Geoderma 321 (2018) 141–150

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Influence of Cu application on ammonia oxidizers in fluvo-aquic soil

Huan He[a,1], Hua Liu[a,1], Tianlin Shen[b], Shaodong Wei[c], Jiulan Dai[a,*], Renqing Wang[a,b]

[a] Environment Research Institute, Shandong University, Jinan 250100, China
[b] Institute of Ecology and Biodiversity, College of Life Science, Shandong University, Jinan 250100, China
[c] Biology, Section for Microbiology, University Park 15, Copenhagen 2100, Denmark

ARTICLE INFO

Handling Editor: Yvan Capowiez

Keywords:
amoA gene
Ammonia-oxidizing archaea
Ammonia-oxidizing bacteria
Copper
Fluvo-aquic soil

ABSTRACT

Nitrification driving by archaeal and bacterial ammonia oxidizers is an essential step of the global nitrogen cycle. This laboratory study screened toxicity of copper (Cu) stress in the fluvo-aquic system, investigated the ecological niche of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) communities and analyzed the response of AOA and AOB communities to Cu stress in the system. We added Cu as $CuCl_2$ solution at 0 mg, 100 mg, 300 mg, 600 mg, and 1200 mg Cu per kg soil in the fresh fluvo-aquic soil. Soil samples were incubated for 6 h, 12 h, 1 d, 3 d, 7 d, 14 d, 28 d, and 56 d in lab condition. The soil potential nitrification rates (PNR) were measured at each stage. Denaturing gradient gel electrophoresis (DGGE) analysis of *amoA* of AOA and AOB were performed at 56 d of Cu incubation. Soil RNA was extracted and the abundance of *amoA* transcripts was analyzed by real time polymerase chain reaction (real-time PCR) at 1, 3, and 7 d. The *amoA* gene abundance analysis and DGGE profile analysis showed that the abundance and diversity of AOA were higher than that of AOB in fluvo-aquic soil. However, the correlations between the soil PNR and the number of *amoA* transcript indicated that the AOB community, rather than AOA community, dominates ammonia oxidation in Cu-polluted fluvo-aquic soil ecosystems. Combining the results of the soil PNR and the abundance and diversity of ammonia oxidizers community, Cu hormetic concentration was identified as a range from 100 to 600 mg kg^{-1}, while the toxic concentration was higher than 600 mg kg^{-1} in fluvo-aquic soil system. Furthermore, the results of *amoA* transcript numbers showed that AOA may be resistant to Cu stress and AOB may be more sensitive to Cu stress under our experiment conditions.

1. Introduction

Heavy metal contamination in agricultural soil has become a serious problem because heavy metals impose permanent pressure on physical and chemical properties of soil, influence adversely fertility of agricultural soil, and affect the organisms and ecosystem processes (Oliveira et al., 2010; Henriques et al., 2015). Among all metals, copper (Cu) has received much attention because of its high toxicity (Sheldon and Menzies, 2005). Cu in agricultural soil was mainly derived from several sources, e.g., the use of pesticides (Bu et al., 2013), sewage irrigation (Han et al., 2006), and atmospheric deposition (Zhang, 2001). It has been recognized that long-term use of Cu-based pesticides would result in Cu accumulation in agricultural soil. A preliminary survey showed that the Cu content was higher than 100 mg kg^{-1} in vineyard soil, which represents the threshold of Cu content as measured by European Commission in 1986 (Council Directive, 1986). When total Cu concentration exceeds this threshold (100 mg kg^{-1}), Cu would negatively affect plant growth and soil functioning (Ippolito et al., 2010). Comparatively, the paddy soil containing from 150 to 650 mg kg^{-1} of Cu has been used for growing cereal grass in Zhejiang province of China (Wang et al., 2007). Therefore, in the laboratory simulation of Cu toxicity, the range of Cu^{2+} concentration 0–800 mg kg^{-1} in soil was frequently used (Hu et al., 2014).

The adverse effects of Cu on soil biological processes and soil microbial communities have been investigated (Brookes and McGrath, 1984). Cu poses a significant risk to certain plant beneficial bacteria (Sharaff and Archana, 2015), brings distinct changes of soil microbial community structure and diversity (Brandt et al., 2010), and affects the function of soil microorganisms related to the global C-cycle and N-cycle (Chaudri et al., 2008). For example, Nunes et al. (2016) reported that, as Cu concentration increased from normal level (13–16 mg kg^{-1}) to high level (400–500 mg kg^{-1}) in grassland soil, a lower relative abundance of Proteobacteria, Actinobacteria and Verrucomicrobia was observed, while the relative abundance of Nitrospira and Acidobacteria became higher. In addition, the same study also reveals that the transcriptional activity of ammonia-oxidizing Nitrospira was enhanced in

* Corresponding author.
E-mail address: daijiulan@sdu.edu.cn (J. Dai).
[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.geoderma.2018.01.037
Received 5 September 2017; Received in revised form 24 January 2018; Accepted 28 January 2018
Available online 20 February 2018
0016-7061/

the presence of high Cu level (500 mg kg^{-1}) in soil (Nunes et al., 2016).

Although Cu is an essential metal required by many cellular enzymes of soil microbes, excessive Cu could be harmful to soil microbes. The proteins involved in energy metabolism would be down-regulated, while the proteins required for Cu resistance ($CopA_1$ and $CopH_4$) are up-regulated (Waldron et al., 2009; Pozo et al., 2010; Gillan et al., 2017). Gillan et al. (2017) have tested soil biological resistance to heavy metals Cu, Zn and Hg by genome sequencing. They found that Cu-translocating P-type ATPase and multicopper oxidase, two critical enzymes involved in Cu homeostasis, were significantly up-regulated when Cu concentration increased from 810 mg kg^{-1} to 3920 mg kg^{-1}. The main purpose of these current researches is to use the metal sensitivity of some soil biological properties to assess and monitor heavy metal pollution (Smolders et al., 2001; Oliveira et al., 2010; Gilliam et al., 2016).

Nitrification, a microbial-mediated process that plays a key role in the natural nitrogen cycle, is sensitive to heavy metals, so it has been widely used in screening Cu toxicity (Sauvé et al., 1999; Khan and Scullion, 2000; Arp and Bottomley, 2006). In addition, Brookes (1995) have suggested that combing microbial activity (e.g., soil respiration, soil potential nitrification rate) and microbial population parameters (e.g., abundance and diversity of microbes) would provide more sensitive indications of heavy metals in soil system. Therefore, combining soil potential nitrification rates (PNR) and the diversity of ammonia oxidizer community is a research strategy to screen Cu toxicity in soil system, since the nitrification process is driven by ammonia oxidizer (Lejon et al., 2010; Mertens et al., 2010).

Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) mediate ammonia oxidation, the first and rate-limiting step of nitrification. A large number of researches have reported that AOA and AOB co-exist in soils environment but questions remain concerning their ecological niche in agricultural soil systems (Di et al., 2010; Wessen et al., 2010; Habteselassie et al., 2013). The niche differentiation of AOA and AOB was usually investigated by measuring the abundance and activity of ammonia monoxygenase (*amoA*) gene, the product of which catalyzes the oxidation of ammonia to hydroxyl amine (Nicol et al., 2008). In soil and marine systems, AOA *amoA* gene abundance is usually higher than that of AOB, suggesting that AOA outnumbers AOB in these systems (Xu et al., 2012). However, the expression level of AOB *amoA* gene is higher than that of the AOB *amoA* gene in some agricultural soil types, indicating that AOB rather than AOA functionally dominates ammonia oxidation (Guo et al., 2012). Therefore, comprehensive analysis of both abundance and activity of *amoA* gene is essential to accurately reflect the ecological niche of AOA and AOB (Yamamoto et al., 2010; Pratscher et al., 2011; Jacquot et al., 2014).

Chinese fluvo-aquic soil, mainly composed of the Yellow river sediments, is widely distributed in the North China Plain (Finzi et al., 2015; Wang et al., 2016). It is one of the most important agricultural soil types and governs crops productivity in North China Plain. Pollution of Cu in fluvo-aquic soil is a growing problem in recent years. However, monitoring methods and monitoring indicators for Cu toxicity in fluvo-aquic soil remain uncertain. In this study, we addressed three issues in fluvo-aquic soil system: 1) ecological niche of AOA and AOB; 2) identification of Cu hormetic concentration and toxic concentration; 3) distinct response of AOA and AOB communities to Cu stress.

2. Materials and methods

2.1. Soil sampling and experimental design

Fluvo-aquic soil was collected from the Ecological Station of China Agricultural University in Huantai (36°57′75″N, 117°59′21″E), Shandong Province, China. This station became an experimental research site in 1996 and has been aiming to develop high-quality, high-yield, cost-effective, ecofriendly, and safe agriculture.

Soil samples were collected from the topsoil (0 to 20 cm) and sieved (1 mm), and aliquots (400 g) were added to Erlenmeyer flasks (1000 mL). These samples were incubated in an artificial climate incubator (HPG-400H, Harbin Donglian Electronic Technology Development Co., Ltd., China) at 25 °C ± 2 °C, at 60% ± 5% maximum water field holding capacity by adding deionized water. Soil samples were homogenized and incubated for one week in order to reduce the variability between samples. Soil physicochemical properties were determined before Cu addition in accordance to the standard methods recommended by the Chinese Society of Soil Science (Lu, 2000). Soil pH was measured using a PHS-3C pH meter (Leici, China) with 1: 2.5 suspension in H_2O. Acetic ammonium saturation was used to determine the Cation exchange capacity (CEC). The distribution of soil particle size was measured using the micro-pipette method (Miller and Miller, 1987). Soil organic matter was quantified via oxidation with potassium dichromate titration of $FeSO_4$. The background Cu in soil was determined using atomic absorption spectrometry (TAS 990, Beijing Purkinje General Instrument Co., China) after digestion in an AIM 600 Block Digestion System with aqua regia reflux. The basic properties of the fluvo-aquic soil are as follows: pH 8.35; 0.81 g kg^{-1} total nitrogen; 26.40 cmol kg^{-1} CEC; 18.26 g kg^{-1} soil organic matter; C:N ratio 13.08; 13.01% sand size (0.05–2 mm) distribution; 78.55% silt size (0.002–0.05 mm) distribution; 18.44% clay size distribution (< 0.002 mm); 19.8 mg kg^{-1} Cu.

Cu was added as $CuCl_2$ solution at 0, 100, 300, 600, and 1200 mg Cu kg^{-1} soil. Four replicates were performed under each concentration level. Subsequently, the soils were thoroughly mixed by hand and incubated for 6 h, 12 h, 1 d, 3 d, 7 d, 14 d, 28 d, and 56 d of exposure to Cu. PNR was measured and EC_{50} value (effect concentration causing a 50% reduction in the PNR) for Cu were determined based on PNR at each stage. Soil DNA extraction, real time polymerase chain reaction (real-time PCR) of *amoA* and denaturing gradient gel electrophoresis (DGGE) analysis of *amoA* were performed at 56 d of Cu incubation. Soil RNA was extracted and reverse transcription of *amoA* was performed at 1, 3, and 7 d. The experimental flow chart was shown in Fig. S1. The DGGE analysis of *amoA* DNA in long-term incubation (about 56 d) of Cu would help us to test the tolerance of ammonia oxidizers to Cu. The genes *amoA* transcripts analysis with short-term incubation (1, 3 and 7 d) of Cu may provide information on activity of ammonia oxidizers and on the ecological niche of ammonia oxidizers communities in fluvo-aquic soil.

2.2. Soil PNR

The PNR of the soil samples was measured using the method by Smolders et al. (2001) with slight modifications. Four replicates were performed for Cu-treated soils and non-treated soils. Each soil sample (dry weight 5 g) was incubated in 20 mL induced solution (PBS: $(NH_4)_2SO_4$: $NaClO_3$ = 10: 1: 1) in a 50 mL Erlenmeyer flask. The soils were incubated at 25 °C for 0 h and 24 h in the dark. After incubation, the NO_2^--N was extracted from the soils with 5 mL of 2 mol L^{-1} KCl. The concentration of NO_2^--N was determined using an autoanalyzer 3 digital colorimeter (Bran + Luebbe, Germany). PNR defined as the yield of nitrite per gram of dry soil per hour, was calculated according to Eq. (1).

$$PNR = V \times (N_2 - N_1)/(T \times m) \quad (1)$$

where V refers to the volume of KCl (ml); N1 and N2 refers to the content of NO_2^--N (mg mL^{-1}) at the beginning of incubation and the end of incubation, respectively; T is the incubation period (hours); m refers to the quantity of soil (g); PNR is the potential nitrification rate (mg g^{-1} DW h^{-1}).

EC_{50} refers to the Cu concentration that inhibits the PNR to half of the control group and it was used to assess the tolerance of soil to Cu. In this study, Cu EC_{50} was obtained through "s" type dose-response curve