Bioresource Technology 221 (2016) 181–187

Contents lists available at ScienceDirect

Bioresource Technology

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

Effects of copper on the abundance and diversity of ammonia oxidizers during dairy cattle manure composting

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HIGHLIGHTS

- Potential ammonia oxidation values were inhibited significantly by Cu addition.
- Cu at 50 and 500 mg kg^{-1} inhibited *amoA* gene abundance in cattle manure compost.
- Cu may affect the AOA and AOB community structure and diversity.

ARTICLE INFO

Article history:
Received 12 July 2016
Received in revised form 31 August 2016
Accepted 4 September 2016
Available online 7 September 2016

Keywords:
amoA gene
Ammonia-oxidizing archaea
Ammonia-oxidizing bacteria
Composting
Copper

ABSTRACT

This study investigated the effects of adding Cu(II) at two exposure levels (50 and 500 mg kg^{-1}, i.e., Cu50 and Cu500 treatments, respectively) on the activity of ammonia-oxidizing microorganisms during dairy cattle manure composting. The results showed that the pH, NH_4^+-N, NO_3^--N, and potential ammonia oxidation values were inhibited significantly by the addition of Cu(II). Furthermore, the abundances of the ammonia-oxidizing archaea (AOA) *amoA* gene and ammonia-oxidizing bacteria (AOB) *amoA* gene were determined by quantitative PCR, and their compositions were evaluated by denaturing gradient gel electrophoresis (DGGE). AOA was the dominant ammonia oxidizing microorganism, of which the abundance was much higher than AOB during composting. Cu50 and Cu500 had significant inhibitory effects on the abundance of the *amoA* gene. The DGGE profile and statistical analysis showed that Cu(II) changed the AOA and AOB community structure and diversity, where *Nitrosomonas* and *Crenarchaeota* dominated throughout the composting process.

1. Introduction

Trace elements are used widely in animal husbandry to achieve the optimum growth rate and because of their antimicrobial properties (Moral et al., 2008). Copper is an important element for promoting growth and improving the feed efficiency (Xiong et al., 2010). In particular, copper sulfate is used widely as a dietary supplement in animal nutrition throughout China and the world. However, copper is adsorbed poorly in the animal gut and much of it is excreted unchanged in the urine and feces (Long et al., 2004), with levels as high as 481.5 mg kg^{-1} in cattle manure in China (Ji et al., 2012). This leads to the application of heavy metal-contaminated manure as fertilizer, which is recognized as the dominant source of copper inputs in agricultural soils (Brandt et al., 2010).

In general, composting is an effective and economic approach for decontaminating and transforming agricultural waste into useful resources via an aerobic, self-heated process, which is driven by the activity of micro-organisms that have different functions during the degradation and stabilization of organic materials, as well as reducing the presence of pathogens and odors (Jarvis et al., 2009). Nitrification has the main role in the turnover of nitrogen during composting (Jarvis et al., 2009) and it controls the subsequent fate of mineralized nitrogen. The oxidation of ammonia is the first and rate-limiting step of nitrification, and determines the transformation balance between oxidized and reduced forms of nitrogen (Zeng et al., 2011; Jarvis et al., 2009). Several studies have examined the roles of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), which are the main organisms responsible for nitrification in compost (Jarvis et al., 2009; Yamamoto et al., 2010; Zeng et al., 2011). However, a previous study indicated that the addition of copper sulfate to compost

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http://dx.doi.org/10.1016/j.biortech.2016.09.016
0960-8524/

via manure caused changes in the microbial community as well as the overall microbial activity pattern (Guo et al., 2012). Furthermore, it was demonstrated that the potential enzymatic activities related to nitrification were altered after the application of copper-contaminated manure to compost (Guo et al., 2012; Li et al., 2015). However, it is still unclear whether the observed changes in ammonia-oxidizing microbes are caused by a general reduction in the abundance of ammonia oxidizers, the loss of specific phylotypes, or changes in the expression levels of the corresponding genes.

Therefore, the aim of this study was to investigate the effects of spiking dairy cattle manure with two different concentrations of copper on the abundance and diversity of ammonia-oxidizing microbes during composting. The *amoA* gene encodes a subunit of the ammonia monooxygenase enzyme responsible for the first step of the nitrification process (Zeng et al., 2011), so this study used the *amoA* genes of AOA and AOB as indicators of nitrification. Previous studies have investigated the effects of copper on AOA and AOB in soil environments (Li et al., 2009; Subrahmanyam et al., 2014), and this study hypothesized that both AOA and AOB may respond in different ways to the addition of copper during composting. The results of this study helped improve the understanding of the ecological effects of copper on the abundance and diversity of ammonia oxidizers, as well as elucidating the relative contributions of AOA and AOB to nitrification during cattle manure composting process in the presence of added copper. This study may provide useful information for further research to enhance the retention of nitrogen of the manure composting containing copper.

2. Materials and methods

2.1. Chemicals and sample collection

Copper sulfate ($CuSO_4$, 99.0% ACS grade) and other inorganic reagents (ACS grade) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). The fresh manure was collected from a dairy farm in Yangling, Shanxi, China. After collection, the manure was mixed well using the collection equipment, placed in a plastic box, and transported to the laboratory. The manure was analyzed in the laboratory to determine its physicochemical properties, including the heavy metal contents. Wheat straw was cut into pieces that measured <1 cm and used as a bulking agent for composting. The dairy cattle manure had a pH of 7.8, organic carbon content of 410.8 g kg^{-1}, and an organic nitrogen content of 1.91 g kg^{-1}. The wheat straw had an organic carbon content of 417.6 g kg^{-1} and an organic nitrogen content of 6.09 g kg^{-1}.

2.2. Design of the composting experiment

Manure and wheat straw were mixed at a ratio of 4:1 (by dry weight, DW) to prepare a compost mixture, where the C/N ratio of the mixture was adjusted to 25:1, and the moisture content was adjusted with water to 55%. Natural ventilation was used to supply oxygen. The Cu content of the compost mixture was 25 mg kg^{-1}. Based on the Cu residue level reported previously in manures (Li et al., 2009, 2014; Ji et al., 2012), additional $CuSO_4$ solution was used at a specific concentration to spike each sample with Cu concentrations of 50 and 500 mg Cu kg^{-1} (DW), thereby obtaining final Cu contents of 75 and 525 mg kg^{-1} (DW), which were designated as the Cu50 and Cu500 treatments, respectively. An equivalent amount of distilled water was added to the control (without the addition of $CuSO_4$). The compost mixtures were packed loosely in foam boxes, which measured 45 × 27.5 × 51.5 cm (length × width × height) and the foam had a thickness of 3.5 cm. To provide aeration, the mixture was turned once every two days during the early phases and twice each week subsequently.

Samples were collected from each treatment after 2, 8, 13, 21, and 35 days. Approximately 50 g of each compost mixture was collected at three different depths in the composting material. Each sample was divided into two parts, where the first was stored at 4 °C for later analysis and the second was stored at −80 °C for DNA extraction.

2.3. Physicochemical parameters and potential ammonia oxidation (PAO) rate analysis

The pile temperature was monitored at 10 cm depth (surface, core, and bottom, respectively.). For total copper content of the raw material determination, 0.5 g dried and ground samples digested with mixture of nitric, hydrochloric, and hydrofluoric acid at a ratio of 1:1:2. The Cu were extracted using diethylenetriaminepentaacetic acid (DTPA) with a solid to liquid ratio of 1:5 (w/v) and DTPA-extractable and total contents of Cu analyzed using a flame atomic absorption spectrometer (Hitachi, Japan). DTPA-extractable copper was defined as Cu(II) (Jordão et al., 2011), and the data showed in Table S1. The pH was measured with a digital pH meter by mechanically shaking the fresh sample as a suspension in water at a ratio of 1:10 (w/v) for 40 min at 200 rpm. NH_4^+ and NO_3^- were extracted with 2 M KCl and determined by flow injection analysis (Systea, Italy). The PAO rates were measured as the accumulated NO_2^- using the chlorate inhibition method, according to Jarvis et al. (2009) and Zeng et al. (2011). The NO_2^- concentration was determined by flow injection analysis (Systea, Italy). The PAO rate was calculated by linear regression based on the accumulated NO_2^- over time.

2.4. Genomic DNA extraction and quantitative PCR (qPCR)

All of the compost samples were dried using a freeze dryer (Songyuan, Beijing, China) until the water content was at the same low level (3–4%). The samples were then crushed and sieved through 1-mm pore filters using an ultra-centrifugal mill (ZM200, Retsch, Germany). Next, the total DNA was extracted from 0.1 g of each freeze-dried compost sample using a Fast DNA Spin Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA), according to the manufacturer's instructions. The concentration and quality of DNA were determined using an Epoch Multi-Volume Spectrophotometer System (BioTek, USA). The extracted DNA was stored at −20 °C for subsequent use.

The primers amoA-AF (5′-ATG GTC TGG CTW AGA CG-3′) and amoA-AR (5′-GCC ATC CAT CTG TAT GTC CA-3′) (Francis et al., 2005), and amoA-1F (5′-GGG GTT TCT ACT GGTGGT-3′) and amoA-2R (5′-CCC CTC KGS AAA GCC TTC TTC-3′) (Witzel and Rotthauwe, 1997), were used to quantify the copy numbers of the AOA and AOB *amoA* genes, respectively. qPCR was performed in triplicate with an iCycler IQ5 Thermocycler (Bio-Rad, USA). Each reaction system comprised a total volume of 20 μL, which contained 10 μL of 2× SYBR real-time PCR premixture (Cwbiotech, Beijing, China), 0.4 μL (10 μM) of each primer, 2 μL of DNA template, and the volume was made up with sterile water. The qPCR conditions were as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s, annealing for 30 s at 61 °C for AOA or 60 °C for AOB, and extension at 72 °C for 32 s. Ten-fold serial dilutions of linearized plasmids containing cloned *amoA* genes were used to produce the standard curves for qPCR. The detection limit was 10^4 copies g^{-1} compost. A melting curve was obtained at the end of the reaction to verify the specificity of the amplicon. The PCR efficiencies and linearity (R^2) for each standard curve were 90.0–101.0% and 0.990–0.992, respectively.