environment [15]. However, none of the diversity indices correlated to recovery despite a marked difference in diversity among treatments. The number of different soil inocula - a *nominal* proxy of the potential initial diversity – was positively correlated to the recovery (Table 3). This weak correlation ($r=0.46$, Table 3) is, however, based on only five treatments which had more than one inoculum. The number of inocula might also indirectly enhanced the recovery by increasing the initial PNR, since both are positively correlated ($r=0.75$, Table 3). Studies in which the diversity was experimentally decreased by inoculating soil dilutions previously showed similar trends. Griffiths et al. [8] observed no consistent effect of the microbial diversity on recovery of the potential nitrification and thymidine incorporation to Cu, and Wertz et al. [37] similarly observed

no effect of decreasing nitrite oxidizers diversity on the recovery of the nitrification after a transient heat stress in soil. In general, a lack of correlation between diversity and recovery when the microbial diversity is either experimentally increased or decreased, is observed among different studies and this might be attributed to the inherently large diversity and, hence, the functional redundancy of the soil microbial community compared to other ecosystems. As such, even the diversity and functional redundancy of the nitrifying community, thought to be limited compared to other microbial communities [13], appears to be sufficient to survive stress and restore the community's function after long term exposure to stress. This can also explain the apparent inherent ability of the ammonium oxidizing community to adapt to Zn in soils as shown in several studies [19-20; 30-31].

Recovery was most strongly explained by the initial PNR (potential nitrification activity). It is not very clear how a potential activity might explain the recovery, the PNR is indeed not the rate of nitrification in soils that are initially contaminated with Zn but it is the potential rate at unlimited NH_4^+ -supply in absence of Zn. All soils were amended with a large NH_4^+ -dose before the start of incubation. Likely, a high potential activity in a soil (high PNR) reflects the presence of active biomass ready to nitrify and grow when conditions are possible, even at slower rates under toxic conditions such as Zn stress, while a low PNR reflects a very weak potential activity for nitrification or growth in any condition. We previously showed faster adaptation of potential nitrification to Zn at increasing nitrification activity [31]. In the latter, activity was increased by adding increasing doses of NH_4^+ -N to the soil. In this study here, differences in initial potential nitrification activity were not due to differences in initial AOB abundance, since the initial AOB abundance did not significantly differ among treatments. Differences in activity are therefore most likely due to differences in the nitrifying community composition. The contribution of AOA

to soil nitrification is still debated [11; 39] and was not studied in the soils used for inoculation. It is therefore unknown whether the lack of correlation of the AOB abundance to the PNR after 2 months incubation and the lack of correlation of the AOB diversity to the recovery is due to differences of the contribution of AOA to nitrification, or of the AOA community composition, its diversity and abundance among the treatments. However, it is thought that AOA are dominant nitrifiers in acidic soils (<5.5) and that AOB preferentially use NH_4^+ , while AOA have more affinity for organic N [26]. The weight of evidence hence suggests that AOB are more important in this study than AOA when assessing potential nitrification. Indeed, the soil used in this study has a pH well above 5.5 and therefore the relative importance of AOB in nitrification is larger [26]. More importantly, NH_4^+ is supplied in excess for assessing PNR favouring AOB activity even in acidic soils [32]. In addition, previous studies in two different acidic soils showed that AOA do not play a role in recovery of nitrification to Zn stress and that the relative abundance of AOA compared to AOB in soil decreased after long term Zn exposure [19; 31; 32]. Nevertheless, study of the contribution of AOA in soil nitrification under different soil conditions deserves dedicated methodologies which discriminate between AOB and AOA (e.g. stable isotope probing, RNA abundance analysis).

This study shows that recovery of potential nitrification to Zn stress is not explained by the AOB diversity. However, potential nitrification activity and initial Zn tolerance are correlated to recovery which are obviously determined by the nitrifying community composition. This study therefore suggests that the potential community activity and composition explain recovery to stress in soil, rather than diversity as such.

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Supplementary Information Figure 1: Two-dimensional presentation of the AOB *amoA* DGGE profiles of the treatments inoculated with the mixtures in relation to the single soil inocula treatments used in the mixture based on nMDS ordination (Bray-Curtis similarity) as measured after 2 months incubation prior to Zn amendment. Mixture 2 showed great resemblance with Teso. In the other mixtures the inoculated soils all contributed to the new established community after two months of incubation. Treatments inoculated with mixtures: filled symbols; single soil inocula treatments: open symbols.

Supplementary Table 1: Overview of the diversity measures (expected and measured based on AOB *amoA* DGGE profiling) of the inoculated treatments before Zn stress (two months after inoculation).

Treatment (inocula)	Expected ¹ AOB Diversity			Measured AOB Diversity				
	Richness	# bands	# inocula	Richness	# bands	Shannon-Wiener		
Mixture 1	83	43	3	33 (4.5)	24 (1)	3.07	(0.29)	
Mixture 2	72	47	3	94 (16.5)	29 (2)	3.28	(0.09)	
Mixture 3	196	105	7	71 nd	25 nd	2.88	nd	
Mixture 4	97	50	3	98 (3.5)	27 (5)	3.44	(0.002)	
Mixture 5	>>	>>	22	114 (51.3)	34 (1)	3.40	(0.44)	
Kövlinge	5	11	1	6 (0.6)	15 (1)	2.48	(0.05)	
Nagyhörcsök	17	11	1	7 (0.6)	16 (1)	2.71	(0.05)	
Kasterlee	46	18	1	10 (0.1)	15	2.66	(0.02)	
Borris	46	18	1	20 (0.1)	21	2.96	(0.005)	
Souli	15	11	1	57 (9.7)	23 (2)	3.03	(0.09)	
Zeveren	31	15	1	67 (1.8)	24	3.07	(0.01)	
Guadalajara	20	14	1	70 (8.2)	24 (1)	3.05	(0.10)	
Teso	36	21	1	81 (19.3)	31 (3)	3.30	(0.12)	

¹The expected AOB diversity is derived from the AOB *amoA* DGGE profiles of the soils before inoculation (see text); >> Richness of mixture 5 could not be estimated, but is assumed to be very high, since it is comprised of 22 soils. The measured AOB diversity is quantified based on AOB *amoA* DGGE profiles two months after inoculation and is an average value of two DNA extracts (standard deviation in brackets) except for mixture 3 (one replicate).