Do ammonia-oxidizing archaea respond to soil Cu contamination similarly as ammonia-oxidizing bacteria?

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Abstract Inhibitory experiments were conducted to investigate the responses of the population sizes of ammonia-oxidizing archaea (AOA) and bacteria (AOB) and the potential nitrification rates (PNRs) to Cu contamination in four Chinese soils. PNR was determined using a substrate-induced nitrification (SIN) assay, and the population size of the nitrifiers represented by *amoA* gene abundances was quantified using a real-time polymerase chain reaction (qPCR) assay. Both population size and PNR of the ammonia oxidizers reduced considerably at high Cu concentrations in all the soils. Bacterial *amoA* gene abundance was reduced by from 107-fold (Hailun soil) to

more than 232-fold (Hangzhou soil) at the highest Cu concentrations (2,400 mg kg⁻¹ Cu for Hailun, Langfang and Guangzhou soils and 1,600 mg kg⁻¹ Cu for Hangzhou soil), while reduction in archaeal *amoA* gene abundance was from 10-fold (Langfang soil) to 89-fold (Hangzhou soil). AOA seemed more tolerant to Cu contamination than AOB. Nitrification rates were inhibited by more than 50% at a Cu concentration of 600 mg kg⁻¹, and by more than 90% at the highest Cu concentrations in all soils. These results indicated that both AOA and AOB can be inhibited by toxic metals, highlighting the need to consider the role of AOA in nitrification in soils.

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Introduction

Nitrification, the microbially-mediated conversion of ammonia (NH₃) to nitrate (NO₃⁻) (via nitrite, NO₂⁻), plays a central role in the global nitrogen cycle (Arp and Bottomley 2006; Jackson et al. 2008). Ammonia oxidation, the first and rate-limiting step of nitrification (Arp et al. 2002), has long been considered to be catalyzed by autotrophic ammonia oxidizing bacteria (AOB) belonging to the gamma- and beta-proteobacteria (Arp and Bottomley 2006). However, recent metagenomic and cultivation-dependent studies

have demonstrated that archaea are also involved in nitrification (Leininger et al. 2006; Nicol and Schleper 2006; Wuchter et al. 2006). As one of the three domains of life, archaea have long been considered as extremophiles, largely restricted to habitats such as sulfidic hot springs and salt brines (Fox et al. 1977; Woese et al. 1990). However, recent studies have shown that archaea are widespread in diverse environments including oceans (Karner et al. 2001) and soils (Leininger et al. 2006). Given their potential role in nitrification, AOA have been the focus of recent interest, especially in soils (Tourna et al. 2008), but also other ecosystems (Nicol and Schleper 2006), and investigation of response of AOA to environmental factors (e.g., Cu contamination) can be a possible step for the validation of their role in soil nitrification.

Much of our understanding on nitrification has focused on the AOB. Correlations between AOB nitrification kinetics and population size in soil have been reported (Okano et al. 2004). Cell-specific

ammonia oxidation rates in soils and sewage, based on the assumption that AOB were the only nitrifiers, fall within the range of those estimated in pure cultures (see Table 1), suggesting no ‘missing’ ammonia oxidation. There are, however, exceptions. For example, in a mixed conifer forest autotrophic AOB could not account for the high nitrification rates observed (Jordan et al. 2005), highlighting the potential role of AOA (Leininger et al. 2006). A contribution of AOA to nitrification in arable soils has also been suggested as a possible explanation for a lack of congruence between AOB numbers and net nitrification rates (see Cavagnaro et al. 2008 and references therein). These studies highlight the need for further investigation of the ecology of AOA and AOB in soils, if we are to understand their relative contributions to nitrification under a range of conditions.

Nitrification is sensitive to environmental factors such as pH, temperature, water content, light, and/or ammonia concentration (Dancer et al. 1973; Kowalchuk and Stephen 2001; Nishio and Fujimoto 1989; Oved et

Table 1 Specific ammonia oxidation rates of pure cultures and nitrifying communities in previous studies

Method	Organism	Oxidation rate (fmol NH ₃ cell ⁻¹ h ⁻¹)	Reference
FA, pure culture	<i>Nitrosospira</i>	4	Belser and Schmidt 1978 Okano et al. 2004
FA, pure culture	<i>Nitrosolobus</i>	23	Belser and Schmidt 1978 Okano et al. 2004
AODC, pure culture	<i>Nitrosospira</i> spp.	4.3 (50) ^a	Jiang and Bakken 1999 Okano et al. 2004
qPCR	Indigenous AOB in sewage	16–43	Daims et al. 2001b
FISH	Indigenous AOB in sediment	2.3±0.4	Daims et al. 2001a
qPCR	Indigenous AOB in activated sludge	7.7	Harms et al. 2003
FISH	Indigenous AOB in activated sludge	1.3–8	Altmann et al. 2003
qPCR	Indigenous AOB in soil	15.6 (0–1) ^c	Okano et al. 2004
FISH	Indigenous AOB in activated sludge	0.03–43.00	Coskuner et al. 2005
DAPI-stained ^b , pure culture	<i>Nitrosopumilus maritimus</i>	~0.17	Könneke et al. 2005
CARD-FISH	<i>Nitrosopumilus maritimus</i> ^c	0.08–0.17 (25°C) ^d	Wuchter et al. 2006
DAPI-stained ^b , pure culture	<i>Nitrosomonas europaea</i>	24.3	Kindaichi et al. 2006
FISH	Indigenous AOB in sewage	0–3.8	Lydmark et al. 2007

Cell abundance estimated by: FA, Fluorescent-antibody staining; AODC, Acridine orange direct counting; CARD-FISH, fluorescence *in situ* hybridization and catalyzed reporter deposition; or qPCR, quantitative polymerase chain reaction;

^a Values in parentheses are experiment duration (hours);

^b DAPI-stained cells were directly counted on filters by fluorescence microscopy;

^c dominated member among the nitrifying crenarchaeal enrichment culture;

^d Value in parentheses are incubation temperatures;

^e Values in parentheses are duration of experiment (days).

al. 2001; Stephen et al. 1998). To date, most studies have focused on the AOB. However, the discovery of AOA (Könneke et al. 2005; Treusch et al. 2005) and discrepancies between nitrification rates of AOB abundance (see above), necessitate simultaneously study of both AOA and AOB, in terms of their abundance, physiology and response(s) to environmental stressors. Nitrification in soils is sensitive to heavy metals, including Cu (Doelman and Haanstra 1984; Oorts et al. 2006; Premi and Cornfield 1969). In fact, the sensitivity of nitrification to heavy metals is commonly used to assess the impact such contaminants in soils (ISO, Geneva, Switzerland; Smolders et al. 2001), providing the basis for the widely used substrate induced nitrification assay (SIN) (Smolders et al. 2004; Broos et al. 2005). While the SIN assay is commonly used to provide guidelines for heavy metal contamination, few studies have quantified the impacts of heavy metal additions on both the AOA and AOB. If the AOA and AOB are simultaneously differentially impacted by Cu addition, then their relative abundance in soils will be an important determining factor in the outcomes (and potentially the validity) of the SIN assay. However, this remains to be seen. Therefore, here we report results of an experiment in which we studied the responses of AOA and AOB to Cu addition in a range of arable soils. Specifically, we aimed to:

- 1) Comparatively assess the impact of Cu addition on the abundance of AOA and AOB in these soils; and
- 2) Compare the potential nitrification rates and abundances of ammonia oxidizers in the SIN assay.

Methods and materials

Soil

Soil was collected from Guangzhou, Guangdong province, Southern China; Hangzhou, Zhejiang province, Southeast China; Langfang, Hebei province, Northern China; and Hailun, Heilongjiang province, Northeast China. The Hailun soil was from grassland and the others from cultivated land. These soils are representative of four major agricultural regions from

across China. The soils were air-dried and sieved (mesh size: 2 mm) and soil physicochemical properties were determined, as follows. Soil pH was measured with a soil/water ratio of 1:5. Soil organic matter content (OMC) was determined by combustion analysis (Marriott and Wander 2006). Total nitrogen content was determined as N_2 by thermal conductivity detection (Matejovic 1997). Cation exchanging capacity (CEC) was measured with 1 M ammonium chloride at pH 7.0 after pretreatment to remove the soluble salts. Soil particle size was analyzed following Tan (2005). Major element concentrations were determined by ICP-AES or ICP-MS after dissolution with *aqua regia* (HNO_3 : HCl -1:3) (Zarcinas et al. 1996).

Soil Cu addition and nitrification assays

Sieved, air-dried soil from Hailun, Langfang and Guangzhou was amended with $CuCl_2$ solution to give nominal concentrations of 0, 37.5, 75, 150, 300, 600, 1,200, 2,400 $mg\ Cu\ kg^{-1}$ dry soil. The Hangzhou soil was amended to give nominal concentrations of 0, 25, 50, 100, 200, 400, 800 and 1,600 $mg\ kg^{-1}$ dry soil. The Cu addition treatments were selected based upon results of preliminary studies (data not shown), in which nitrification rates were reduced by $\geq 90\%$. After amendment, soils were thoroughly mixed by hand. The control and Cu-amended soils were left to equilibrate overnight at 100% MWHC (maximum water holding capacity), then air-dried and stored at room temperature before use.

The control and Cu-amended soils were used to establish a controlled microcosm study. The measurement of PNR in soils followed the SIN assay described by Smolders et al. (2004). Six replicates of 7 g soil for each Cu treatment were incubated in a 50 ml centrifuge tube under dark aerobic conditions at $25 \pm 2^\circ C$, maintained at $50 \pm 5\%$ of MWHC by addition of deionized water. There were 192 microcosm tubes in total. After 14-day preincubation, one tube of each Cu treatment was extracted with 35 ml 1.0 M KCl and mineral N pools (NH_4^+ -N, and NO_2^- -N plus NO_3^- -N) determined using a flow injection autoanalyser (SKALAR, Holland). To each of the remaining (five) tubes in each treatment, 0.5 ml of 0.044 M $(NH_4)_2SO_4$ was added, and incubated for a further 7 days. At this time, two replicates were analyzed for NH_4^+ -N and NO_2^- -N plus NO_3^- -N (as

above) and the remaining 3 replicates were used for DNA extraction (see below). Data were used to calculate potential nitrification rates (see below).

Soil solution extraction and analysis

Soil solutions in all treatments (one replicate for each treatment) were extracted at the beginning of SIN assay using the method of Thibault and Sheppard (1992). Cu^{2+} activities in soil solutions was measured using a cupric ion selective electrode (Cu-ISE, Orion 94–29) coupled with a reference electrode (Orion 900200) and a mV meter (Orion 720) (Ma et al. 2006). pH, electrical conductivity (EC) and dissolved organic carbon (DOC) were measured following conventional procedures (Tan 2005). Elemental concentrations in soil solutions were measured either by inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS) depending on their concentrations in the solution, following the methods of Khan et al. (2008).

DNA extraction and real-time PCR

DNA was extracted from ca. 0.5 g (fresh weight) of soil using a Bio 101 FastDNA SPIN Kit for soil (Bio 101 Inc., Calif., USA), following the manufacturer's instructions. The concentration and quality of the extracted DNA was determined by spectroscopic analysis (Nano-drop, Wilmington, Delaware, USA) and agarose gel electrophoresis. DNA was stored at -20°C .

Copy numbers of the functional ammonium mono-oxygenase (*amoA*) from AOA and AOB were determined by real-time PCR using an iCycler iQ 5 thermocycler (Bio-Rad, Calif., USA). To optimize the qPCR reaction system, some of the DNA extracts were diluted 100-fold or 10-fold and used as template.

The 25- μl reaction mixtures included 1 μl of template DNA, 12.5 μl of SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 500 nM of the primers, Arch-amoAF (STAATGGTCTGGCTTAGACG) and Arch-amoAR (GCGGCCATCCATCTG TATGT) for archaeal *amoA* (Francis et al. 2005), and AmoA-1F* (GGGGHTTYTACTGGTGGT) and AmoA-2R (CCCCTCKGSAAAGCCTTC TTC) for bacterial *amoA* (Stephen et al. 1999). For archaeal *amoA* amplification, the protocol was as follows: 95°C for 5 min; 30 cycles consisting of 94°C for 45 s, 53°C for 60 s, and 72°C for 60 s; and 72°C for 15 min. For bacterial *amoA* amplification the protocol was: 94°C for 3 min, 40 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, followed by melting curve analysis was from 65 – 98°C , 0.2°C per read, 6 s hold. Fluorescence was read during each cycle at 83°C .

A standard curve of *amoA* genes of known copy numbers was prepared as described by the et al. (2007). Ten-fold serial dilutions of a known copy number of the plasmid DNA were used to perform real-time PCR assays in triplicate to generate standard curves. The amplification efficiencies ranged from 89.8% to 98.5% for AOB and from 94.1% to 102.3% for AOA, and the linear coefficient correlation (R^2) of standard curves ranged from 0.989 to 0.996 for AOB and from 0.990 to 0.994 for AOA.

Data analysis and calculations

Differences in AOA and AOB *amoA* copy numbers between unamended and amended soils were compared using t-tests, using SPSS 11.5 (SPSS Inc., Chicago, USA). PNR, defined as the yield of nitrite plus nitrate per kg of dry soil per day, were calculated from the difference between the concentrations of nitrite plus nitrate at day-0 and day-7 after ammonium addition (Oorts et al. 2006; Smolders et al. 2001).

Table 2 Selected properties of soils from Hailun, Langfang, Hangzhou and Guangzhou in China

Soil	pH	PNR ₀ (mg N kg ⁻¹ d ⁻¹)	CEC (cmol+kg ⁻¹)	OMC %	Total N	Al	Ca	Fe	K	Soil texture (%)			Mg (Mg kg ⁻¹)	Cu (Mg kg ⁻¹)
										Clay	Silt	Sand		
Hailun	6.6	2.9	33.6	3.0	0.25	3.2	0.7	2.7	0.4	40.4	26.7	32.9	5430	18.3
Langfang	8.8	10.2	6.4	0.6	0.06	0.9	1.3	1.6	0.2	10.1	3.7	86.2	4946	9.5
Hangzhou	6.8	8.0	12.8	2.5	0.25	2.3	0.6	2.4	0.3	38.9	36.3	24.8	4472	47.2
Guangzhou	7.3	10.6	8.3	1.5	0.13	2.1	0.2	1.8	0.1	25.3	12.4	62.3	630	10.8

PNR data was used to generate log-log dose-response curves automatically by the software for each soil. The percent effective doses (ED_x) of a toxicant, in this case Cu were calculated. ED_{50} calculation was performed by regression from the dose-response curve automatically and ED_{10} , ED_{20} and ED_{90} values were calculated from fitted equations manually.

Results

Soil and soil solution physico-chemistry

Physicochemical properties of the four soils are given in Table 2. The physio-chemical properties of studied soils covered a wide range in pH (6.6–8.8), CEC (6.4–33.6 $\text{cmol} + \text{kg}^{-1}$), clay content (10.1–40.4%), background Cu (9.5–47.2 Mg kg^{-1}) and so on.

Cu amendment resulted in a significant, non-linear (exponential, $R^2=0.980\text{--}0.998$), increase in Cu^{2+} concentration in the soil solution, for all soil types (Fig. 1). Soil solution pH decreased with increasing Cu doses by 0.84 units in the Langfang soil to 2.89 units in the Guangzhou soil, at the highest Cu doses; this change in pH was paralleled by an increase in elemental P, S, Ca, Na, Ni and Zn concentrations, EC and DOC (data not shown).

amoA gene abundance

Bacterial *amoA* gene abundance ranged from 3.74×10^9 copies g^{-1} of dry soil (Langfang, 150 mg kg^{-1} Cu) to less than 10^4 copies g^{-1} of dry soil (Guangzhou, $2,400 \text{ mg kg}^{-1}$ Cu) (Fig. 2). Archaeal *amoA* gene abundance ranged from 2.97×10^9 copies g^{-1} of dry soil

Fig. 1 Responses of Cu^{2+} in soil solutions to Cu addition to the soils from Hailun, Langfang, Hangzhou and Guangzhou

Fig. 2 Responses of archaeal (AOA) and bacterial (AOB) *amoA* gene abundances and potential nitrification rates (PNR) to Cu contamination in soils from Hailun, Langfang, Hangzhou and Guangzhou, China. Abundances of bacterial *amoA* genes were below the detection limit (10^4 copies g^{-1} of dry soil) at a Cu concentration of $1,600 \text{ mg kg}^{-1}$ in Hangzhou soil and at Cu concentrations $\geq 1,200 \text{ mg kg}^{-1}$ in Guangzhou soil. Values for gene abundance are means ($n=3$) $\pm$ standard errors. Superscript letter (α) indicates significant differences ($P < 0.05$) compared with the unamended controls (t-test). Values for PNR are means of the two replicates. For ease of comparison, unamended controls are arbitrarily placed at 1 on the (log scale) x-axis

(Hailun, unamended) to 1.86×10^6 copies g^{-1} of dry soil (Guangzhou, 2,400 Mg kg^{-1} Cu). In unamended soil, the range in bacterial *amoA* gene abundance was large, from 1.26×10^9 copies g^{-1} of dry soil (Langfang) to 2.32×10^6 copies g^{-1} of dry soil (Hangzhou), but variation was less in archaeal *amoA* abundance, from 2.97×10^9 copies g^{-1} of dry soil (Hailun) to 1.64×10^8 copies g^{-1} of dry soil (Guangzhou) (Fig. 2).

amoA gene copy numbers changed with Cu addition. With the exception of the Langfang soil, bacterial *amoA* copy numbers were lower than archaeal *amoA* copy numbers in all Cu addition treatments, with the greatest overall difference in the Hangzhou soil. Both the archaeal and bacterial *amoA* gene copies generally decreased with increased Cu addition in all soils. For example, in Hangzhou soil, bacterial and archaeal gene abundances were reduced from around 10^6 copies g^{-1} (unamended) of dry soil to $<10^4$ copies g^{-1} of dry soil (1,600 mg kg^{-1} Cu) and from 10^9 copies g^{-1} of dry soil (unamended) to nearly 10^7 copies g^{-1} of dry soil (1,600 mg kg^{-1} Cu), respectively. Bacterial *amoA* gene abundance was reduced from 107-fold (Hailun soil) to more than 232-fold (Hangzhou soil) at the highest Cu concentrations, while reduction in archaeal *amoA* gene abundance was much less, from 10-fold (Langfang soil) to 89-fold (Hangzhou soil).

Substrate-induced nitrification

Substrate Induced Nitrification (SIN) determined following standard eco-toxicology protocols differed nearly 4-fold, with the Guangzhou soil having the greatest (10.6 mg N kg^{-1} d $^{-1}$), and the Hailun soil the lowest (2.85 mg N kg^{-1} d $^{-1}$) rates. Nitrification rates

were inhibited by more than 50% at a Cu concentration of 600 mg kg^{-1} , and by more than 90% at the highest Cu concentrations in all soils (Table 3). The ED₅₀ values were ranked Hangzhou > Hailun > Guangzhou > Langfang, which was different from the ranking for the PNR.

Discussion

This study quantified the responses of AOA and AOB to Cu contamination in a range of agricultural soils in China. The population sizes of AOA and AOB were relatively large, and while they all decreased with Cu addition, responses varied considerably between soils. The impact of Cu on AOA and AOB not only resulted in changes in population sizes, but also activity, with a reduction in potential nitrification rate (PNR) with increasing Cu addition. Together these data highlight the need to consider both the AOB and AOA in nitrification in soils, and the impacts of heavy metal contamination on these important functional groups of soil microorganisms.

AOA and AOB *amoA* copy numbers varied significantly between soils, ranging from 10^4 – 10^9 and 10^6 – 10^9 per g dry soil, respectively. These *amoA* copy numbers are consistent with published values for both AOA (Leininger et al. 2006; Wuchter et al. 2006) and AOB (Cavagnaro et al. 2007; Cavagnaro et al. 2008; He et al. 2007; Okano et al. 2004) in agricultural soils. Where no additional Cu was added to the soils, neither AOA nor AOB copy numbers were consistently higher or lower than one another, highlighting the importance of edaphic factors in

Table 3 ED_x (mg kg^{-1}) for Cu in soils from Hailun, Langfang, Hangzhou and Guangzhou in China

Soil	ED ₁₀		ED ₂₀		ED ₅₀		ED ₉₀	
	95%CI		95%CI		95%CI		95%CI	
Hailun	68.8	10.8–493.4	101.6	29.0–567.0	371.8	102.6–1347.9	903.1	224.9–17925.2
Langfang	68.6	18.4–81.3	96.1	38.1–118.1	171.5	119.2–246.7	513.8	414.2–1392.5
Hangzhou	150.6	34.6–354.4	216.8	73.0–426.8	392.0	224.8–683.6	942.0	541.3–3562.5
Guangzhou	192.7	2.7–1392.4	233.3	10.9–1213.3	333.8	89.9–1238.9	673.9	235.4–13804.4

PNR₀: potential nitrification rate in the unamended soil samples. Mean value of the two replicates

ED_x: x percent effective dose

95%CI: 95% confidence interval

determining the abundance of the different groups of nitrifiers. However, it should be stressed that, at high Cu doses ($>600 \text{ mg kg}^{-1}$), the significant increase in H^+ and other toxic ions (e.g., Zn^{2+} and Ni^{2+}) induced by Cu addition as well as Cu^{2+} may also contribute the observed toxicity to nitrifying activity and populations (Oorts et al. 2006). Therefore, need exists for a further investigation of the relative contribution of Cu amendment and covarying toxic ions to observed toxicity.

The addition of Cu to each of the soils resulted in a clear increase in available Cu, as expected (Ma et al. 2006). The increase in available Cu was paralleled by a concomitant decrease in both AOA and AOB *amoA* copy numbers, as with AOB in other soils amended with Zn (Xia et al. 2007). The data presented here permit the calculation of ED values (Smolders et al. 2001) for Cu. ED values indicated that the toxicity to ammonia oxidizers occurred at a Cu dose of 200 mg kg^{-1} and more than 90% was inhibited at a Cu dose of $1,000 \text{ mg kg}^{-1}$ in all of the soils studied here. At these high Cu doses, SIN was also very low (see below).

We hypothesized that AOA may be less sensitive to Cu addition as archaea are reported to tolerate extreme conditions. Typically, archaeal membranes are less permeable to ions than bacterial membranes because of the chemical structure of their membrane lipids (Kandle and König 1998). This characteristic may provide archaea with the ability to survive under stress (e.g. energy or osmotic stress) (Valentine 2007). In the soils studied here, Cu doses up to $1,200 \text{ mg kg}^{-1}$ appeared to impact AOA and AOB similarly. However, at very high Cu addition treatments ($> 1,200 \text{ mg kg}^{-1}$), AOB populations were almost entirely eliminated (in all soils) while AOA persisted in two of the soils studied here (Hangzhou, and Guangzhou). This suggests that AOA in these soils may be more tolerant to Cu contamination. This response warrants further detailed investigation.

A clear relationship between AOB numbers and net nitrification has been reported in some soils (Okano et al. 2004), but not others (e.g. Jordan et al. 2005; Cavagnaro et al. 2008). Similarly, heavy metal (including Cu) addition has been shown to decrease soil nitrification rates (Nies 1999; Oorts et al. 2006; Smolders et al. 2001). Here there was a clear relationship between PNRs and both AOA and AOB population sizes, as indicated by multi-regression analysis between PNR and *amoA* gene abundance

(data not shown). Similar results has been demonstrated for AOA and AOB following fertilizer and zinc addition (He et al. 2007; Xia et al. 2007). This further highlights the role of both AOA and AOB in nitrification in these soils. What remains to be seen however, is if the decrease in AOA and AOB population sizes was accompanied by a change in community composition. Such responses has been reported for total bacterial diversity following Cu contamination (Smit et al. 1997).

Taken together, this study highlights the importance of Cu contamination on the functional ecology of Nitrifiers. The reduction in AOA and AOB populations reported here may be an underestimation, due to the detection of DNA from recently dead cells (Bakken 1997; Cavagnaro et al. 2008; Chabrierie et al. 2003), or from slow-growing or dormant cells (Balaban et al. 2004). Thus, studies of RNA from AOA and AOB in these and other soils may be especially valuable in further exploring this issue. Due to difficulties associated with the establishment of AOA pure cultures from soils, and the lack of information about the relative importance of AOA and AOB in soil nitrification, inhibition assays may yield new insights into the relative contribution of AOA and AOB to nitrification in soils. Our data further highlight the need for an integrated approach that links important soil processes with the organisms that undertake them.

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