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Copper pollution decreases the resistance of soil microbial community to subsequent dry–rewetting disturbance

Jing Li[1], *Jun-Tao Wang*[1], *Hang-Wei Hu*[2], *Yi-Bing Ma*[3], *Li-Mei Zhang*[1], *Ji-Zheng He*[1,2,*]

1. *State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*

2. *Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010, Australia*

3. *National Soil Fertility and Fertilizer Effects Long-term Monitoring Network, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

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ABSTRACT

Dry–rewetting (DW) disturbance frequently occurs in soils due to rainfall and irrigation, and the frequency of DW cycles might exert significant influences on soil microbial communities and their mediated functions. However, how microorganisms respond to DW alternations in soils with a history of heavy metal pollution remains largely unknown. Here, soil laboratory microcosms were constructed to explore the impacts of ten DW cycles on the soil microbial communities in two contrasting soils (fluvo-aquic soil and red soil) under three copper concentrations (zero, medium and high). Results showed that the fluctuations of substrate induced respiration (SIR) decreased with repeated cycles of DW alternation. Furthermore, the resistance values of substrate induced respiration (RS-SIR) were highest in non-copper-stressed (zero) soils. Structural equation model (SEM) analysis ascertained that the shifts of bacterial communities determined the changes of RS-SIR in both soils. The rate of bacterial community variance was significantly lower in non-copper-stressed soil compared to the other two copper-stressed (medium and high) soils, which might lead to the higher RS-SIR in the fluvo-aquic soil. As for the red soil, the substantial increase of the dominant group WPS-2 after DW disturbance might result in the low RS-SIR in the high copper-stressed soil. Moreover, in both soils, the bacterial diversity was highest in non-copper-stressed soils. Our results revealed that initial copper stress could decrease the resistance of soil microbial community structure and function to subsequent DW disturbance.

Introduction

Terrestrial ecosystems are increasingly influenced by intensive anthropogenic perturbations and climatic changes (Singh et al., 2010), which are predicted to profoundly affect belowground nutrient allocation and lead to increased fluctuations in precipitation magnitude and frequency at regional and continental scales (Dore, 2005). Soil drying-rewetting (DW) is a common environmental disturbance in agricultural practices exemplified by the frequencies and intensities of irrigation (Shi and Marschner, 2014). Moreover, dry–wet alternations are frequently encountered in paddy

* *Corresponding author*. E-mail: jzhe@rcees.ac.cn (Ji-Zheng He).

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soils in different seasons, where the field is maintained under flooded conditions in the crop-growing season and under drained conditions in the off-crop season (Kimura and Asakama, 2006). The significant changes in soil conditions due to DW alternations might considerably affect the soil-dwelling microorganisms, which are essential to the sustainability of ecosystem functioning and services. However, our knowledge is lacking regarding the responses of soil microbial communities to the DW alternations, which strongly affects our ability to predict the responses of ecosystem functions to future environmental disturbance.

In recent years, the influence of DW disturbance on soil microbial activity has been frequently investigated (Fierer and Schimel, 2002; Borken and Matzner, 2009). For example, soil nitrification activity was assumed to be inhibited by osmotic stresses due to limited diffusion of substrates in dry soil (Hu et al., 2015a, 2015b). Some microorganisms can withstand the stress and survive by accumulating osmoregulatory solutes under water-limited conditions (Yao et al., 2011). After the rewetting of dry soil, the water potential sharply decreases, which leads to the death of microorganisms by bursting of cells, while microbes with high tolerance quickly release the accumulated solutes to survive (Shi and Marschner, 2014; Hu et al., 2015a, 2015b). It is generally accepted that soil DW events can cause a short-lived flush of carbon, nitrogen and phosphorus nutrients into the soil solution, which increases substrate and nutrient availability to the surviving microbes and stimulates their microbial activity (Shi and Marschner, 2014). Despite these studies on the impacts of DW disturbance on microbial activity, however, few studies had comprehensively investigated the shifts of soil microbial community composition, diversity and function in response to the DW disturbance.

Previous studies suggested that the resistance of a stressed microbial community to a subsequent disturbance depends on the nature of the disturbance (Tobor-Kaplon et al., 2005). Margesin et al. (2011) argued that if the primary and subsequent disturbances were similar, the resistance and resilience of soil microbial community would be stronger. The similarity of the primary and subsequent disturbances might contribute to the development of co-tolerance of microbial communities (Philippot et al., 2008). However, how the resistance of a stressed microbial community will respond to a new disturbance remains largely unknown. Copper is released into the environment by anthropogenic activities, such as sewage irrigation, mining activities, and intensive use of pesticides and herbicides (Smith, 2009). Approximately 3.4 million tons of copper is released to the terrestrial surface every year (Zhou et al., 2000), thus it is necessary to study how the soil microbial community is affected by copper pollution in response to DW disturbance, especially repeated DW alternations.

The main objective of this study was to explore the responses of the bacterial community and activity in copper-contaminated soils to subsequent DW disturbance. As an essential component of soil communities, soil bacteria play pivotal roles in nutrient cycling processes, and they are largely responsible for the functioning of farmland ecosystems (Hu et al., 2015a, 2015b). The activities of soil microorganisms mostly contribute to soil respiration, yet most microorganisms in the soil are dormant, which leads to low soil respiration (Lin and Brookes, 1999). Soil respiration can be stimulated by adding easily decomposable substrates. We therefore measured substrate induced respiration (SIR) instead of soil basal respiration to characterize the microbial activity. The changes in the bacterial diversity and community composition were also investigated in response to repeated DW alternations. Soils collected from field sites with contrasting soil properties were incubated in a microcosm experiment with ten cycles of DW alternation. We hypothesized that: (1) the resistance of SIR to disturbance might fluctuate in response to repeated DW transitions and be higher in the copper-stressed soils compared to non-copper-stressed soils; (2) the soil bacterial community might significantly change during a period of repeated DW alternations, and the shifts in the bacterial community might determine the resistance of SIR to the disturbance.

1. Materials and methods

1.1. Field description and soil sampling

Soil samples were collected from two long-term experimental sites located in Dezhou (37.33°N, 116.63°E) of Shandong province, and in Qiyang (26.75°N, 111.88°E) of Hunan province, China. The soil in Dezhou is classified as fluvo-aquic soil while the soil in Qiyang as red soil, and the two soils have distinct soil properties, as described previously (Li et al., 2015). Soil pH in Dezhou and Qiyang was measured as 7.9 and 4.3, respectively. The fluvo-aquic soil is a sandy soil with a ratio of clay: silt: sand of 18:18:64, while the red soil is clayey, with a ratio of clay: silt: sand of 46:35:19. In both sites, different amounts of copper chloride powders were mixed thoroughly with surface soil and then applied back into the plots in July 2007. There were eight different copper treatments with the copper levels of 0, 50, 100, 200, 400, 800, 1600, and 3200 mg/kg soil in the fluvo-aquic soil, and 0, 12.5, 25, 50, 100, 200, 400, and 800 mg/kg soil in the red soil. The plots had been planted with maize-wheat rotations under conventional agricultural management practices since 2007. Soil samples were collected from the top 10 cm by mixing five soil cores for each plot in July 2012 and stored at 4°C before the microcosm experiment.

1.2. Soil microcosm incubation

According to a previous study on the effects of copper addition on bacterial communities in the same two long-term experimental sites located in Dezhou (37.33°N, 116.63°E) and Qiyang (26.75°N, 111.88°E) (Li et al., 2015), the added copper concentrations could be classified into three categories including zero, medium and high concentrations based on the significant changes of bacterial community composition, that is, 0, 800 and 3200 mg/kg added copper in the fluvo-aquic soil compared with 0, 200 and 800 mg/kg added copper in the red soil. The treatment without copper addition (Cu0) was regarded as the control in both soils, and DW alternations were performed in soils with three different copper levels. In total, soil microcosms were constructed with eight treatments including Cu0,

Cu0-S (S standing for DW stress), Cu800-S, and Cu3200-S in the fluvo-aquic soil and Cu0, Cu0-S, Cu200-S, and Cu800-S in the red soil. Soils were adjusted to 40% water-holding capacity (WHC), and pre-incubated in loosely-capped vials at 25°C for 15 days. Thereafter, 168 microcosms (8 treatments × 7 time points × 3 replicates) were established in 100-mL vials containing 10 g of soils and loosely capped to allow air exchange. The DW disturbance consisted of ten DW cycles (1 c–10 c) of drying for 24 hr (fan-forced air at 25°C) and rewetting (adding sterilized water to 60% WHC) for 48 hr (Degens et al., 2001). This regime provided enough time for microorganisms to recolonize the soil after each drying event. Soil water content was determined after drying or rewetting immediately by oven drying at 105°C for 8 hr, and it was below 5% after drying and recovered to 23%–32% after rewetting in the two soils (Fig. S1). Microcosms were incubated at 25°C in the dark for 30 days, and destructively sampled after 0, 1 c, 2 c, 4 c, 6 c, 8 c, and 10 c (7 time points). The sampled soils were transferred into serum bottles to measure the SIR (Li et al., 2014). Partial soil samples were freeze-dried and stored at −80°C before deoxyribonucleic acid (DNA) extraction.

1.3. SIR

Soil samples were incubated in 150 mL serum bottles sealed with rubber stoppers and aluminum caps at 25°C. A mixture of the three substrates $C_6H_{12}O_6$, $(NH_4)_2SO_4$ and KH_2PO_4 with a ratio of 80:13:2 was thoroughly ground and mixed in a mortar (ISO, 17155:2002). Then the mixture was homogeneously added into the soil with a ratio of 1% (*i.e.* 1 g of mixture was added into 100 g of dry soil). CO_2 was collected in air bags every hour after the substrate addition. A minimum of three hourly measurements was conducted to calculate the average values of the CO_2 concentrations on a gas chromatograph (Agilent 7890A GC System).

1.4. Soil DNA extraction and quantitative PCR (qPCR)

Total genomic DNA was extracted from 0.25 g of soil samples using the MoBio Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol, with slight modifications, such that the FastPrep bead beating system (Bio-101, Vista, CA, USA) was used at a speed of 5.5 m/sec for 45 sec at the initial cell-lysis step. The concentration and quality of extracted DNA were checked photometrically using a NanoDrop® ND-2000c UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The bacterial 16S rRNA gene abundance was quantified on an iCycler iQ 5 thermocycler (BioRad Laboratories, Hercules, CA, USA) using the primer pairs BACT1369F and PROK1492R with the probe TM1389F (Suzuki et al., 2000). The 25 μL reaction mixture contained 12.5 μL of Premix Ex Taq (Takara Biotechnology, Dalian, China), 0.25 μL of each primer (10 μmol), 0.5 μL of probe (10 μmol) and 1 μL of five-fold diluted DNA template (1–10 ng). Amplification conditions were as follows: 95°C for 10 sec, 35 cycles of 15 sec at 95°C and 1 min at 56°C. Standard curves were developed using ten-fold serial dilutions of plasmids containing the correct insert of the bacterial 16S rRNA gene, and the PCR efficiency for different assays ranged between 90% and 105%.

1.5. High-throughput sequencing of bacterial communities and data processing

The V4–V5 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 515f and 907r (Zhou et al., 2011). The PCR reactions in a 20 μL mixture contained 0.4 μL of FastPfu Polymerase (TransGen Biotech, China), 4 μL of 5 × FastPfu Buffer, 2 μL of dNTPs (2.5 μmol/L), 0.6 μL of each primer (5 μmol/L), five-fold diluted template DNA (~15 ng) and sterilized water. Thermal-cycling conditions were as follows: an initial denaturation of 5 min at 95°C, 26 cycles of 30 sec at 95°C, 30 sec at 55°C, 45 sec at 72°C, with a final elongation of 72°C for 10 min. The PCR products were purified using a Wizard SV Gel and PCR Clean-up system (Promega, San Luis Obispo, CA, USA). The concentrations of PCR products were fluorometrically quantified by the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) before being sequenced on the Miseq platform (Illumina, San Diego, CA, USA) at Majorbio, Shanghai, China.

Raw sequences generated through the Miseq paired-end sequencing were demultiplexed and processed in QIIME 1.7.0 (Caporaso et al., 2010a, 2010b). Chimeric sequences were identified and removed using UPARSE (Edgar, 2013), and UPARSE was also used to bin the quality reads into operational taxonomic units (OTUs) at the 97% similarity level on the Bio-Linux platform (Field et al., 2006). A set of representative sequences for each OTU were aligned against the Greengenes database (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010b), and sequences were classified using the latest Greengenes database *via* the RDP classifier (Wang et al., 2007). Resampling according to the minimum sequence numbers across all samples was performed before the downstream analyses to minimize effects of sampling effort on the bacterial diversity (Caporaso et al., 2010a). The bacterial alpha diversity was characterized by calculating the Shannon index in QIIME 1.7.0. Principal coordinate analysis (PCoA) was used to visualize the shifts in bacterial communities during DW alternations based on the Bray–Curtis dissimilarity matrices.

1.6. Statistical analyses

The bacterial 16S rRNA gene copies were log-transformed prior to statistical analysis to satisfy the normality assumptions. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (homogeneous variance) and Welch's *t* tests (non-homogeneous variance) was conducted to compare the SIR and the bacterial abundance across different copper-stressed treatments and different DW cycles. Two-way ANOVA was used to analyze the effects of DW and copper stress on SIR and bacterial abundance. Permutation multivariate analysis of variance (PerMANOVA) was used to evaluate the significance of the Bray–Curtis dissimilarity matrices of bacterial communities. We calculated the resistance of SIR using the resistance index (RS) (Orwin and Wardle, 2004), according to the following equation:

$$RS(t_0) = 1 - \frac{2|D_0|}{(C_0 + |D_0|)}$$

where D_0 is the difference between the control (C_0; values of the SIR at day 0 among different copper stressed treatments) and the disturbed soils (P_0, values of the SIR at 1 c, 6 c, 10 c of DW alternations among different copper stressed treatments). This index has the advantage of being standardized by the control, being bounded between −1 (less resistance) and +1 (maximal resistance), and it remains bounded even when extreme values are encountered (Orwin and Wardle, 2004). To determine the rate of community variance, a linear model was fitted with the differences between the bacterial communities at 1 c, 6 c, 10 c and those at day 0 based on the Bray–Curtis dissimilarity. The slope of the regression is the rate of change, and a 'steeper' slope (larger absolute value) indicates a more rapidly changing community (Shade et al., 2011). All linear models were fitted in the SPSS environment for statistical computing.

A structural equation model (SEM) was constructed to explore the direct and indirect relationships of the soil bacterial community, the resistance of SIR (RS-SIR), copper stress (Copper), and DW disturbance (DW). In the model, the DW was a categorical exogenous variable with four levels: 0, 1, 6, and 10 (Delgado-Baquerizo et al., 2014), and the bacterial community was characterized by the Bray–Curtis dissimilarity matrix. Prior to the SEM procedure, the interrelationships were investigated among the aforementioned variables by the Mantel test in QIIME 1.7.0. Based on *a priori* and theoretical knowledge, we assumed a conceptual model whereby the shifts of bacterial community, copper stress and DW disturbance could affect the variations of RS-SIR, and copper stress and DW disturbance could impact the bacterial community. The maximum likelihood estimation method was used to compare the SEM with the observation. Model adequacy was determined by χ^2 tests, goodness-of-fit index (GFI), Akaike Information Criteria (AIC), and root mean square error of approximation (RMSEA), and the conceptual model was revised according to these indices. Adequate model fits were indicated by a non-significant χ^2 test ($p > 0.05$), high GFI (>0.90), low AIC, and low RMSEA (<0.05) (Grace, 2006). SEM analysis was performed using AMOS 17.0 (Amos Development Corporation, Meadville, PA, USA).

2. Results

2.1. Variations in SIR, bacterial abundance and diversity

Wet-up resulted in an immediate CO_2 pulse from the soil, and the preceding copper stress significantly affected the amount of CO_2 released during the incubation. The SIR values at 1 c strongly increased relative to that at 0 day in different copper-stressed treatments in the fluvo-aquic soil (Fig. 1a, $p < 0.05$). The Cu0-S treatment released 2.0 times more CO_2 (from 4.8 to 14.6 μL/(g·day)) than that at 0 day, while the Cu800-S and Cu3200-S treatments released 5.5 more CO_2 (from 1.9 to 12.2 μL/(g·day)) and 8.3 times (from 0.6 to 5.5 μL/(g·day)), respectively. The SIR values significantly decreased at 6 c in all copper-stressed treatments ($p < 0.05$). After ten DW cycles, the SIR value in the Cu0-S treatment had no significant difference with that at day 0. However, the SIR values increased 3.8 times and 11.2 times in the Cu800-S and Cu3200-S treatments, respectively. Two-way ANOVA analysis showed that both copper and DW stress significantly affected the SIR ($p < 0.001$, $p < 0.001$), and the interactions between these two stressors also exerted significant influences on the SIR ($p < 0.01$). Similar to the fluvo-aquic soil, the SIR values in the Cu0-S and Cu800-S treatments at 1 c increased 1.7 and 8.6 times compared to those at day 0 in the red soil, respectively (Fig. 1b). As for different copper-stressed treatments, the SIR values also sharply decreased at 6 c. At the end of ten DW cycles, only the Cu800-S treatment obviously released 5.6 times more CO_2 than that at day 0. The SIR was also significantly affected by the copper and DW stress ($p < 0.001$, $p < 0.001$).

The bacterial abundances displayed no significant variation tendencies during the ten DW cycles in both soils ($p = 0.053$, $p = 0.051$, Figs. S2a and S2b), but they were significantly impacted by different levels of copper stress ($p < 0.001$, $p < 0.01$). The variations were negligible relative to the baseline of the bacterial abundance (ranging from 5.53×10^9 to 2.22×10^{11} copies/g dry soil). The diversity characterized by Shannon index varied slightly during ten DW alternations in the fluvo-aquic soil (Table S1). It was significantly higher in the Cu0-S than that in the Cu800-S and Cu3200-S treatments. As for the red soil, marginal decreases were observed with increasing cycles of DW alternations in all copper-stressed treatments. The diversity was also significantly higher in the Cu0-S than that in the Cu800-S treatment.

2.2. Resistance of SIR and rates of bacterial community variance

We calculated the RS indexes for SIR (RS-SIR) at 1 c, 2 c, 4 c, 6 c, 8 c, and 10 c, and selected the soil samples at inflection points (1 c, 6 c, 10 c) to analyze the bacterial diversity, community composition and rates of community variance. According to the values of RS-SIR, the Cu0-S treatment varied from −0.33 to 0.50, while in the Cu800-S and Cu3200-S treatments the ranges of RS-SIR were −0.69 to −0.50 and −0.84 to −0.59 in the fluvo-aquic soil (Fig. 2a), respectively. The values of RS-SIR were significantly higher in the Cu0-S treatment than those in the Cu800-S and Cu3200-S treatments at all tipping points ($p < 0.05$). As for the red soil, the variations of the RS-SIR in the Cu0-S and Cu200-S treatments ranged from −0.25 to 0.84 and from 0.30 to 0.87, which were significantly higher than that in the Cu800-S treatment (−0.79 to −0.55) (Fig. 2b, $p < 0.05$). In both soils, the SIR in the non-copper-stressed treatments was most resistant to the DW disturbance, and the SIR in the high copper-stressed treatments was least resistant, yet the responses of SIR in the medium copper-stressed treatments were different. The values of RS-SIR significantly changed with increasing cycles of DW disturbance ($p < 0.05$), which displayed increasing tendencies from 1 c to 6 c and began to decrease after 6 c.

The rates of bacterial community variance might partially explain the variations of RS-SIR. To calculate the rate of change, linear models were constructed with the differences between the bacterial communities at 1 c, 6 c, and 10 c and those at day 0 based on the Bray–Curtis dissimilarity (Fig. 3a, b). The slope of the regression was the rate of community variance. All the linear models were significantly fitted in the fluvo-aquic soil

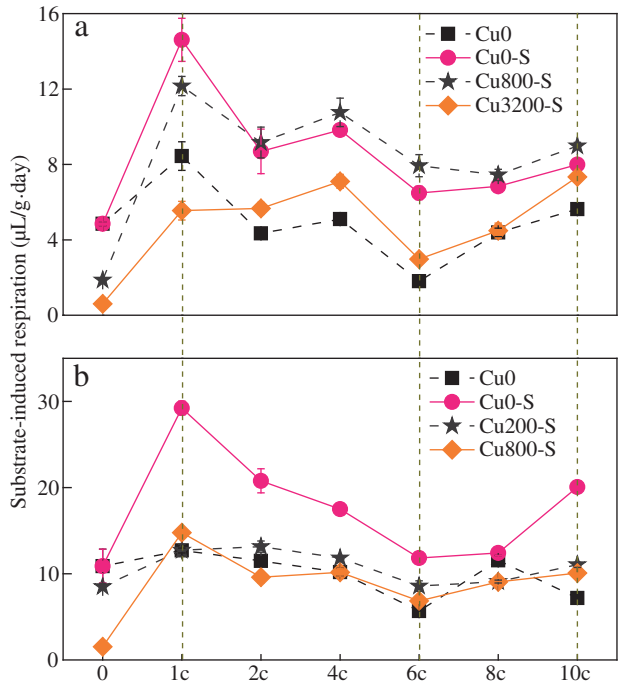

Fig. 1 – Changes in the substrate induced respiration (SIR) of different copper-stressed treatments with repeated dry-wet alternations in the fluvo-aquic soil (a) and red soil (b). Dark yellow dashed lines indicate the tipping points. Error bars represent standard errors (n = 3). Cu0: without Cu addition; Cu200, Cu800, Cu3200: with 200, 800, and 3200 mg/kg Cu addition; S: DW stress; c: cycle.

($p < 0.05$). The slopes in Cu800-S and Cu3200-S were 0.171 and 0.142, which were almost 2 times higher than that (0.089) in Cu0-S (Fig. 3a). The linear models also fitted well with the Bray–Curtis similarities in the red soil ($p < 0.001$) except for the Cu0 treatment. However, the slopes differed slightly among copper-stressed treatments in the red soil (Fig. 3b).

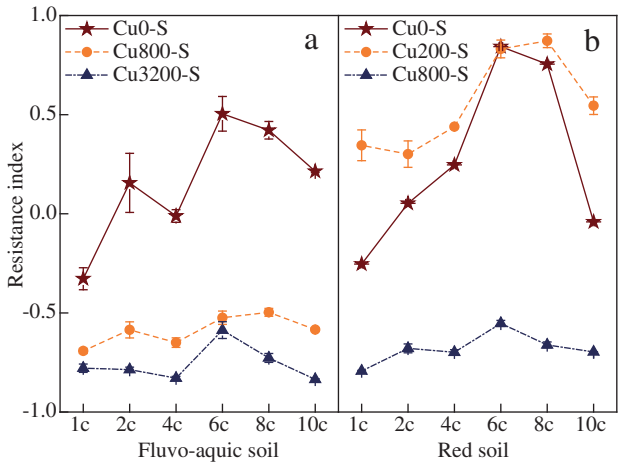

Fig. 2 – Changes in the resistance index of different copper-stressed treatments at the tipping points including 1 c, 6 c and 10 c in the fluvo-aquic soil (a) and red soil (b). Error bars represent standard errors (n = 3). Cu0: without Cu addition; Cu200, Cu800, Cu3200: with 200, 800, and 3200 mg/kg Cu addition; S: DW stress; c: cycle.

2.3. Shifts in the bacterial community compositions

The PCoA analysis revealed that the bacterial communities obviously changed with increasing cycles of DW alternations in both soils (Fig. S3), which was corroborated by PerMANOVA analysis ($p < 0.05$). The relative abundance of different groups in the Cu0 treatments in both soils displayed no significant changes during the DW alternations compared to the other copper stressed treatments in both soils.

The bacterial communities were dominated by the *Acidobacteria* phylum (mostly *Acidobacteria-6* class), followed by the *Proteobacteria* phylum (primarily *alpha*, *beta* and *gamma* classes), *Bacteroidetes* phylum (mostly *Cytophagia* class) and *Actinobacteria* phylum (mostly *Actinobacteria* class) in the fluvo-aquic soil (Fig. 4a). The fluctuations of different bacterial groups in relative abundance were significantly higher in the Cu3200-S treatment than those in the Cu0-S and Cu800-S treatments. The relative abundances of *Acidobacteria-6* decreased with increasing cycles of DW alternation in the Cu0-S, Cu800-S, and Cu3200-S treatments, and the decrease was mostly (from 23.0% to 6.8%) observed in the Cu3200-S treatment. Similarly, *Betaproteobacteria* also showed significantly decreasing tendencies in all the copper-stressed treatments, and the decrease was most prominent in the Cu3200-S treatment (16.7%). The variations in the relative abundances of *Actinobacteria* were the reverse, and the increase (22.6%) was mostly found in the Cu3200-S treatment. A significant decrease (20.6%) was observed in the relative abundance of *Cytophagia*, while *Alphaproteobacteria* increased by 29.9% in the Cu3200-S treatment. The variation of *Gammaproteobacteria* fluctuated in response to the DW disturbance in the Cu0-S and Cu800-S treatments, increasing to the maximum at 1 c or 6 c, then decreasing to the initial state at day 0 at the end of DW disturbance.

The bacterial community compositions in the red soil were different from those in the fluvo-aquic soil, which were dominated by *WPS-2* phylum, followed by *Actinobacteria* phylum (mostly *Actinobacteria* and *Thermoleophilia* classes), *Firmicutes* phylum (mostly *Bacilli* class), *Chloroflexi* phylum (mostly *Ktedonobacteria* class), *Acidobacteria* phylum (mostly *Acidobacteria* class), and *Planctomycetes* phylum (mostly *Planctomycetia* class) (Fig. 4b). Decreasing tendencies were found in the relative abundances of *Acidobacteria* and *Ktedonobacteria* classes in response to the DW disturbance in all the copper-stressed treatments, however, the shifts of *Planctomycetia* were in reverse. As two classes of the *Actinobacteria* phylum, *Actinobacteria* and *Thermoleophilia* displayed different variations. *Actinobacteria* decreased first, then recovered after 10 c, while *Thermoleophilia* decreased without recovery. The relative abundance of *Bacilli* reached a peak at 1 c, and the increase was 32.6% in Cu0-S, 35.6% in Cu200-S and 8.6% in the Cu800-S treatment ($p < 0.01$), followed by decreasing to the initial state after 10 c. The fluctuation of *WPS-2* was greatest in the Cu800-S treatment with 33% of the increase occurring at 6 c, and the increase was also significant in the other two treatments.

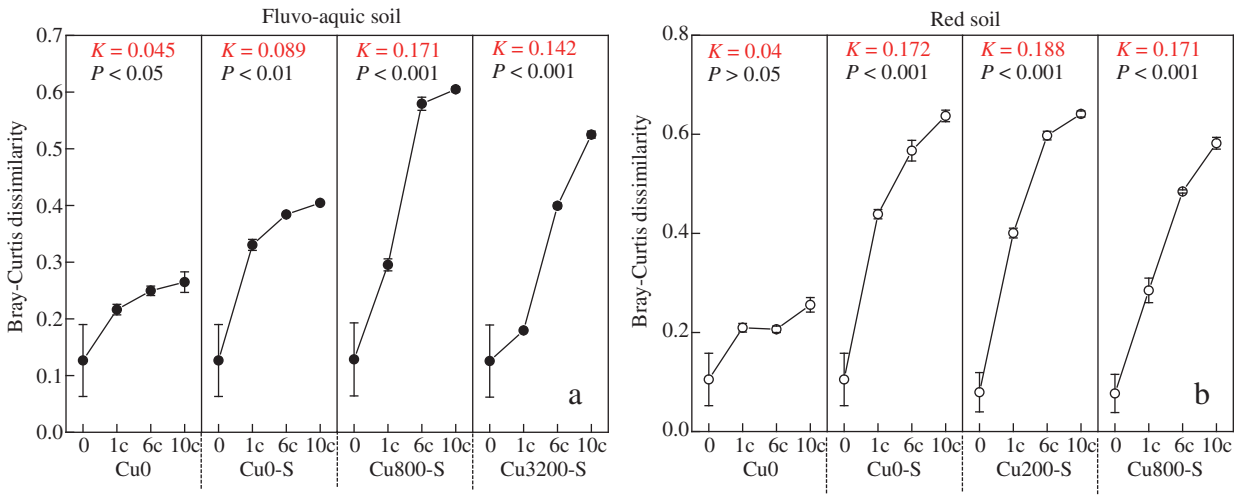

Fig. 3 – The Bray–Curtis dissimilarities between bacterial communities of different copper-stressed treatments at the tipping points including 1 c, 6 c and 10 c in the fluvo-aquic soil (a) and red soil (b). The slope of the lines indicates the rate of change of the bacterial community. Error bars represent standard errors (*n* = 3). Cu0: without Cu addition; Cu200, Cu800, Cu3200: with 200, 800, and 3200 mg/kg Cu addition; S: DW stress; c: cycle.

2.4. *Impacts of the bacterial communities on the resistance of SIR*

The shifts of soil bacterial community showed direct and significantly positive effects on the RS-SIR in the fluvo-aquic soil ($\lambda = 0.30$, $p < 0.001$, Fig. 5a). Copper stress significantly and negatively impacted the RS-SIR ($\lambda = -0.28$, $p < 0.001$, Fig. 5a), however, DW disturbance did not exert significant influence on the RS-SIR ($\lambda = -0.06$, $p = 0.152$, Fig. 5a). Both copper and DW disturbance displayed significant positive effects on the shifts of the soil bacterial community ($\lambda = 0.85$, $p < 0.001$; $\lambda = 0.22$, $p < 0.001$, Fig. 5a).

As for the red soil, the shifts of the soil bacterial community also showed a direct positive effect on the RS-SIR ($\lambda = 0.50$, $p < 0.001$, Fig. 5b), while copper and DW disturbance negatively impacted the RS-SIR ($\lambda = -0.26$, $p < 0.001$; $\lambda = -0.15$, $p < 0.001$, respectively, Fig. 5b). Similar to the fluvo-aquic soil, both disturbances showed significant positive effects on the shifts of the soil bacterial community ($\lambda = 0.65$, $p < 0.001$; $\lambda = 0.49$, $p < 0.001$, Fig. 5b).

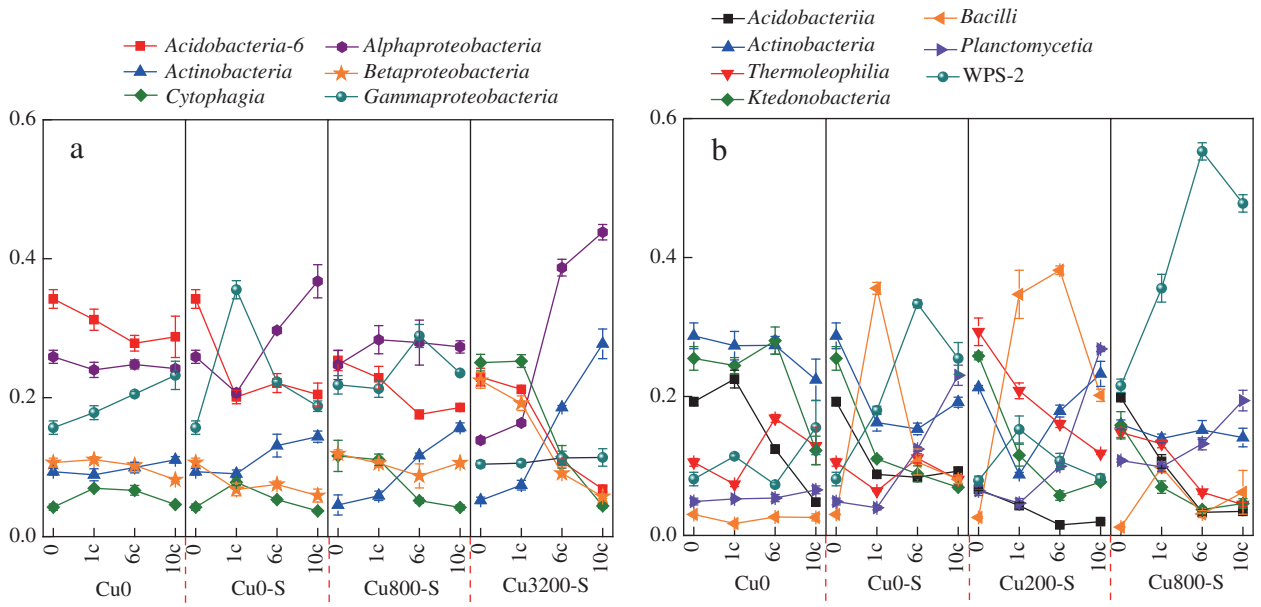

Fig. 4 – Shifts in the bacterial community composition of different copper-stressed treatments at the tipping points including 1 c, 6 c and 10 c in the fluvo-aquic soil (a) and red soil (b). Error bars represent standard errors (*n* = 3). Cu0: without Cu addition; Cu200, Cu800, Cu3200: with 200, 800, and 3200 mg/kg Cu addition; S: DW stress; c: cycle.

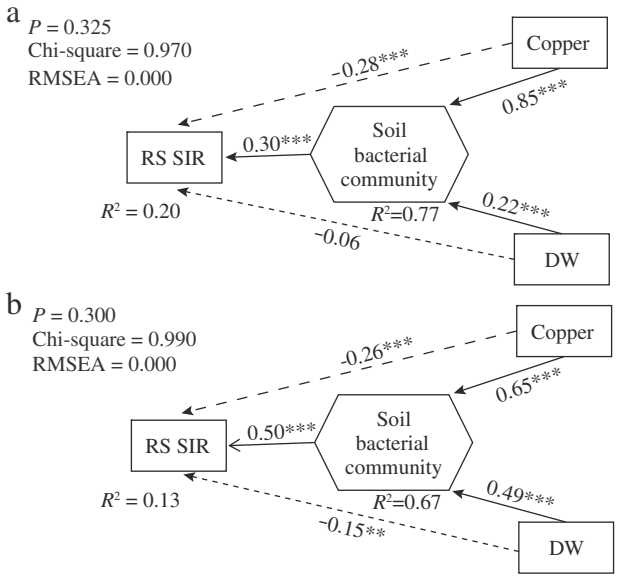

Fig. 5 – Structural Equation Model (SEM) showing the causal relationships among bacterial community, resistance indexes for SIR (RS-SIR), copper stress (Copper), and dry-rewetting disturbance (DW) in the fluvo-aquic soil (a) and red soil (b). The final model fits the data well (a): maximum likelihood, χ^2 = 0.970, d*f* = 1, *p* = 0.325; goodness-of-fit index (GFI) = 0.999; Akaike Information Criteria (AIC) = 18.97; root square mean errors of approximation (RMSEA) = 0.000. (b): maximum likelihood, χ^2 = 0.990, degree of freedom (d*f*) = 1, *p* = 0.300; goodness-of-fit index (GFI) = 0.999; Akaike Information Criteria (AIC) = 20.98; root square mean errors of approximation (RMSEA) = 0.000. Solid and dashed lines indicate positive and negative pathways, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. SIR: substrate induced respiration.

3. Discussion

3.1. Changes in the magnitude of SIR decreases with repeated dry–wet alternations

The flush of nutrients into soil solution in a short time upon rewetting resulted in the instantaneous increase of soil respiration (Shi and Marschner, 2014). The respiration flush often decreases with increasing cycles of DW alternation (Fierer et al., 2003; Wu and Brookes, 2005). These findings were supported by our results, that the increases of SIR were largest at 1 c in different copper-stressed treatments, and the fluctuations decreased with continuous DW alternations. Decrease in respiration rates was accompanied by changes in microbial functioning due to shifts in the microbial community structure induced by DW stress (Fierer and Schimel, 2002). This conclusion was supported by the evidence in our study that the bacterial community composition notably changed in response to frequent DW alternations (Figs. 4 and S2). The resistance of SIR was lowest at 1 c, and increased to the peak at 6 c before declining, which suggested that resistance did not increase with increasing cycles of DW alternation. SEM analysis ascertained that the DW disturbance indirectly affected the resistance of SIR by directly impacting the bacterial community, therefore, the increase of resistance might be attributed to the adaption of the microbial community to the DW disturbance by changing the community structure (Fierer et al., 2003).

3.2. Resistance of SIR to DW disturbance was relatively low in the copper-stressed soils

There has been some debate about the impacts of initial stress on the soil microorganisms in response to subsequent stress (Tobor-Kaplon et al., 2006; Li et al., 2014). In this study, our results demonstrated that the SIR in the copper-stressed soils (medium and high) were less resistant to subsequent DW disturbance than in the non-copper-stressed soil (zero) in the fluvo-aquic soil. As for the red soil, the resistance of SIR in the high copper-stressed soils was also significantly lower than that in the zero and medium copper-stressed soils. This result was in line with previous studies, that previously stressed soils were more affected by a subsequent disturbance than unstressed soils (Tobor-Kaplon et al., 2006). We speculated that organisms in the metal-contaminated soils might allocate more energy to detoxification and damage repair; therefore, it was harder for stressed soils to cope with additional disturbance (Kuperman and Carreiro, 1997). However, the resistance of SIR in the medium copper-stressed soils to DW disturbance was different in the two soils, which might be attributed to the extractable copper concentrations. The extractable copper concentration was 406 mg/kg in the medium (Cu800-S) copper-stressed treatment in the fluvo-aquic soil (Table S2), which was similar to that (378 mg/kg) in the high (Cu800-S) copper-stressed treatment in the red soil (Table S2); therefore, the resistance of SIR in the medium copper-stressed treatment in the fluvo-aquic soil was significantly lower than that in the non-copper-stressed treatment. Moreover, the extractable copper concentration was 96.7 mg/kg in the medium (Cu200-S) copper-stressed treatment in the red soil, and the copper concentration might not be toxic enough to negatively affect the resistance of SIR.

Our results demonstrated that initial copper stress decreased the resistance of SIR to subsequent DW disturbance. The shifts of microbial community diversity might influence the resistance of SIR (Bell et al., 2005). The bacterial diversity was higher in the non-copper-stressed treatments compared to the copper-stressed treatments in both soils, which might contribute to the high resistance of SIR. A study involving pure or mixed boreal forest in different soil types subjected to stress from wildfire or harvesting found evidence that the resistance of the microbial biomass to experimental disturbances (dry–wet, Cu and HCl) was greatest in mixed forest, probably owing to high resource diversity leading to greater microbial diversity and more resistant taxa (Royer-Tardif et al., 2010).

The rates of community variance in the copper-stressed treatments were significantly higher than that in the non-copper-stressed treatment in the fluvo-aquic soil, which might also lead to the low resistance of SIR observed in the copper-stressed treatments. The SEM analysis corroborated

the finding that the shifts of bacterial communities determined the resistance of SIR. Although the rates of community variance differed slightly between different copper-stressed treatments in the red soil, the resistance of SIR was significantly lower in the high copper-stressed treatment compared to the other two treatments. This might be explained by the substantial increase of the dominant group *WPS-2* in the high copper-stressed soil. The results of SEM analysis also supported the supposition that the changes of the bacterial community positively impact the resistance of SIR. Exceeding the extremity of ecological response for a microbial community might result in the decrease in the resistance of SIR (Hoover et al., 2014). Extremity in ecological responses is predicted to occur when systems cross extreme response thresholds, that is, the tolerance of one or more species in a community is exceeded, and dominant species would determine the extremity of the ecological responses (Hoover et al., 2014). If only rare species are affected, the effects on ecosystem function could be small; conversely, significant changes in dominant species could lead to large effects (Hillebrand et al., 2008). Therefore, the DW disturbance exerted largely negative effects on the resistance of SIR in the high copper-stressed treatment in the red soil. In summary, three factors including low bacterial diversity, high rates of community variance and substantial variations in the dominant group might determine the low resistance of SIR in response to DW disturbance in copper-stressed soils.

3.3. Bacterial communities respond sensitively to DW disturbance

The microbial diversity was insensitive to the DW disturbance compared to the microbial community composition (Fierer et al., 2003). Our results supported this conclusion, that bacterial diversity and abundance varied slightly during DW alternations while the bacterial community composition significantly changed. The SEM analysis confirmed that the DW disturbance significantly impacted the bacterial community. Frequent DW alternations might alter the specific composition of microbial communities by selecting for microbes surviving rapid changes in water potential (Van Gestel et al., 1993).

Actively growing microbes have been found to be more susceptible to DW stress than slower growing microbes (Fierer et al., 2003). In the fluvo-aquic soil, *Acidobacteria-6*, *Betaproteobacteria* and *Cytophagia* decreased significantly in relative abundance and exhibited lower resistance to the DW disturbance. In contrast, *Actinobacteria* and *Alphaproteobacteria* displayed higher resistance. They were tolerant to the rapid changes in soil water potential, probably owing to thicker and more rigid cell walls, and they possess compatible solutes which enhance osmoregulatory capabilities (Schimel et al., 1999). Alternatively, these microorganisms are capable of rapid growth on the labile substrates released into the soil during the frequent DW events (Denef et al., 2001). However, the *Gammaproteobacteria* were not resistant to the DW disturbance, but were resilient. The recovery of *Gammaproteobacteria* might be ascribed to the decrease in released substrates from cell lysis with increasing DW alternations (Wu and Brookes, 2005; Shi and Marschner, 2014). As for the red soil, *Acidobacteria* and *Ktedonobacteria* were less resistant than *Planctomycetia* and *WPS-2* to the DW disturbance. The relative abundance of *Bacilli* sharply increased at 1 c and became the dominant group in the bacterial communities, and these changes might lead to a flush of soil respiration (Barnard et al., 2015). *Bacilli* belongs to Gram-positive bacteria with thick cell walls, which might explain the strong resilience to the DW disturbance (Schimel et al., 1999). The two classes of *Actinobacteria* responded differently; neither *Thermoleophilia* nor *Actinobacteria* were resistant, but *Actinobacteria* were resilient. At the phylum level, the relative abundance of *Acidobacteria* decreased with increasing DW alternations in both soils, which was opposite to the finding in a previous study that *Acidobacteria* increased in relative abundance after DW disturbance (Barnard et al., 2015), and the difference might be ascribed to differences in the cycles of DW alternation. Different bacterial groups responded differently to the DW disturbance, which was probably due to contrasting bacterial life-strategies related to water availability (Barnard et al., 2013).

4. Conclusions

In conclusion, we found that initial copper stress decreased the resistance of soil microbial function (exemplified by SIR) to DW disturbance. The explanation might be ascribed to the low bacterial diversity, high rates of community variance and large fluctuation of dominant group triggered by initial copper stress, which was corroborated by the model in which the shifts of microbial communities determined the resistance of SIR. Moreover, the resistance of SIR did not increase with increasing cycles of dry–wet alternation. This study provided evidence on the effects of initial stress on the soil microbial community and function in response to subsequent stress. Simultaneously, we speculated that DW-induced changes to microbial community structure may affect microbial functioning, providing a possible mechanism for the changes in soil processes exposure to numerous DW cycles.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.10.009.

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