Dynamics of the nitrous oxide reducing community during adaptation to Zn stress in soil

Authors: Stefan Ruyters^{*}, Jelle Mertens, Isabelle T'Seyen, Dirk Springael and Erik Smolders

Address: Department of Earth and Environmental Sciences, Division of Soil and Water Management, K.U.Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

*Corresponding author

email address: struyters@yahoo.com

Abstract

Laboratory studies show that the nitrous oxide (N₂O) reduction rate in soil is strongly inhibited by trace metal contamination; however, this effect appears transient. Here we assess if this recovery is due to microbial adaptation associated with shifts in community composition. Soils were spiked with zinc chloride (0-5000 mg Zn kg⁻¹) in a factorial design with 3 application rates of organic matter (OM), i.e. 0, 2 and 4 g milled hay kg⁻¹, to accelerate growth and, potentially, adaptation rate. The soil treatments were incubated outdoors with free drainage during 1 year and periodically sampled. The potential N₂O reduction rate, measured in an anaerobic laboratory assay, was inhibited by Zn during the first 2 months after spiking with 50% inhibition at 500-1000 mg Zn kg⁻¹. After 6 months exposure, the N₂O reduction rate recovered to at least 80% of the rate in the control treatment in the series receiving OM up to the largest Zn dose, but strong inhibition remained in the series which did not receive OM. In this series recovery was only observed after 12 months exposure. Soil pore water Zn concentrations did not explain the recovery of the N₂O reduction rate in the control series suggesting that recovery is due to adaptation and not to reduced Zn bioavailability. The faster recovery in the series receiving OM was partially, but not fully related to the effects of OM on Zn bioavailability. The recovery at all Zn and OM treatments co-varied with a recovery of *nosZ* gene abundance from about 1×10⁷ copies g⁻¹ soil in the soil treatments with decreased activity to 5×10⁸ copies g⁻¹ soil in the other soil treatments. The *nosZ* gene DGGE profile of the soil communities revealed minor changes in the *nosZ* containing community. This study strongly suggests that the transient effects of trace metal inhibition of N₂O reduction is due to the development of a Zn tolerant denitrifying community.

Introduction

Short term studies have revealed a marked inhibition of N₂O reduction in soil, when only moderately contaminated with trace metals. In soils spiked with Zn²⁺ salts, the N₂O reduction decreased by 50% at 230-370 mg Zn kg⁻¹, concentrations that cover the range of soil Zn limits in the EU (De Brouwere et al., 2007). No effects were found on the total denitrification activity (N₂O + N₂ production) at those Zn concentrations. Such findings might be of environmental concern taking into account that 65% of global N₂O emissions originate from soil (Smith and Conen, 2004) and that N₂O contributes to the increase of greenhouse gasses in the atmosphere (IPCC, 2007). The effects of metals on N₂O reduction, however, appear transient. For example, N₂O reduction decreased over time in Zn spiked soils and effects were not detectable in field contaminated soils (De Brouwere et al., 2007). Decreasing toxicity of trace metals to soil microbial processes has been attributed to microbial adaptation (Giller *et al.*, 1998). Community adaptation may be explained by selective growth of tolerant groups and selective decay of sensitive groups (Diaz-Raviña and Bååth, 1996). As a result, the microbial activity restores due to the development and activity of a tolerant microbial community. Soil nitrifying communities have been shown to adapt to Zn additions and develop Zn tolerance within 1-2 years of exposure (Mertens et al., 2009). On the other hand, adaptation and Zn tolerance of the N₂O reducing community is observed within 2 months after Zn addition (Holtan-Hartwig et al., 2002; De Brouwere et al., 2007). These results suggest that adaptation occurs considerably faster for denitrification than for nitrification. The adaptation rate may relate to the microbial diversity and the growth rate, since the heterotrophic denitrifying populations have a greater diversity and growth rate compared to the slow growing and less diverse nitrifying populations (Zumft, 1999; Koops and Pommerening-Roser, 2001). This hypothesis has been tested for the nitrifying community showing an increased adaptation rate with increasing microbial activity, potentially

corresponding with increasing growth rate (Ruyters et al., 2010). Adaptation and tolerance development of nitrifying communities co-occurred with a changing community structure, although changes in abundance were not observed (Ruyters et al., 2010). Holtan-Hartwig et al. (2002) showed an increased N₂O reducing community tolerance to Zn 2 months after a single contamination event, but no data are available about changes of the denitrifying community composition and abundance.

The use of molecular tools is very well suited to understand the link between the denitrification activity and the denitrifying community structure (Philippot and Hallin, 2006). Only few studies have focused on the effect of increased trace metal concentrations on the denitrifying community structure. Philippot et al. (2008) did not observe effects of increased Hg concentrations (100 mg kg⁻¹) on the denitrifying community structure within 120 days exposure monitoring the *narG* gene responsible for NO₃⁻ reduction. Throbäck et al. (2007) observed a decreased denitrification activity during 90 days incubation co-occurring with a reduced *nirK* abundance (responsible for the reduction of NO₂⁻ to NO) and increased *nirK* diversity upon Ag contamination (100 mg Ag kg⁻¹). No similar studies have been performed using the *nosZ* gene that encodes the enzyme responsible for the reduction of N₂O to N₂, although N₂O reduction is the most metal sensitive process in denitrification.

The objectives of this study were to examine (i) whether the recovery of the N₂O reduction on the long term is explained by microbial adaptation and not by reduced Zn bioavailability and (ii) whether adaptation is associated with changes in the *nosZ* containing community structure and *nosZ* abundance. A 1-year experiment was conducted using soil spiked with increasing Zn concentrations (0-5000 mg Zn kg⁻¹) and adaptation of the N₂O reduction was monitored periodically using an anaerobic laboratory assay. To examine the potential accelerating effect of

the growth rate on the adaptation rate we applied 2 concentrations of organic matter (OM) as milled hay to the soil treatments.

Materials and methods

Soil sampling

Uncontaminated top soil (0-20 cm) was sampled from grassland in Zeveren (Belgium) in June 2007, immediately sieved (<4 mm) and stored fresh in plastic barrels at 4 °C in darkness until use. Selected soil characteristics were measured as described previously (Smolders et al., 2004). The soil is a sandy loam soil with a cation exchange capacity of 21 cmol_c kg⁻¹, 3.5% organic matter, soil pH (0.01 M CaCl₂) of 5.6, C/N ratio of 10 and a total background Zn concentration of 60 mg kg⁻¹.

Experimental set-up

The experimental design was a full factorial experiment with 5 Zn rates performed in duplicate divided in 3 series. The sampled uncontaminated soil (also denoted as the original soil) was contaminated with increasing Zn doses by adding dissolved ZnCl₂ to final measured total Zn concentrations of 60 (control soil - no added Zn), 220, 723, 1320 and 5000 mg Zn kg⁻¹ dry soil (hereafter experiment 1). Soil samples of this Zn series are designated as Zn0 to Zn4 with increasing Zn concentration. Perforated plastic pots (volume 2 dm³) filled with 1.5 kg soil were incubated in open air. The pots with free drainage were installed in a container filled with sand to insulate soil and were periodically weeded. The pH decrease due to Zn²⁺ addition was corrected by mixing CaO in the soil at the start of the experiment with doses estimated from measured pH and soil titration. The soil pH (CaCl₂, 0.01M, 5g in 25 ml) remained at 5.44 on average (± 0.12), among all treatments and sampling times. Two months after Zn addition treatments were divided in a control Zn series without extra organic matter addition (OM0 series) and two Zn series with

extra organic matter applied as milled hay (OM1: 2 g hay kg⁻¹ dry soil; OM2: 4 g hay kg⁻¹ dry soil). The hay application was repeated 8 months after Zn addition, i.e. 6 months after the first hay application.

Soil was sampled immediately after Zn addition ('T0') and after 2 months ('T1' – i.e. before the first hay application), 6 months ('T2' – i.e. 4 months after the first hay application) and 12 months ('T3' – i.e. 4 months after the second hay application) exposure to Zn. Prior to sampling, the soil in the pots was homogenized. To monitor the immediate effect of OM after Zn addition soil was contaminated with 5 Zn doses in a second experiment (hereafter experiment 2) with a comparable set-up to the first experimental set-up (pH controlled with CaO) and immediately treated with organic matter (OM1 and OM2). One week and two months later pore water Zn concentrations were determined. N₂O reduction activities were only determined one week after Zn and OM applications.

At each sampling period soil pH (0.01 M CaCl₂) and total Zn concentrations (determined using aqua regia digestion) of all treatments were determined. N₂O reduction activities and pore water Zn concentrations (determined using double chamber method; Smolders et al., 2004) of all treatments of the OM0 series (T0, T1 and T3) and treatments Zn0, Zn2 and Zn4 of the OM0 (T2) and OM1 and OM2 series (T2 and T3) were determined. DNA analysis (DNA extraction, PCR-DGGE and qPCR) was performed at each sampling period on treatments Zn0, Zn2 and Zn4 of all OM series.

Measuring the Potential Nitrous Oxide Reduction Rate (PNORR)

Soil denitrification potentials are commonly assayed with the acetylene inhibition method measuring the N₂O production at unlimited supply of carbon and nitrate (Groffman et al., 2006). However, previous studies showed that the total denitrification activity (N₂O and N₂ production)

was not influenced by trace metal additions in contrast with N₂O reduction (De Brouwere et al., 2007). The determination of soil N₂O reduction rates is challenging, since this occurs often parallel with N₂O production from NO₃⁻ reduction. The determination of N₂O reduction rates requires mass balance calculations and measurements excluding atmospheric N₂. To overcome these problems, a new test was developed that measures the N₂O reduction rate in soil after endogenous NO₃⁻ is depleted. The N₂O produced after this first incubation is removed by flushing. Subsequently, N₂O is added to the soil samples and the rate of N₂O reduction is determined. This is measured under non-limiting N₂O concentrations, therefore the assay is termed the Potential Nitrous Oxide Reduction Rate (PNORR). Note, however, that no extra carbon was provided. Practically, fresh soil was aerobically preincubated for 5 days (25°C) at pF=2 after sampling. One gram of soil was subsequently added to airtight 12 ml tubes and the atmosphere of the tubes was evacuated and flushed with He for 8 min. Three replicate tubes were set up per treatment replicate. The tubes were incubated for 7 days at 20°C until all endogenous NO₃⁻ was metabolized. This was checked by destructive sampling of analytical replicates and extraction of soil nitrate using 1 M KCl and measuring the NO₃⁻ concentration colorimetrically (Skalar, SA40). The headspace of residual tubes was evacuated and flushed with He for 8 min to remove produced gaseous N compounds. Afterwards, 20 mg N₂O-N kg⁻¹ soil was injected in the tubes (0.5 ml of 17% N₂O in He) and the tubes were incubated at 20°C. The N₂O concentration in the headspace was measured at the start and a second time after 10-20 hours incubation on a gas chromatograph provided with a Porapak Q column and an Electron Capture Detector (GC-ECD; GC8AIE, Shimadzu). The PNORR was calculated as the difference of the average N₂O concentration between the start and the second observation. For each treatment replicate three analytical replicates were measured. The PNORR is expressed as the average rate resulting from 2 treatment replicates per unit of soil weight (mg N₂O-N kg⁻¹ soil h⁻¹). Maximal N₂O depletion

was 85% of the initial value. The standard deviation of the PNORR between the 3 analytical replicates was about 11% of the mean value and the standard deviation of the different slopes for the treatment replicates (n=2) was about 6% of the mean value.

DNA extraction, PCR-DGGE and *nosZ* gene copy quantification

DNA was extracted and purified as described by Uyttendaele et al. (2006). Briefly, total DNA was extracted using mechanical (bead beating), enzymatic (lysozyme) and chemical lysis (20% sodium dodecyl sulphate). DNA was purified using chloroform-isoamylalcohol 24:1 and the Wizard[®] DNA clean-up kit with vacuum manifold (Promega). Purified DNA was stored in sterile water at -20°C. Fragments of the *nosZ* gene were amplified with primers nosZ2F and nosZ2R' (Hallin et al., 2009), targeting nitrous oxide reducers. PCR conditions were as described by Hallin et al. (2009). A GC clamp was attached at the 5' end of the reverse primer. The PCR product size was checked using standard agarose gel electrophoresis. Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting analysis of PCR amplified *nosZ* gene fragments was performed using 6% polyacrylamide gels with a linear gradient of 40-65%. Gels were run as described by Mertens et al. (2006).

Copy numbers of *nosZ* gene fragments were quantified in duplicate per DNA extract on a Rotor-Gene 3000 real-time PCR cyclet (Corbett Research). Primers nosZF and nosZ1622R (Throbäck et al., 2004) were used. Real time PCR reactions were performed in a total volume of 15 µl adding 3 µl DNA as template and 7.5 µl SYBR Green Mastermix (Qiagen). Primer concentrations were 0.43 µM for nosZF and 0.27 µM for nosZ1622R. Thermal cycling conditions were as follows: 15 min 95°C; 45 cycles of 15 s 95°C, 15 s 60°C, 30 s 72°C; 5 min 72°C. Dissociation curve analysis and standard agarose electrophoresis was performed to confirm amplification specificity. Dilution series of purified PCR products from cloned *nosZ* gene of

Paracoccus denitrificans LMG 4049^T (Geets et al., 2007) were used as qPCR standards. The efficiency of the qPCR assay was about 80%. The qPCR data are expressed as the average value of the 2 treatment replicates.

Statistics

Dose-response curves were fitted by log-logistic modeling (Doelman & Haanstra, 1989) using the Marquardt method (proc NLIN, SAS[®]9.1; NC, USA). ANOVA was performed to detect effects of Zn on the PNORR and *nosZ* gene abundance. Significant ($P < 0.05$) inhibitory effects of increasing Zn concentrations on individual *nosZ* gene copy numbers were detected using Student's *t*-tests. Cluster analysis of DGGE profiles was performed using the GelCompar II software 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). A similarity matrix for the profiles was calculated based on the Pearson product-moment correlation coefficient (Uyttebroek et al., 2006).

Results

Soil properties as affected by treatments and incubation time

The measured total Zn concentrations at T0 were 60 (control soil - no added Zn), 220, 723, 1320 and 5000 mg Zn kg⁻¹ dry soil. At T2 (6 months), total Zn concentrations in soil decreased by maximally 50% from initial measured values due to leaching outdoors and remained at a similar level at later sampling periods. Milled hay applications did not affect total Zn concentrations. Zinc concentrations in pore water at T0 increased from 0.6 mg Zn l⁻¹ in treatment Zn0 to 5000 mg Zn l⁻¹ in treatment Zn4. After two months incubation, Zn concentrations in pore water decreased in all Zn treatments of the control series (OM0) from initial values to maximally 200 mg Zn l⁻¹ at T1 and T2 and 400 mg Zn l⁻¹ at T3 in treatment Zn4. Milled hay applications decreased pore water Zn concentration with a factor 10 (in treatment Zn2) to 20 (in treatment Zn4) at T2 and T3

compared to the corresponding Zn treatments of the OM0 series. As will be discussed (see section 4 Discussion), this striking effect of OM on Zn solubility is explained by the reduced soil solution pH and reduced Ca^{2+} activity in OM amended soils. Organic matter applications immediately after Zn addition (experiment 2) did not affect pore water Zn concentrations (data not shown). However, two months later pore water Zn concentrations decreased up to a factor 10 in the Zn4 treatment.

Denitrification in Zn and OM amended soil treatments

One week after Zn addition, the PNORR decreased progressively with increasing Zn concentrations in the OM0 series ranging from $1.24 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil h}^{-1}$ in treatment Zn0 to $0 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil h}^{-1}$ in treatment Zn4 (Figure 1 A). The EC50 value based on measured total Zn concentrations was $1066 \text{ mg Zn kg}^{-1}$ and did not change significantly after 2 or 6 months incubation (Figure 1 B-C and Table 1). After 12 months Zn exposure the PNORR was only significantly ($p < 0.05$) inhibited in the Zn3 treatment. In contrast, PNORR was fully recovered in the Zn1, Zn2 and Zn4 treatment (Figure 1 D). The PNORR of the Zn0 treatment after 12 months exposure was halved compared to the PNORR in the Zn0 treatment at T0 which might be attributed to depletion of substrate.

Organic matter (hay) application did not affect the Zn toxicity at 1 week after spiking (experiment 2) since the EC50 values did not significantly differ compared to those of the OM0 series showed in Table 1. The EC50 values were $826 \text{ mg Zn kg}^{-1}$ in the OM1 and $600 \text{ mg Zn kg}^{-1}$ OM2 series. Organic matter applications always increased the absolute values of N_2O reduction activities. For example, the PNORR doubled in the Zn0 treatments of series OM1 ($0.80 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil h}^{-1}$) and OM2 ($0.92 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil h}^{-1}$) compared to the Zn0 treatment of the OM0 series after 6 months of Zn exposure (i.e. 4 months after hay application). At 6 months after

Zn addition, PNORR of the Zn2 and Zn4 treatments recovered to 90% (OM1) or 80% (OM2) of the values of the respective Zn0 treatment. Thus, recovery from Zn stress was faster in the OM1 and OM2 series compared to the OM0 series (Figure 1 C). Full recovery was observed after 12 months exposure to Zn concentrations up to 2770 mg Zn kg⁻¹ (Figure 1 D). Absolute PNORR values recorded for the Zn0 treatments in the OM0, OM1 and OM2 series during the course of the experiment are summarized in Table 2.

The dose-response curves based on pore water Zn concentrations showed similar trends as for total soil Zn concentrations for the OM0 series at T2 (EC50 27 mg Zn l⁻¹) and T3 (full recovery) (Figure 2 A and Table 1). This shows that the gradual recovery of the PNORR in the OM0 treatments is not related to gradual immobilization (fixation) of Zn, strongly suggesting that adaptation reactions occurred. In Figure 2 C PNORR was related to pore water Zn concentrations for the three series at 6 months Zn exposure. The pore water Zn concentrations partially explained the almost complete recovery of the PNORR in the OM1 and OM2 series at 6 months Zn exposure. However, this aspect will be discussed in detail below (see Discussion).

Table 1: Overview of the EC50 values during the course of the experiment expressed on total (mg kg⁻¹) and pore water (mg l⁻¹) Zn concentrations. 95% confidence intervals are given in brackets.

Incubation Period	EC50	
	mg Zn kg ⁻¹	mg Zn l ⁻¹
1 week (T0)		
OM0	1066 [764-1488]	250 [119-522]
2 months (T1)		
OM0	550 [130-2321]	3 [1-8]
6 months (T2)		
OM0	376 [348-405]	27 [11-63]
OM1	>2500	>10
OM2	>2500	>10
12 months (T3)		
OM0	>2560	>386
OM1	>2850	>50
OM2	>2650	>50

Table 2: Overview of the recorded values of the Potential Nitrous Oxide Reduction Rate (PNORR) (mg N₂O-N kg⁻¹ soil h⁻¹) in the Zn0 treatment of the series with (OM1 and OM2) and without (OM0) organic matter application at different sampling periods during the experiment. Standard deviations of the treatment replicates (n = 2) are given in brackets.

Incubation Period	PNORR	
1 week (T0)		
OM0	1.08	(0.03)
2 months (T1)		
OM0	1.12	(0.06)
6 months (T2)		
OM0	0.43	(0.08)
OM1	0.80	(0.02)
OM2	0.92	(0.03)
12 months (T3)		
OM0	0.55	(0.06)
OM1	0.95	(0.02)
OM2	0.94	(0.23)

Figure 1: The Potential N₂O Reduction Rate (PNORR, mg N₂O kg⁻¹ h⁻¹) in response to soil Zn contamination (mg Zn kg⁻¹) at 4 sampling times after Zn contamination (± 1 standard deviation; $n = 2$) of the OM0 series (○) and of 2 series amended with milled hay (OM1: ▽ and OM2: □). Lines are calculated dose response curves at sampling times 1 week and 2 months (A and B), but are only indicative of the trend at sampling times 6 months and 12 months (C and D). Recovery of the denitrifying activity occurred faster when organic matter was applied. Absolute PNORR values of the Zn0 treatments of the respective series at all sampling times are given in Table 2.

Figure 2: The Potential N₂O Reduction Rate (PNORR, mg N₂O kg⁻¹ h⁻¹) in response to soil Zn contamination. Panels A and B show the effects of time on Zn toxicity in absence of organic matter application (OM0). Panels C and D show the effects of organic matter application on Zn toxicity 6 months after Zn contamination. Zn concentrations are expressed as pore water Zn concentration (A and C) and as rescaled 'available' pore water Zn concentration (B and D) to exclude differences in toxicity of soluble Zn due to the large differences in pore water pH, Ca²⁺ and Mg²⁺ activities among the organic matter treatments as explained in the text. Adaptation to Zn occurred within 6 months for the organic matter amended series (OM1 ▽ and OM2 □; D) and only within 12 months incubation for the OM0 series (○; A and B). Lines are only indicative of the trends.

Changes in *nosZ* gene abundance and *nosZ* diversity

The original community contained 1.1×10^8 *nosZ* gene copies g^{-1} soil. After six months Zn exposure, the *nosZ* gene abundance decreased significantly ($p < 0.05$) by a factor 40 in treatment Zn2 and by a factor 60 in treatment Zn4 compared to the Zn0 treatment in the OM0 series (Figure 3 A). In the OM1 and OM2 series, the *nosZ* gene copy numbers of both Zn2 and Zn4 treatments were not significantly different from the corresponding gene copy number of the Zn0 treatments. After 12 months of Zn exposure, the *nosZ* gene copy number was also restored in the Zn2 and Zn4 treatment of the OM0 series (Figure 3 B). The *nosZ* gene abundance correlated well with the denitrifying activity (PNORR) ($R^2 = 0.74$) for the data at T2 and T3 of the OM0 series.

Figure 3: The *nosZ* gene abundance (log *nosZ*, in black) correlates with the Potential N_2O Reduction Rate (PNORR, in grey). A decrease of the PNORR in the Zn amended treatments (Zn2 and Zn4) of the control series (OM0) after 6 months Zn exposure is associated with a decrease of the *nosZ* gene abundance. After 12 months Zn exposure PNORR as well as *nosZ* gene abundance of the Zn2 and Zn4 treatment restored to the value in the Zn0 treatment. Means indicated with the same character within each graph are not significantly different ($p > 0.05$).

Changes in the *nosZ* containing community structure were detectable for the highest contaminated treatment (Zn4) of the OM0 series after 12 months of incubation (Figure 4). The intensity of the dominant bands in the original community profile decreased in the Zn4 treatment, while another band became more dominant. This change of the DGGE profile was already observed after 6 months incubation in the Zn4 treatment of the OM1 (not shown) and OM2 series (Figure 4). These three DGGE profiles showed 60% ($\pm 11\%$) similarity, while 43% ($\pm 16\%$) similarity with all other DGGE profiles was observed. Organic matter applications did not affect the *nosZ* diversity in the Zn0 and Zn2 treatments.

Figure 4: The *nosZ* gene containing community structure during 12 months Zn exposure with (OM2) and without (OM0) organic matter application. The band on the DGGE gel with increased intensity in the Zn4 treatments is indicated on the figure.

Discussion

The EC50 of N₂O reduction did not change significantly during the first 6 months of Zn exposure without organic matter application ranging from 500-1000 mg Zn kg⁻¹. These results differ from the results obtained by De Brouwere et al. (2007) using soil from the same location. Initially, De Brouwere et al. (2007) measured an EC50 of 231 mg Zn kg⁻¹. However, these authors used a different method to measure N₂O reduction, which might affect toxic thresholds. According to that study denitrification restored within 2 months incubation (EC50 > 1600 mg Zn kg⁻¹), while adaptation in the present study was only observed after 12 months Zn exposure (EC50 > 2560 mg Zn kg⁻¹). This slower adaptation might be attributed to the lower incubation temperature (average temperature of 15 °C during the first three months of incubation vs. 25°C in the study of De Brouwere et al. (2007)). Diaz-Raviña and Bååth (1996) showed that adaptation of the microbial community to metal toxicity occurs slower at a lower temperature.

The EC50 values based on pore water Zn concentrations instead of total Zn concentrations exclude Zn ageing processes and are a better predictor of Zn toxicity since it reflects the bioavailable Zn (Mertens et al., 2007). As shown in Figure 2 A the EC50 based on pore water Zn concentrations increased from 25 mg Zn l⁻¹ after 6 months incubation to >386 mg Zn l⁻¹ after 12 months incubation. This largely suggests that the recovery of the denitrification within 12 months Zn exposure is due to the adaptation of the denitrifying community and not to Zn immobilization processes.

We previously showed that substrate amendment accelerates adaptation of the nitrifying community to Zn (Ruyters et al., 2010). Brandt et al. (2009) recently showed that carbon amendment stimulated tolerance development of the soil microbial community to sulfadiazine additions and attributed the accelerated adaptation to increased microbial activity. In the present study, the microbial activity was stimulated by hay applications. Nitrous oxide reduction was

almost fully recovered within 6 months including the highest contaminated treatment for both organic matter applications (Figure 1 C). This was not observed in the series without OM applications, confirming the accelerating effect of a stimulated activity on the recovery of the denitrification. Experiment 2 showed that hay addition did not restore denitrification within one week after Zn exposure indicating that the recovery is not merely an effect from higher carbon substrate availability.

The pore water Zn concentrations of the Zn2 and Zn4 treatments amended with organic matter (OM1 and OM2 series) decreased compared to those of the corresponding Zn treatments of the OM0 series by at least a factor of 10. This apparent Zn immobilization process requires attention as it might partially explain the faster recovery of the PNORR in the OM amended series. Simultaneously with the Zn concentration, pore water Ca concentrations equally decreased sharply. For example, in the Zn2 treatments 6 months after spiking, Ca^{2+} concentrations were 12 mM in OM0 decreasing to 0.6 mM in OM1 or 0.8 mM in OM2. These changes are related to (i) the effect of fresh plant residues on solution pH and (ii) more intensive soil leaching due to the effect of OM application on soil structure. Plant residues contain high concentrations of K^+ salts of organic acids such as oxalate, malate etc., which increase soil pH. Also, further decomposition of organic acid consumes protons due to the decarboxylation of organic acid anions (Mokolobate and Haynes, 2001) and the corresponding pH increase occurs between 14 and 42 days after residue application (Xu et al., 2006). The soil used in this study has a variable soil charge since the CEC is dominated by organic matter (see 2.1 soil sampling). As a result, proton consumption due to organic matter decomposition increases the effective CEC, thereby provoking sorption of Ca^{2+} , Mg^{2+} and Zn^{2+} on the soil, whereas K^+ remains in solution due its lack of selective sorption. The soil pH, measured in 10 mM CaCl_2 extracts, does not detect this pore water pH change, since Ca^{2+} exchanges with H^+ on the soil. The soil solution Ca^{2+} concentration of the OM1 and OM2

treatments are >10 fold below that in the 10 mM CaCl₂ solution used for pH measurements. As a result, the soil pH (CaCl₂ 0.01M) is below soil solution pH for the OM1 and OM2 treatments. The pore water (pw) pH can be estimated from the Ca²⁺ pore water concentration and the soil pH_{CaCl2} (0.01M CaCl₂) with the lime potential equation (Bolt and Bruggenwert, 1976, p82), i.e. $pH_{pw} = pH_{CaCl2} - 0.5 \times \log(Ca^{2+}_{pw} (M)/0.01)$. This reveals that the application of organic matter increased pore water pH by 0.5 pH units compared to the treatments that received no OM.

It is well established in aquatic toxicology that the toxicity of soluble Zn²⁺ increases with decreasing concentrations of Ca²⁺, Mg²⁺ and H⁺ (increasing pH). These interactions are included in the Biotic Ligand Model concept (Di Toro et al., 2001). This means that the toxicity of soluble Zn cannot be compared among treatments that differ in pH and Ca²⁺ and Mg²⁺ concentrations such as the treatments of the OM0, OM1 and OM2 series. The cationic interactions for Zn²⁺ toxicity on soil nitrifying microorganisms have been measured in pure cultures and these interactions were confirmed in soil (Mertens et al., 2007). These interactions are summarized in an empirical Freundlich equation and were used here to correct soluble Zn concentrations for differences in pore water pH and Mg²⁺ and Ca²⁺ concentrations across the treatments, i.e. pore water Zn concentrations were rescaled to ‘available’ Zn concentration (Figure 2 B and D). This rescaling is only an approximation since the interactions are not proven for denitrifying microorganisms. However, the changes are manifest compared to unscaled concentrations and reveal that the recovery of the denitrification in the OM series is not only the result of a decreased Zn bioavailability, but can at least partially be attributed to adaptation of the N₂O reducing community within 6 months Zn exposure. This rescaling hardly varies the data of the treatments of OM0 (Figure 2B), because time or OM effects on pore water pH or Ca²⁺ were minimal. This analysis strongly suggests that adaptation is accelerated in the presence of higher organic matter levels.

As stated by Philippot and Hallin (2005) the denitrifying community is well suited for understanding the relationship between community structure and activity. Consequently, several studies have linked activity with the denitrifying community composition using functional denitrification genes as molecular markers. Henry et al. (2008) and Miller et al. (2008) reported an increased denitrifying activity upon carbon additions with only a minor impact on the denitrifying community. Miller et al. (2009) showed that the *nosZ* gene abundance increased upon carbon substrate additions by providing liquid manure, but not by means of crop residue applications (Miller et al., 2008). Similarly in the present study, the *nosZ* gene abundance did not differ in the Zn0 treatments between soils amended or not with hay although the PNORR varied about a factor of 2 after 12 months (Table 2). Substrate depletion might explain this discrepancy since it decreases activity without leading to immediate and excessive cell death and, thus, decreased *nosZ* gene abundance. In contrast, inhibition of the nitrous oxide reduction due to Zn co-occurred with a decrease of the *nosZ* gene abundance, whereas the recovery of the nitrous oxide reduction process in all three OM series was related to a recovery of the *nosZ* gene abundance to the level in the control treatment (Figure 3). These results show a clear relationship between changes of the denitrification activity and changes of the denitrifying community size due to Zn contamination. Changes of the nitrous oxide reducing community structure, as revealed with DGGE, are rather modest compared to similar studies on the nitrifying community (Mertens et al., 2006; Ruyters et al., 2010). Hence, the recovery of denitrification activity after an initial acute Zn exposure is mainly explained by changes in community size rather than changes of the community structure.

In conclusion, this study confirms that trace metal inhibition of N₂O reduction is a transient effect and that recovery was correlated with the development of a Zn adapted denitrifying community. This process was accelerated in the presence of higher organic matter levels.

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