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# Response of humic-reducing microorganisms to the redox properties of humic substance during composting

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ABSTRACT

Humic substance (HS) could be utilized by humus-reducing microorganisms (HRMs) as the terminal acceptors. Meanwhile, the reduction of HS can support the microbial growth. This process would greatly affect the redox conversion of inorganic and organic pollutants. However, whether the redox properties of HS lined with HRMs community during composting still remain unclear. This study aimed to assess the relationships between the redox capability of HS [i.e. humic acids (HA) and fulvic acids (FA)] and HRMs during composting. The results showed that the changing patterns of electron accepting capacity and electron donating capacity of HS were diverse during seven composting. Electron transfer capacities (ETC) of HA was significantly correlated with the functional groups (i.e. alkyl C, O-alkyl C, aryl C, carboxylic C, aromatic C), aromaticity and molecular weight of HA. Aromatic C, phenols, aryl C, carboxylic C, aromaticity and molecular weight of HS were the main structural features associated with the ETC of FA. Ten key genera of HRMs were found significantly determine these redox-active functional groups of HS during composting, thus influencing the ETC of HS in composts. In addition, a regulating method was suggested to enhance the ETC of HS during composting based on the relationships between the key HRMs and redox-active functional groups as well as environmental variables.



## 1. Introduction

Humic substances (HS) and humic-reducing microorganisms (HRMs) are the major participants in humus respiration. HS can be reduced by HRMs (Stevenson, 1994), and reduced HS can be served as an electron donor and transfer electrons to a large variety of inorganic as well as organic contaminants with higher redox potential (Yuan et al., 2011; Zhu et al., 2013, 2014), including U (VI) (Gu and Chen, 2003; Wang et al., 2015a), Cr (VI) (Ding et al., 2014), nitrobenzenes (Yuan et al., 2017) and chlorinated compounds (Leitão, et al., 2016; Zhang et al., 2015). This so-called electron shuttling between HS and HRMs were recognized to facilitate and increase the contaminants reduction rates (Klüpfel et al., 2014; Lovley et al., 1996; Jiang and Kappler, 2008).

HS are chemically heterogeneous polyfunctional organic molecules and constitute redox-active polymeric organic compounds formed during the degradation of organic matter (OM) such as lignin, proteins and carbohydrates (Aiken et al., 1984). The electron transfer capacities (ETC) of HS have been ascribed primarily to their functional groups (Visser, 1964; Zhang et al., 2015). Quinones as the redox-mediating functional groups are known to accept and donate elections, i.e., to participate in redox reactions in HS, which stimulated the transfer of electrons during the redox reactions evaluated (Smith et al., 2015; Visser, 1964). Moreover, the electron spin resonance measurements spectra obtained for the HS were consistent with semiquinones being the main organic radicals (Scott et al., 1998). Previous studies have also suggested that the ETC of various HS samples was associated to phenolic moieties and aromaticity of the samples (Aeschbacher et al., 2012; Chen et al., 2003; Yang et al., 2015). These studies documented that quinones or quinone-like functional groups were the major functional groups responsible for the ETC of HS samples.

It was well recognized that HRMs could be capable of transferring electrons to redox active functional groups in HS coupled to

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the oxidation of acetate and hydrogen, and that the reduction of HS can support HRMs growth (Martinez et al., 2013). The ubiquity of HRMs were observed based on a wide diversity of environments in which microbial humus or quinone-reducing activities were observed (Weber et al., 2006). A broad phylogenetic diversity of HRMs were detected across a range of chemical and physical conditions, including hyperthermophilic, thermophilic, psychrophilic, acidophilic and alkaliphilic conditions, demonstrating that the ubiquity of this type of microbial metabolism (Weber et al., 2006; Xi et al., 2016).

Composting is a biological transformation process to stabilize different organic solid waste materials. During composting, active fraction in organic substances such as polysaccharides, aliphatics and proteins can be used as food by microorganisms (Xi et al., 2016), and a higher concentration of organic macromolecules such as aromatic carbon (C), polyphenolic structures of HS could be formed (Saidpullicino et al., 2007; He et al., 2014; Wang et al., 2015a). Several studies have been demonstrated that HS in composts can function as electron shuttles to participate in microbial reduction (He et al., 2014; Yang et al., 2015; Yuan et al., 2016). Previous study have been documented that composts contain a widespread variety of HRMs with the increasing abundance and capacity of reducing HS or Fe (III) (Xi et al., 2016). Although various literatures have already studied on the microbial interactions with HS (Qian et al., 2011), and on HS application as redox mediator for bioremediation purposes (Fp and Cervantes, 2009), the effect of HRMs community dynamic on the redox properties within HS has not been studied in the environment, especially in composts.

In this study, the aims were to: (1) confirm the redox-active functional groups of HS that associated with the ETC of HS, (2) determine the key HRMs affecting these redox-active functional groups of HS, and (3) propose a regulating method for enhancing the ETC of HS according to the effect of environmental variables on the key HRMs community during composting. This work would be critical to develop adaption strategies in remediating contaminated soils or maintaining composting environmental sustainability.

## 2. Material and methods

### 2.1. Composting process and analysis

Composting experiment was performed at Shanghai Songjiang Composting plant, China. Seven compost sources were chicken manure (CM), dairy cattle manure (DCM), fruits and vegetables (FVW), weeds waste (WW), corn straw waste (SW), green waste (GW), sewage sludge (SS). Each compost pile was more than 2t, and the piles were turned mechanically every 7d. Substrates used for composting were sawdust (SD) or 1% urea to obtain a C/N of 25–35:1. SD was obtained from a local wood processing industry in suburbs of Shanghai. The size of SD is powdery particles of sawn wood, with an average diameter of about 0.5 mm. At the completion of the compost on day 40, the C/N of the composts was 11.72 (CM), 13.21 (DCM), 14.97 (WW), 14.25 (FVW), 15.33 (SW), 16.0 (GW), 11.02 (SS), respectively. The humidity was maintained at around 65–70%. Seven composts attained a thermophilic temperature of 58 to 62 °C that was followed by a cooling period for 30 days. Triplicate composite samples were collected at different points from the top to the bottom of the piles at three main stages, i.e., mesophilic phase (1d), thermophilic phase (7d) and mature phase (23d).

1 g of each sample was processed for the isolation of HRMs immediately. Subsamples were air-dried, ground to pass through a 0.25 mm sieve, and stored in a desiccator for further analysis. Physical-chemical parameters including temperature, C/N, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), OM, ammonia nitrogen ($NH_4^+$-N), nitrate nitrogen ($NO_3^-$-N), moisture and pH were analyzed. In addition, biological indicator, i.e., germination index (GI), was also investigated. The data of these physical-chemical parameters were shown in Table S1. Detailed analytical methods and part of data (CM, DCM and SS) have been published in our previous studies (Wang et al., 2015b; Xi et al., 2016).

### 2.2. Enrichment of HRMs and diversity analysis

The analog anthraquione-2, 6-disulfonate (AQDS) (0.5 mmol $L^{-1}$) was selected as the electron acceptor to enrich the HRMs in composts. Lactate (5 mmol $L^{-1}$) was added as carbon source in the substrate. After almost 7–8 transfers, a stable microbial culture was obtained. HRMs diversity was characterized by sequencing 16S rRNA gene sequences amplified by PCR from bulk extractions, using the Illumina Miseq 2500 platform. Detail methods of the enrichment of HRMs and 16S rDNA sequencing have been recently published (Xi et al., 2016).

### 2.3. Sequence accession numbers

The data reported in this paper have been deposited under accession KX243554–KX244319 in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra).

### 2.4. Humic substances extraction

HS was exhaustively extracted from composts and quantified through a method based on the International Humic Substances Society (IHSS) protocol (Sparks et al., 1996). The extracted HS were then separated into humic acid (HA) (precipitated) and fulvic acid (FA) (supernatant) fraction by acidifying the extract to pH 1 with 6 M hydrochloric acid (HCl) and subsequently centrifuging the extract at 5000 rpm. The HA fraction was suspended in a solution of 0.1 M HCl/0.3 M hydrogen fluoride (HF) to remove mineral impurities and dialyzed until the elimination of chloride ions. The FA fraction was purified with adsorption resin XAD-8, and the alkaline eluate was made to pass through a cation exchange resin.

### 2.5. Electrochemical measurements

Electrochemical experiments were conducted by the electrochemistry workstation CHI660D (Chenhua, Shanghai, China) with a conventional three-electrode cell in a anoxic condition ($N_2$, atmosphere at 25 °C±1 °C). The detailed method of electrochemical analysis has been described in Aeschbacher et al. (2010) and Tan et al., (2017). Briefly, a graphite plate electrode was used as the working electrode, and the Pt net and $Hg/Hg_2Cl_2$ electrodes were chosen as the counter and reference electrodes, respectively. 1,1′-ethylene-2,2′-bipyridyldiylium dibromide (DQ) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were used as mediated electrochemical reducation (MER; at Eh = −0.49 V) and oxidation (MEO; at Eh = +0.61 V), respectively. The number of the transfered electrons was quantified by integration of reductive and oxidative current responses. The values of ETC including electron-accepting capacities (EAC) and electron-donating capacities (EDC) of HA (or FA) was calculated using the following fomullar.

$$\mathrm{EAC} = \frac{\int \frac{I_{red}}{F} dt}{m_{HS}} \quad (1)$$

$$\mathrm{EDC} = \frac{\int \frac{I_{ox}}{F} dt}{m_{HS}} \quad (2)$$

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