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Impact of copper on the diazotroph abundance and community composition during swine manure composting

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ABSTRACT

Biological nitrogen fixation is a major pathway in ecosystems. This study investigated the effects of adding Cu at different levels (0, 200, and 2000 mg kg^{-1}) on the diazotroph community during swine manure composting. Quantitative PCR and high-throughput sequencing were used to analyze the abundances of diazotrophs and the community composition based on the *nifH* gene. The *nifH* gene copy number was relatively high in the early stage of composting and Cu had a significant inhibitory effect on the *nifH* copy number. Furthermore, Cu decreased the diversity of *nifH* and changed the microbial community structure in the early stage. The *nifH* genes from members of Firmicutes and *Clostridium* were most abundant. Co-occurrence ecological network analysis showed that the Cu treatments affected the co-occurrence patterns of diazotroph communities and reduced the associations between different diazotrophs. Interestingly, Cu may weaken symbiotic diazotrophic interactions and enhance the roles of free-living diazotrophs.

1. Introduction

Copper (Cu) is a trace element that is used as a feed additive to prevent diseases and promote animal growth (Gant et al., 2007; Højberg et al., 2005). Thus, it is used widely in the modern livestock industry, especially in pig farms (Xiong et al., 2010). However, trace elements are absorbed poorly by animals and most are excreted in their original form in animal urine and feces (Cang et al., 2004), thereby resulting in massive amounts of trace element residues in animal manure. Cu has been found in swine manure at levels ranging from 50.0 to 2016.7 mg kg^{-1} in China (Xiong et al., 2010). It is a common practice in China to spread animal manure directly onto agricultural land, which may cause severe toxicity in plants and high levels of this trace element can enter the food chain (Kalyanaraman and Sivagurunathan, 1993).

Composting is a well-known method for stabilizing organic waste and it can also reduce the bioavailability or transform the chemical forms of heavy metals (Chen et al., 2010). Compost can be used as an organic fertilizer after converting biodegradable components into nuisance-free, sanitary, and humus-like materials (Bernal et al., 2009). Microorganisms play key roles in the composting process where the succession of the microbial community is important for the effective management of composting. The appearance of some microorganisms improves the quality of the compost product, such as those involved with nitrogen fixation (Pepe et al., 2013). In biological nitrogen fixation, nitrogen gas (N_2) can be reduced to bioavailable ammonium (NH_4^+) by diazotrophs using nitrogenase. Diazotrophs include free-living bacteria, such as *Azospirillum*, *Cupriavidus*, and some sulfate-reducing bacteria, and symbiotic diazotrophs such as *Rhizobium* and *Frankia* (Chen et al., 2008; Postgate, 1998; Sellstedt and Richau, 2013). Furthermore, symbiotic N_2 fixation bacteria dominate in the soil, where they can enter the plant roots and form root-nodule symbiotic relationships with plants (Tu et al., 2016). The *nifH* gene family has been employed widely to study diazotrophic microbial communities in various environments because they are an important source of nitrogen inputs in many ecosystems (Bellenger et al., 2014). Recently, some studies have considered the changes in diazotrophs during composting. For example, Beauchamp et al. (2006) showed that carbohydrate was amended in compost, which could increase the diazotroph activity in the compost. Pepe et al. (2013) showed that diazotrophs were dominant in the initial stage of composting and they could increase the total nitrogen content when added to compost. Sun et al. (2016a) demonstrated that the *nifH* gene abundance declined significantly during the later stage of composting when *Azoarcus* and some sulfate-reducing bacteria were the dominant diazotrophs. In addition, the presence of Cu in raw materials can affect the activities of enzymes (Li et al., 2014), the ammonia oxidation process (Yin et al., 2016), and copper resistance in the microbial community (Yin et al., 2017) during composting.

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However, the changes in the diazotroph community are still unclear and only limited information is available regarding the effects of Cu on diazotrophs.

The present study investigated the effects of spiking swine manure with different concentrations of copper on the abundance and diversity of diazotrophs during composting. Quantitative PCR (qPCR) and high throughput sequencing were used to evaluate the effects of Cu on the abundance of the *nifH* gene as well as the diversity and structure of the diazotroph community during composting. Network analysis was used to determine the co-occurrence patterns of diazotrophs during the composting process. The results of this study may facilitate further research into improvements in the quality of swine manure compost containing copper.

2. Materials and methods

2.1. Preparation of the compost

The swine manure had a total nitrogen content of 26 g kg^{-1}, organic carbon content of 380.2 g kg^{-1}, pH of 7.6, and total copper content of 121 mg kg^{-1}. The wheat straw had a total nitrogen content of 6.5 g kg^{-1} and an organic carbon content of 496.3 g kg^{-1}. All of the composting trials were performed in 500-mL cylindrical plastic compost reactors (height = 143 mm, diameter = 85 mm, and caliber = 68 mm) containing 150 g of swine manure mixed with straw as a substrate, where the C:N ratio of the mixture was adjusted to 20:1. Copper sulfate solution was mixed with each sample to spike them at Cu concentrations of 0, 200 (highest concentration in pig feed (Huang et al., 2007)), and 2000 (highest concentration in pig manure (Xiong et al., 2010)) mg Cu kg^{-1}, which were designated as the control, Cu200, and Cu2000, respectively, and the three treatments were performed in 45 plastic containers (15 for each treatment). The experimental design was described previously by Li et al. (2014) and the Cu residue levels in swine manure were determined by Xiong et al. (2010). The reactors were placed in an incubator to control the temperature of the compost. The temperature was artificially controlled in an incubator in the following two phases: the early phase (comprising the mesophilic phase (20–55 °C) from 0 to 5 days and the thermophilic phase (55 °C) from 6 to 13 days) and late phase (comprising the cooling phase (50–40 °C) from 14 to 21 days and the maturity phase (decreased to 20 °C) from 22 to 35 days), as described previously (Li et al., 2014). Three plastic containers for each treatment were sampled as triplicates on days 2, 7, 14, 21, and 35.

2.2. Physicochemical parameters

Before determining the pH and electrical conductivity (EC), each sample was homogenized for 30 min in 1:10 (w/w) distilled water and the supernatant was then tested with a Thermo Orion 3-star pH-meter (San Diego, CA, USA) and a conductivity electrode (DDS-11A, Shanghai). After determining the pH and EC, the suspension was tested using a TOC Analyzer (Elementar, Germany) to measure the water soluble carbon (WSC) contents. Total organic carbon was measured by incinerating the dried samples at 550 °C for 24 h in a muffle furnace and the total nitrogen content (TN) was determined with a Kjeldahl analysis system (FOSS, Denmark). The NH_4^+ and NO_3^- contents were analyzed colorimetrically using a flow injection analysis (Systea, Italy) by mechanically shaking the fresh sample as a suspension in 2 M KCl at a ratio of 1:50 (w/v) for 60 min at 150 rpm. Bioavailable Cu was extracted using 1 M $MgCl_2$ solution as described by Li et al. (2014), and each extract was analyzed with a Model Z-2000 Series Polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan).

2.3. Genomic DNA extraction and qPCR detection of nifH gene

The compost samples were pretreated before extracting the DNA, where they were freeze-dried using a freeze dryer (Songyuan, Beijing, China) and sieved through 1-mm pore filters in an ultra-centrifugal mill (ZM200, Retsch, Germany). DNA was extracted using a FastDNA Spin Kit for soil (MP Biomedicals, LLC, Illkirch, France). The *nifH* gene copy numbers were determined by qPCR (Bio-Rad CFX Connect™) in triplicate for each sample with the *nifH* gene primer set comprising PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and PolR (5′-ATSGCCATCATYTC-RCCGGA-3′) (Poly et al., 2001). The qPCR conditions comprised an initial hold for 10 min at 95 °C, followed by 40 cycles for 10 s at 95 °C, 30 s at 60 °C, and then 32 s at 72 °C. A melting curve was obtained at the end of the reaction to verify the specificity of the amplicon. The standard curve indicated a PCR amplification efficiency of about 83% and linearity of 0.99.

2.4. Illumina HiSeq sequencing analysis of the nifH gene

The *nifH* gene was analyzed by high-throughput sequencing using the Illumina HiSeq platform at Biozeron (Shanghai, China). The primer set comprising nifHF/nifHR (Braun et al., 1999) was used for PCR amplification. A unique barcode was added to each sample at the 5′-end of the forward primer to distinguish the sample.

After sorting the sample sequences according to their barcodes, the barcode and primer sequences were deleted. The remaining sequences were then converted into amino acid sequences using the FunGene Pipeline via the Ribosomal Database Project server (http://fungene.cme.msu.edu/FunGenePipeline/) (Pereira et al., 2013). Sequences with translated proteins that did not match the *nifH* protein sequence or that contained termination codons were discarded. The remaining high-quality sequences were grouped into operational taxonomic units (OTUs) at 97% identity, as explained by Pereira et al. (2013). Representative sequences in the OTUs were taxonomically classified by BLAST algorithm-based search via GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Co-occurrence network construction and data analysis

Relative abundance profiles were generated for *nifH* OTUs in order to identify the co-occurrence relationships of diazotrophs. To construct highly confident co-occurrence ecological networks, a correlation matrix (R matrix) and significance matrix (P matrix) were obtained using R (Version 3.3.1) by calculating all of the possible pairwise Pearson's rank correlations between all OTUs. A Pearson's correlation coefficient cutoff of 0.60 was employed for network construction. The ecological networks were then visualized using Cytoscape (Banerjee, 2016). OTUs with the maximum betweenness centrality scores were considered keystone species (Banerjee, 2016).

Correlation analyses and Tukey's multiple comparisons were performed using SPSS 19.0 (SPSS Inc., USA). Circos graphs, non-metric multidimensional scaling (NMDS), and heatmaps were obtained using R (Version 3.3.1).

3. Results and discussion

3.1. Changes in parameters during composting

The pH increased gradually in the control during the first week and the maximum value (pH 9.14) was reached on day 7. The pH decreased to 8.51 in the final compost, which is within the optimal environmental range for microorganisms. In addition, the pH was slightly lower in Cu2000 compared with the other treatments after 14 days, which might be ascribed to the large amounts of Cu^{2+} that can be precipitated as $Cu_2(OH)_2CO_3$ (malachite) and $Cu(OH)_2$ in alkaline conditions (Ma et al., 2006), thereby reducing the amount of OH^- and decreasing the pH value in Cu2000. The maximum EC values found in the three treatments on day 2 ranged among 4.66–6.13 mS cm^{-1} and they decreased gradually until the end of the composting process. In addition,