Co-tolerance to zinc and copper of the soil nitrifying community and its relationship with the community structure

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Abstract

Soil microbial communities can develop trace metal tolerance upon soil contamination with corresponding metals. A few studies have reported co-tolerance in such cases, i.e. tolerance to other metals than those to which the microbial community had been exposed to. This study was set-up to test for co-tolerance of nitrifying communities to zinc (Zn) and copper (Cu) and to relate tolerances to shifts in community structure using *amoA* AOB (ammonia oxidizing bacteria) DGGE. Seven sets of soils, each representing a Cu or Zn contamination gradient were sampled from four locations. At two locations, both Cu and Zn had been added as single contaminants. Increased Zn and Cu tolerance of the nitrifying communities was consistently observed in response to corresponding soil contamination. Co-tolerance to Zn was obtained in two of the three Cu gradients and that to Cu in one of the four Zn gradients. DGGE analysis and sequencing showed that contamination with either Zn or Cu selected for identical AOB phylotypes in soils at one location but not at the other location. The nitrifying community structures in soils from different locations did not become more similar upon Zn exposure than those in corresponding uncontaminated soils. Hence, trace metal tolerance development was not due to the emergence of specific AOB phylotypes, but due to the emergence of different AOB phylotypes bearing tolerance mechanisms for Zn, Cu or both metals.

Introduction

Previous studies showed that trace metal contamination decreased the microbial function on the short term. EC50 values for nitrification range from 150-1200 mg kg⁻¹ depending on the soil for both Zn and Cu (Smolders et al., 2003, Oorts et al., 2006). These EC50 values are close to the background values of Zn (10-300 mg kg⁻¹) and Cu (1-239 mg kg⁻¹) naturally occurring in soil. However, it is well known that the soil microbial community function may recover from

increased trace metal concentrations by tolerance development of the microbial community (Giller et al., 1998). This development of trace metal community tolerance is attributed to the extinction of sensitive populations and the selection and proliferation of metal tolerant populations (Díaz-Raviña and Bååth, 1996) resulting in a changed community structure and a recovery of the soil microbial function (Frostegård et al., 1996; Bååth et al., 1998; Witter et al., 2000). This has been shown in Zn and Cu contaminated soils for the general microbial community as well as for specific groups such as the nitrifying community (Mertens et al., 2006; Mertens et al., 2010; Ruyters et al., 2010).

Trace metal tolerance mechanisms are present in nearly all bacterial phyla due to horizontal transfer of chromosomal or plasmid encoded mobile genetic elements (Bruins et al., 2000) and are obviously also found in soil bacteria (Ryan et al., 2005). An important group of resistance mechanisms are P-type ATPases which are efflux systems transporting cations over the cell membrane, e.g. the *czc* system removing Cd^{2+} , Zn^{2+} and Co^{2+} (Nies, 1999). Park and Ely (2008) determined 27 genes that were up-regulated by Zn in *Nitrosomonas europaea*. These included for example mercury resistance genes and inorganic ion transport genes. Thirty genes were down-regulated, e.g. amino acid transporter genes.

Coping with stress is energy demanding and therefore a tolerant microbial community may be more vulnerable to additional stress as shown by Tobor-Kaplon et al. (2006a) in Zn contaminated soils. Alternatively, a tolerant community might better cope with other stresses than a non-tolerant community if the additional stress occurs through a similar mechanism as the first one (Tobor-Kaplon et al., 2006b). An example of such co-tolerance process is the development of tolerance to other trace metals than those to which the microbial community had been exposed to due to, e.g., similar physiological tolerance mechanisms (Bruins et al., 2000). Only few studies

are available on the occurrence of co-tolerance in microbial communities for multiple trace metals in soil. Using the thymidine incorporation technique, Díaz-Raviña et al. (1994) showed that soil contaminated with either Cd, Ni, Pb, Zn or Cu did not only induce tolerance to the corresponding metal, but also to other trace metals. Rusk et al. (2004) showed that the soil nitrifying community in Pb contaminated soils also developed co-tolerance to Zn and in Zn contaminated soils to Pb. Co-tolerance to multiple metals might be due to non-specific efflux systems, intracellular protein binding (e.g. metallothioneins for Cu^{2+} and Zn^{2+}) or to closely linked plasmid or genomic gene clusters encoding different metal tolerance mechanisms. For example, plasmid pMOL30 isolated from *Alcaligenes eutrophus*, encodes for the *czc* system and Cu tolerance (Mergeay, 1991).

Little is known about the relation between the development of co-tolerance and changes of the microbial community. The general objective of this study was to determine co-tolerance of the nitrifying community in soil to zinc (Zn) and copper (Cu) in relation to Zn and Cu exposure scenarios and to analyze the effects of Zn and Cu on the microbial community structure. We assessed if changes of the soil nitrifying community were similar in two soils contaminated with either Zn or Cu, i.e. starting with the same community composition and structure, but contaminated with a different metal. In addition, we questioned whether the bacterial nitrifying community that is metal tolerant would converge to a similar community structure in different soils that inherently have a different native community composition and structure. Only the Ammonia Oxidizing Bacteria (AOB) were considered in the community analysis, since previous studies showed that Ammonia Oxidizing Archaea (AOA) play a minor role in recovery of the nitrification after Zn contamination (Mertens et al., 2009; Ruyters et al., 2010). It is unknown if the same is true for adaptation to Cu contamination.

Materials and methods

Soil sampling

Eight sets of soils, each representing a Zn or Cu concentrations gradient or a corresponding pair of contaminated and uncontaminated (control) soil were collected at 4 different locations (Table 2). Major soil characteristics for the corresponding uncontaminated (control) soils are summarized in Table 1. Zinc contaminated soils from Zeveren (Zev-Zn-Field) (Belgium) and De Meern (DM-Zn-Field) (The Netherlands) were sampled in October 2009 in grassland transects towards a galvanized pylon. The Hygum soil set (Denmark) was sampled in June 2007 near a former wood treatment plant contaminated with CuSO_4 over 80 years ago (Hy-Cu-Field). Other soil sets were derived from artificially contaminated sites or lab experiments. An uncontaminated Zeveren soil, sampled in June 2007, was artificially spiked with increasing doses of ZnCl_2 (60-2500 mg Zn kg^{-1} ; Zev-Zn-Sp) (Ruyters et al., 2010) or 1 dose of CuCl_2 (2400 mg Cu kg^{-1} ; Zev-Cu-Sp) and had been incubated outside with free drainage for more than 2 years. The soil pH of Zev-Cu-Sp was lower than the soil pH of the control (4.7 vs. 5.5). Spalding soil (Australia) was artificially spiked with increasing concentrations of either ZnSO_4 (Spal-Zn-Sp) or CuSO_4 (Spal-Cu-Sp) and incubated outside during 4 years (Broos et al., 2007). Soil pH decreased in Spal-Cu-Sp 2800 from 5.5 in the control soil to 4.8 in the Cu spiked soil. All soils were sieved (< 4 mm) and stored fresh in plastic bags at 4 °C (soils from Zeveren, De Meern, Hygum) or were air dried stored at room temperature (soils from Spalding) pending analyses. Prior to testing metal tolerances and DNA extraction, soils were remoistened and aerobically incubated for 2 weeks at 25 °C except for the freshly sampled Zn contaminated soils from Zeveren and De Meern (1 week incubation).

Table 1: Background Zn and Cu concentrations and selected properties of the uncontaminated soils from the four locations.

Site	Background (mg kg ⁻¹)		pH ^(a)	C _{org} ^(a) (%)	CEC ^(a) (cmol kg ⁻¹)	Sampling date
	Zn	Cu				
Zeeveren	50	15	5.5	3.5	21.1	June 2007
De Meern	140	60	5.0	10.2	29.6	October 2009
Spalding	60	80	5.5	2.2	15.9	2009
Hygum	40	20	5.8	2.1	6.7	June 2007

(a) pH, C_{org} and CEC were determined as described by Smolders et al. (2004)

Table 2: Overview of the soil sets selected for Zn or Cu tolerance testing either contaminated with Zn or with Cu. A distinction is made between the contamination type: gradual contaminated over time in the field ('field') and long term aged after artificial spiking with metal salts ('Sp'). As such, seven sets are distinguished differing in location, trace metal or contamination type.

Site	Soil Set	Zn (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Exposure time (years)
Zeeveren	Zev-Zn-Field	370	bg	>10
Belgium		800 ^(a)	bg	>10
		1200 ^(a)	bg	>10
	Zev-Zn-Sp	100	bg	2
		700	bg	2
		1300	bg	2
		2500 ^(a)	bg	2
	Zev-Cu-Sp	bg	2400 ^(a)	2
Spalding	Spal-Zn-Sp	1850 ^(a)	bg	3
Australia	Spal-Cu-Sp	bg	475	2
		bg	875	2
		bg	1850	2
		bg	2800 ^(a)	4
De Meern	DM-Zn-Field	600	bg	>10
The Netherlands		1000	bg	>10
		1700	bg	>10
		4000 ^(a)	bg	>10
Hygum	Hy-Cu-Field	bg	140	>80
Denmark		bg	400	>80
		bg	1500 ^(a)	>80

(a) Soils were selected for co-tolerance testing

bg: Background concentration as given in Table 1

Trace metal tolerance

Zinc or Cu tolerance of the soil nitrifying community was determined using the spike-on-spike assay described by Mertens et al. (2006). Briefly, ZnCl_2 or CuCl_2 (50 mg ml^{-1}) was added to soil samples suspended in CaCl_2 0.01 M (1:10 solid:liquid ratio) – these suspensions mimic an extraction of the soil microbial community - to final concentrations of 0 (in duplicate), 15, 30, 75, 150, 300 and $500 \text{ mg metal added l}^{-1}$. Ammonium chloride solution (10 mg ml^{-1}) was added to a final concentration of $10 \text{ mg NH}_4^+ \text{-N l}^{-1}$. The pH in suspension was adjusted immediately after N addition and daily during the 3-day test to the pH of the corresponding control soil (± 0.1) using 0.1 M NaOH or HCl if required. Nitrate concentrations were measured colorimetrically in a 1 M KCl soil extract (SA 40, Skalar, The Netherlands) at the start of the experiment and after 3 days incubation at $20 \pm 1 \text{ }^\circ\text{C}$ with head over end agitation (28 rpm). The Potential Nitrification Rate (PNR) was calculated as the rate of increase in nitrate concentration per unit of soil dry weight over time ($\text{mg NO}_3^- \text{-N kg}^{-1} \text{ d}^{-1}$). The PNR in suspension was related to Zn concentrations in the CaCl_2 soil extract. The metal concentrations in the CaCl_2 0.01 M extract were measured at day 3 after centrifugation in the filtered and acidified supernatant (3000 g, 15 min) with ICP-OES (Perkin Elmer Optima 3300 DV, Norwalk, CT, USA). Metal concentrations of the control suspensions (no extra metal added) increased with increasing total soil metal concentration in the soil sets and this increase was more pronounced in the spiked soil sets. Dissolved organic carbon (DOC) concentrations were measured in a filtered ($0.45 \text{ }\mu\text{m}$) soil extract (catalytic combustion with IR detection; Multi N/C 2100, AnalyticJena). The Zn and Cu speciation in all suspensions was calculated using the Windermere Humic Acid Model (WHAM) v.6.0.13 (Tipping, 1998). Input data were the metal concentration, NH_4^+ and Ca^{2+} concentration, the corresponding Cl^- concentration in solution, solution pH and reactive dissolved organic matter (DOM). Reactive

DOM was calculated by multiplying the DOC concentration by 1.3, assuming that DOM consists of 50% DOC and that 65% of the DOM is reactive as fulvic acid (Weng et al., 2002). Dose-response curves, based on the Potential Nitrification Rate values and the metal free ion concentrations, were fitted by log-logistic modeling (Doelman and Haanstra, 1989) using the Marquardt method (proc NLIN, SAS[®] 9.2; NC, USA). Trace metal tolerance was expressed as the free Zn²⁺ or Cu²⁺ concentrations at which the PNR was 50% reduced compared to the control treatment (EC50) and are derived from the dose-response curves.

DNA extraction and amoA AOB profiling

Soil DNA was extracted and purified with the MO BIO Powersoil DNA Isolation Kit according to the manufacturer's protocol (MO BIO laboratories, California, USA). PCR amplification of AOB *amoA* gene fragments using primers GC-*amoA*-1F* and *amoA*-2R (Rotthauwe et al., 1997; Stephen et al., 1998) was performed as described by Mertens et al. (2009). The PCR product size and integrity was checked using standard agarose gel electrophoresis. Denaturing Gradient Gel Electrophoresis (DGGE; Ingeny, Goes, The Netherlands) profiling analysis of the *amoA* gene fragments was performed using 6% polyacrylamide gels with a linear gradient of 40-60% as described by Mertens et al. (2006).

Cloning and sequencing

PCR products obtained from selected soil samples (Spalding Zn 1850 and Spalding Cu 2800; Zevenen Zn 2500 and Zevenen Cu 2400) were cloned into the plasmid vector pCR[®] 2.1-TOPO[®], using the TOPO TA cloning kit according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium). Transformants were analyzed for the presence of the *amoA* gene by PCR amplification using the *amoA* primers described above. The obtained PCR product was purified with the

QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit using both the GC-*amoA*-1F* and *amoA*-2R primers and analyzed with the ABI 3100/3130 DNA-Sequencer (Applied Biosystems).

Data analysis

Metal tolerance is defined as the EC50 value of the fitted dose-response curve (mg trace metal l⁻¹ suspension). Significantly increased EC50 values compared to those of the corresponding control soil represent significant increased metal tolerance and were detected by a one-sided t-test ($p < 0.05$). Metal tolerances (EC50 values) were not compared among soils of different sites, since the solution based thresholds are also affected by pH and soil pH values differed between soils. Pairwise comparison of DGGE profiles was based on the Pearson product-moment correlation coefficient using the GelCompar II software 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). Negative correlation values are trimmed to zero, because they do not have any biological meaning. Correlations are expressed as percentage values and are frequently used as similarity coefficients comparing DGGE profiles (Fromin et al., 2002). Preliminary DGGE gels showed at least 90% similarity between replicate samples (Ruyters et al., 2010 for the Zev-Zn-Sp set, Mertens et al., 2010 for the Spal-Cu-Sp and Hy-Cu-Field sets and data not shown for the other sets) and therefore only one replicate was loaded. The community structure data were reduced to a 2 dimensional space by nMDS ordination based on the Bray-Curtis distance measure (PC-ORD v5) (Ramette, 2007). ANOVA was performed on both ordination axes to test if the *amoA* AOB DGGE profiles of different soils converged when becoming Zn tolerant. Increased Zn tolerance was classified as a nominal factor. Sequences were aligned with MEGA version 5.0 (Kumar et al., 2004) based on the CLUSTALW algorithm (Thompson et al., 1994) using default parameters. A phylogenetic tree was generated with MEGA 5.0 with the obtained sequences using the

neighbor-joining algorithm. Cu tolerance of the nitrifying community of the Cu contaminated Spalding and Hygum soil sets (Spal-Cu-Sp and Hy-Cu-Field – Mertens et al., 2010) and Zn tolerance of the nitrifying community of the Zn contaminated Spalding sets (Spal-Zn-Sp 1850 – Mertens et al., 2009) were determined previously, and values were adopted from those studies. The *amoA* DGGE profiling of those soils was repeated in this study.

Results

Metal tolerance and co-tolerance

Table 3 gives an overview of the Zn and Cu tolerances in all soil sets. The Zn EC50 values (Zn tolerance) increases in each of the 4 Zn sets and tolerance increased with increasing soil Zn. In the Zeveren soil, artificial Zn contamination (Zev-Zn-Sp) followed by more than 2 years incubation increased Zn tolerance (Table 3). Spiking followed by 2 years incubation also increased Zn tolerance in the Spalding soil sets (Spal-Zn-Sp) (Table 3) as previously shown by Mertens et al. (2009). Five Zn contaminated soils which showed significantly increased Zn tolerance and the corresponding control soils were selected for Cu tolerance testing. Only the Zn tolerant community of the Zeveren soil (Zev-Zn-Sp, 2500 mg Zn kg⁻¹) showed increased Cu tolerance compared to the corresponding control soil community (Table 3).

Copper contamination increased Cu tolerance in all Cu contaminated sets of this study. (Table 3). Increased Cu tolerance of Cu contaminated soils was previously reported by Mertens et al. (2010) for the artificially Cu contaminated Spalding sets (Spal-Cu-Sp) and the field contaminated Hygum sets (Hy-Cu-Field) (Table 3). Two of the three soils with significantly increased Cu tolerance also showed increased Zn tolerance (Zev-Cu-Sp 2400 mg Cu kg⁻¹ en Spal-Cu-Sp 2800 mg Cu kg⁻¹; Table 3).

Table 3: Zn tolerances (EC50, mM Zn²⁺) and Cu tolerances (EC50, µM Cu²⁺) of the nitrifying communities in the soil series. Significant increased EC50 values (p < 0.05) compared with the EC50 of the corresponding control soil are in bold and show increased tolerance to the soil contamination. EC50 values which denote co-tolerance are in bold and underlined.

Zeveren					Spalding				
Metal concentration			EC50		Metal concentration			EC50	
	Zn (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn ²⁺ (mM)	Cu ²⁺ (µM)		Zn (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn ²⁺ (mM)	Cu ²⁺ (µM)
control	50	15	0.17	3.8	control	60	80	0.32	6.3 ^(b)
Zev-Zn-Field	370	15	0.43	nd	Spal-Zn-Sp	1850	80	>1.5^(a)	0.79
Zev-Zn-Field	800	15	1.7	8.7					
Zev-Zn-Field	1200	15	0.73	3.3	Spal-Cu-Sp	60	475	nd	7.9 ^(b)
					Spal-Cu-Sp	60	875	nd	19 ^(b)
Zev-Zn-Sp	100	15	0.22	nd	Spal-Cu-Sp	60	1850	nd	441^(b)
Zev-Zn-Sp	700	15	0.55	nd	Spal-Cu-Sp	60	2800	<u>3.1</u>	551^(b)
Zev-Zn-Sp	1300	15	0.63	nd					
Zev-Zn-Sp	2500	15	1.7	<u>19</u>					
Zev-Cu-Sp	50	2400	<u>1.2</u>	126					

Table 3 Continued

De Meern					Hygum				
Metal concentration		EC50			Metal concentration		EC50		
Zn (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn ²⁺ (mM)	Cu ²⁺ (μM)		Zn (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn ²⁺ (mM)	Cu ²⁺ (μM)	
control	140	60	0.076	63	control	40	20	0.12	6.3 ^(b)
DM-Zn-Field	600	60	0.35	nd	Hy-Cu-Field	140	20	nd	4.7 ^(b)
DM-Zn-Field	1000	60	0.17	nd	Hy-Cu-Field	400	20	nd	9.5 ^(b)
DM-Zn-Field	1700	60	0.24	nd	Hy-Cu-Field	1500	20	0.12	27 ^(b)
DM-Zn-Field	4000	60	3.8	31					

(a) Adopted from Mertens et al. (2009)

(b) Adopted from Mertens et al. (2010)

nd: not determined

Nitrifying community structure

Changes of the soil nitrifying bacterial community structure between not contaminated and contaminated treatments were detected in all four Zn contaminated sets of soil. This was detected for both Zeveren soil sets (Zev-Zn-Field and Zev-Zn-Sp) in soils with Zn concentrations from 370 mg Zn kg⁻¹ and 1300 mg Zn kg⁻¹, respectively, and for the De Meern soil sets (DM-Zn-Field) from 1700 mg Zn kg⁻¹ (Supplementary Figure 1, Panel A). Maximally 53-67% pair-wise similarity was observed between the corresponding control soil and those treatments. Zinc exposed soils from Spalding (Spal-Zn-Sp) also showed a distinctly different *amoA* DGGE profile compared with the corresponding control soil (15% similarity).

Differences of the AOB community structure between the Cu contaminated soil and the corresponding control soil were observed in all Cu sets (Supplementary Figure 1, Panel B). The pair-wise similarity of the AOB community of the Cu contaminated soils with the corresponding control soil was low: 3% for Zeveren Cu 2400, 20% for Spalding Cu 2800 and 46% for Hygum Cu 1500.

In Figure 1 the AOB community profiles of all treatments are displayed after nMDS ordination with respect to their community similarity (Bray-Curtis). A shift of the community similarity relative to the corresponding control soil is observed within each contaminated soil sets showing an effect of trace metal contamination on the community structure (Figure 1). The *amoA* DGGE profiles of Zn contaminated soils from different locations did not cluster as can be seen on this graph, even if Zn concentrations are comparable. No effect of increased Zn tolerance on both ordination axes was detected ($p > 0.05$) showing the development of different soil nitrifying and Zn tolerant communities at the different locations in response to Zn contamination.

Figure 1: Graphical display of the *amoA* AOB DGGE profiles based on nMDS ordination (Bray Curtis). A shift of the *amoA* AOB DGGE profile of Zn or Cu contaminated soils compared to their respective control soil (black symbols) is observed. Gray shaded symbols indicate tolerance to the soil contamination. Underlined name tags indicate co-tolerance to Zn and Cu. Zevenen: O; Spalding: □; De Meern: Δ; Hygum: ▽.

Co-tolerance was observed in the Zevenen soil artificially contaminated with 2500 mg Zn kg⁻¹, in the same soil artificially contaminated with 2400 mg Cu kg⁻¹ and in the Spalding soil contaminated with 2800 mg Cu kg⁻¹. Figure 2 compares the soil nitrifying communities in the Zevenen and Spalding soils contaminated with either metal. Pair-wise similarity between the nitrifying community structure of the Zn and Cu contaminated Spalding soils was 83%. DNA sequences of the dominant band of both *amoA* DGGE profiles appeared to be identical (Figure 2 and Figure 3) (submitted to the GenBank database by Mertens et al. (2009) under accession number EU515200). The soil nitrifying communities of the Zevenen soil contaminated with either Cu or Zn showed 71% similarity, but dominant bands represented different sequences (see Figure 2 and Figure 3; submitted to the GenBank database under accession numbers HQ687667 (Zev-Zn-2500-Sp) and HQ687668 (Zev-Cu-2400-Sp)).

Figure 2: *amoA* AOB DGGE profile in the Zeven and Spalding soil either contaminated with Zn or Cu. Dominant bands have identical sequences in the Spalding soils, but different sequences in the Zeven soils (Figure 3). Sampling series are indicated at the top. Control soils (designated with 'C') and Zn or Cu concentrations (mg kg^{-1}) are given under each DGGE profile.

Figure 3: Phylogenetic tree (neighbor joining) of sequenced *amoA* fragments corresponding to the bands indicated in Figure 2. The sequence obtained from Spal-Cu-2800 Sp is identical to that of Spal-Zn-1850 Sp, whereas the sequence obtained from Zev-Cu-2400 Sp is different from that of Zev-Zn-2500 Sp. *amoA* fragment sequences from *Nitrospira*, *Nitrosomonas* and *Nitrosolobus* spp. are given as reference. Accession numbers are given between brackets.

Discussion

Increased trace metal tolerance of the microbial community in soil has previously been shown to develop on the long term and was reconfirmed here for Zn and Cu. Development of tolerance at the community level is the net result of phenotypic changes, genotypic changes and the selection of tolerant species over sensitive species (Millward and Klerks, 2002). Exposure of the same soil nitrifying community to Zn or Cu resulted in the development of an increasingly similar community structure (Figure 2). This resemblance was striking for the Spalding soil community in which identical dominant phylotypes emerged upon Zn or Cu exposure. Resemblance increased to a lesser extent between the Zn and Cu contaminated Zeveren soil, as dominant phylotypes differed. The dominant phylotypes which emerged after metal exposure differed between both the Spalding and Zeveren soils. Based on changes of PLFA abundance after metal amendment (e.g. Zn, Pb, Cu, Ni, Cd) to forest mor layers Åkerblom et al. (2007) and Frostegård et al. (1993) similarly concluded that changes of the microbial community structure are independent of the type of metal added. In contrast, Cu contamination induced different changes of the community compared to Zn contamination in an arable soil (Frostegård et al., 1993).

Co-tolerance - tolerance of the soil bacterial community to other trace metals than the one to which the microbial community has been exposed to – to Zn and Cu was observed in three soil sets: in the artificially Cu contaminated Spalding and Zeveren soil sets, and in the artificially Zn contaminated Zeveren (not the Spalding) soil set. Nevertheless, co-tolerance to Cu was not observed in the Zeveren soil field contaminated with 1200 mg Zn kg⁻¹. The absence of co-tolerance might be attributed to the lower total and CaCl₂ extractable metal concentration in the field contaminated Zeveren soil (1200 mg Zn kg⁻¹; 4 mg Zn²⁺ l⁻¹) compared to the artificially spiked soil (2500 mg Zn kg⁻¹; 23 mg Zn²⁺ l⁻¹). Díaz-Raviña et al. (1994) previously showed that

the extent of co-tolerance to different metals is dependent on the metal concentration in soil. In addition, tolerance measurements in the present study are based on statistical methods on the microbial activity, including not only the presence of tolerant strains, but also their activity, which might explain that co-tolerance is not consistently observed. This might also explain the absence of Cu tolerance in the Zn contaminated Spalding soil sets despite the emergence of identical phylotypes in the co-tolerant Cu contaminated Spalding soil. The absence of co-tolerance to Zn and Cu has been previously reported in Cu or Zn contaminated forest soils (Niklinska et al., 2006). Similarly to our study, Díaz-Raviña et al. (1994) observed more pronounced Zn co-tolerance in Cu contaminated soils than Cu co-tolerance in Zn contaminated soil.

Only AOB phylotypes bearing tolerance mechanisms for the metal they have been exposed to are considered to survive in soil after long term metal exposure. Since co-tolerance to Zn and Cu is observed in both the Zevenen soils spiked with Zn or Cu, it is most likely that the identified dominant phylotypes bear tolerance mechanisms for both metals. Co-tolerance might be due to similar mechanisms for both trace metals (e.g. non-specific trace metal efflux systems) or due to closely linked gene clusters encoding specific tolerance mechanisms for Zn and Cu. This study suggests that the development of co-tolerance is not necessarily associated with the selection of the same phylotypes after Zn or Cu exposure, but might also be due to the selection of different phylotypes bearing Zn and Cu tolerance mechanisms. Indeed, Ryan et al. (2005) showed the occurrence of bacterial strains tolerant to multiple trace metals (Co, As, Ni, Zn and Cu) in a multiple trace metal contaminated soil (4.5 mg l⁻¹ Zn, 1.3 mg l⁻¹ Cu, 0.4 mg l⁻¹ As, 0.4 mg l⁻¹ Cd and 4 µg l⁻¹ Hg). In fact, only 4% of the isolated strains were tolerant to only 1 metal showing that co-tolerance is widespread among bacterial strains.

The occurrence of trace metal tolerance of the soil bacterial community is commonly observed in different soils (Díaz-Raviña and Bååth, 1996; Almås et al., 2004) and has been shown previously for the soil nitrifying community in particular (Fait et al., 2006; Mertens et al., 2006; Mertens et al., 2010). We raised the question if a similar contamination event selects for the same tolerant nitrifying populations in soils of different origin with a different native community. The soil nitrifying bacterial community is a rather select group of β -Proteobacteria (Koops and Pommerening-Roser, 2001). Therefore, specific AOB phylotypes which are tolerant to metals might become dominant after a similar contamination event. In contrast, Mertens et al. (2009) suggested that Zn tolerance is widespread throughout the *Nitrosospora* lineage, which may imply a divergent development of a tolerant community across soils. Divergence might be explained by horizontal gene transfer of genes encoding for tolerance mechanisms. In the present study it can be seen from both the DGGE profiles (Supplementary Figure 1) and the ordination based on Bray-Curtis similarity (Figure 1) that the *amoA* AOB DGGE profiles did not converge to a similar community profile in response to Zn or Cu contamination or due to the development of Zn or Cu tolerance. For example, when comparing artificial contaminated soils from Zevenen and Spalding only 23% similarity based on the Pearson coefficient is observed in Zn tolerant soils. Apparently, trace metal tolerance development was not due to the emergence of specific AOB phylotypes, but due to the emergence of different AOB phylotypes bearing tolerance mechanisms for Zn, Cu or both metals. This suggests that changes of the community structure upon trace metal contamination were determined by the initial community composition and structure and during exposure shaped by the type of metal and the dose. Anderson et al. (2009) previously showed that the bacterial community of different soils did not converge to a similar community structure after metal amendment acknowledging that other factors may also be important in shaping the community.

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Supplementary Information

Supplementary Figure 1: *amoA* AOB DGGE profiles of soil samples with increasing metal contamination. Panel A shows changes of the *amoA* AOB community upon Zn contamination and Panel B upon Cu contamination. Metal concentrations (mg kg⁻¹) are given under the DGGE profiles (C: control).