

**Methodological
interference of
biochar**

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Methodological interference of biochar in the determination of extracellular enzyme activities in composting samples

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Abstract

Biochar application has received increasing attention as a means to trap recalcitrant carbon and enhance soil fertility. Hydrolytic enzymatic assays, such as β -glucosidase and phosphatase activities, are used for the assessment of soil quality and composting process, which are based on use of *p*-nitrophenol (PNP) derivatives as substrate. However, sorption capacity of biochar can interfere colorimetric determination of the hydrolysed PNP, either by the sorption of the substrate or the reaction-product of hydrolysis into biochar surface. The aim of the present work is to study the biochar sorption capacity for PNP in biochar-blended composting mixtures in order to assess its impact on the estimation of the colorimetric-based enzymatic assays. A retention test was conducted by adding a solution of known amounts of PNP in universal buffer solution (pH = 5, 6.5 and 11, corresponding to the β -glucosidase, acid and alkaline phosphatase activity assays, respectively), in samples taken at the initial stage and after maturation stage from 4 different composting piles (two manure composting piles (PM: poultry manure, CM: cow manure) and two other similar piles containing 10 % of additional biochar (PM + B, CM + B)). The results show that biochar blended composts (PM + B, CM + B) generally exhibited low enzymatic activities, compared to manure compost without biochar (PM, CM). In terms of the difference between the initial and maturation stage of composting process, the PNP retention in biochar was shown more clearly at maturation stage, caused by an enlarged proportion of biochar inside compost mixture after the selective degradation of easily decomposable organic matter. The retention of PNP was more pronounced at low pH (5 and 6.5) than at high pH (11), 3 reflecting on pH dependency of sorption 49 capacity of biochar and/or PNP 50 solubility.

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1 Introduction

Agricultural use of biochar has been paid attention as an alternative strategy for mitigation of greenhouse gas (GHG) emission as well as improvement of soil properties. In addition, high sorption character of biochar, similarly to activated carbon, makes it possible to contribute to reduction of several hazards (heavy metals, pesticide, and hydrocarbon) in soil (Yang et al., 2009). Furthermore, it has been reported the suitability of biochar as an additional component for enhancing the composting quality by reducing the nitrogen volatilization due to sorption on surface of biochar (Steiner et al., 2010) and improving compost quality (Dias et al., 2010; Jindo et al., 2012).

In terms of the decomposition of organic matter during composting, enzymatic activities such as β -glucosidase and phosphatase are useful tool to reflect dynamics of biodegradation process and provide valuable information about stability and maturity of the compost (Vuorinen, 2000; Mondini et al., 2004). These hydrolytic enzymes are measured by colorimetric determination of *p*-nitrophenol (PNP) which is formed as the reaction-product of hydrolysis of different *p*-nitrophenol derivatives used as a substrate: nitorophenyl- β -D-glucopiranoside (PNG) for β -glucosidase activity, and *p*-nitrophenyl phosphatase (PNPP) for alkaline and acid phosphatase activities. By contrast, *p*-nitrophenol is a well-known toxic compound in industrial sector, and is treated by absorption in activated carbon (Tang et al., 2007; Ivančev-Tumbas et al., 2008). Furthermore, the biochar, which has similar absorption character to activated carbon, interferes with the extraction of soluble organic compounds, leading to underestimation of soil microbial activities (Chan et al., 2007). Even though several works on the relation between microbial measurements and biochar exposure has been reported (Durenkamp et al., 2010; Bailey et al., 2011; Luo et al., 2013), further research are required for understanding the biochar interaction from the chemical, physical and biochemical point of view. Thies and Rillig (2009) proposed the utilization of spiking assays with specific molecules as internal standard to overcome potential interferences in the estimation of the microbial parameters.

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The aim in present work was to study the influence of biochar as a composting component on the retention of the PNP generated from three colorimetric-based enzymatic assays (alkaline and acid phosphatases and β -glucosidase). The retention of PNP was tested in two different composting mixtures (poultry manure (PM) and cow manure (CM)) and other two similar composting mixtures containing biochar as additional component (PM + B, CM + B).

2 Materials and methods

2.1 Biochar preparation

The production of biochar, made from broad-leaved tree (*Quercus serrate* Murray), was carried out using a Japanese traditional kiln at atmospheric pressure and a temperature range of 400–600 °C. To analyze the physical properties of biochar, we grounded and sieved the biochar less than 0.5 mm in diameter. The main characteristics of the obtained biochar are: pH (H₂O) = 7.23; C = 791.5 g kg⁻¹; O = 91.5 g kg⁻¹; H = 18.9 g kg⁻¹; ash = 78.7 g kg⁻¹; N = 37.6 g kg⁻¹; P = 2.3 g kg⁻¹; K = 14.1 g kg⁻¹; Surface area = 255.0 m² g⁻¹; methylene blue (MB) absorption capacity: 8.3 mg g⁻¹; iodine adsorption capacity: 100 mg g⁻¹.

2.2 Raw materials and composting process

Composting was carried out at Kanagi experimental farm of Hirosaki University. Two composting mixtures were prepared following initial proportion of organic waste: CM – cattle manure (100.9 kg) mixed with apple pomace (76.8 kg), rice straw (9.7 kg) and rice bran (12.7 kg), PM – poultry manure (35.2 kg) mixed with apple pomace (141.8 kg), rice straw (9.9 kg) and rice bran (13.0 kg). Another two composting mixtures (CM + B and PM + B) were prepared by enriching the initial composting mixtures CM and PM with 20 kg of biochar. The organic waste mixtures were composted in cone shaped

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windrows with regular turnings and continuous monitoring of pile temperature and moisture. The principal physicochemical properties of the composting mixtures are described in Table 1, and further information on the composting process and characteristics of the composting mixtures can be found elsewhere (Sonoki et al., 2012).

5 The composting process lasted approximately 3 months for all piles. A representative sample of each organic material was taken at the initial stage (I) and after maturation stage (M). These samples were collected from different spots of piles, mixed together, air dried and grounded to 0.5 mm.

2.3 Thermogravimetric analysis (TGA)

10 Thermal analysis of the organic material was measured using a SDT-2960 simultaneous DSC-TGA thermal analyzer (TA instruments) under static air atmosphere as follows: a temperature equilibrating at 30 °C followed by a linear heating rate of 5 °C min⁻¹ from 30 to 105 °C, an isotherm for 10 min and then continued ramping of 5 °C min⁻¹ from 105 to 680 °C. An index of thermal lability of the organic matter (W_2/W_1), shown
15 in Table 1, was calculated from the ratio: mass loss at 350–550 °C (W_2)/mass loss at 110–350 °C (W_1) (Plante et al., 2009).

2.4 Enzymatic analysis

Alkaline and acid phosphatase and β -glucosidase activities were determined following the methods reported by Tabatabai and Bremmer (1971), and Eivazi and Tabatabai
20 (1988) respectively using 0.5 g of organic material, and 2 mL of modified universal buffer (MUB) containing the following substrate: alkaline phosphatase activity assay was performed at pH 11 using *p*-nitrophenyl phosphatase (PNPP) as substrate, meanwhile acid phosphatase activity assay was performed with the same substrate at pH 5.5; β -glucosidase activity was assayed at pH 6 using *p*-nitrophenyl β -D-glucopiranoside (PNG) as substrate. In the three cases, the suspensions were incu-
25 bated at 37 °C for 1 h. Enzymatic reactions were stopped by cooling in ice for 15 min.

Then, 0.5 mL of CaCl₂ 0.5 M and 2 mL of NaOH 0.5 M (for phosphatases) or 2 mL of Tris (hydroxymethyl) aminomethane-sodium hydroxide (THAM-NaOH) 0.1 M pH 12 (for β -glucosidase) were added. The *p*-nitrophenol (PNP), formed as product reaction from the three enzymatic assays, was determined colorimetrically at a wavelength of 398 nm.

2.5 PNP retention assay during the enzymatic activity analysis

To study the retention of PNP during the analysis of the different enzymatic activities, following spiking assay was performed: instead of adding the substrates (PNG and PNPP) at the beginning of the procedure, reaction-product (PNP) was added with difference concentration (0, 50, 100 and 150 mgL⁻¹) to buffer solution (pH = 5, 6,5 and 11, corresponding to the β -glucosidase, acid and alkaline phosphatase activity assays, respectively). This procedure allows evaluating the retention of PNP by the biochar during the analysis. Controls were performed similarly by adding the same amounts of PNP after the incubation period and before the measurement of the absorbance in the calibrated spectrophotometer (with an external PNP standard solution). These results were shown in Fig. 2 (CM and CM + B) and Fig. 3 (PM and PM + B). Lately, the PNP retention was calculated by fitting the amount of PNP measured after the enzymatic determination (PNP_{exp}) and the amount of PNP added in the control (PNP_{add}) to a linear equation (PNP_{exp} = $k \times$ PNP_{add}), where k was the slope of the linear fitting. The percentage of PNP recovered in the enzymatic determination was calculated as 100 · k , whereas the percentage of PNP retention was calculated as 100 · (1 - k). PNP retention assays were performed in duplicate for all treatments and shown in Table 2.

3 Results and discussion

3.1 Characteristics of the composting mixtures

Different composting mixtures were selected at different stages of the composting process to cover the range of organic matter stabilization degree. The different nature of

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the organic matter at different stages of the biodegradable process and the property of the recalcitrant biochar was assessed by thermogravimetry (Lyons et al., 2006; Tsui and Juan, 2010; Many et al., 2013). Basically, the TGS-DSC diagrams are characterized by two main mass losses, showing two exothermic peaks, and these are respectively corresponded to volatilization of light compounds such as aliphatic molecules or carbohydrates and another to oxidation of high molecular weight components (Fig. 1). Comparing the graph shapes between the samples from initial stage (Fig. 1a and c) and from maturity stage (Fig. 1b and d), the second wave of peak, generated by mass loss at 350–550 °C, was pronouncedly shown at maturity stage, due to the selective degradation of labile organic materials during the composting process. As a consequence, the index of lability of W_2/W_1 in all samples of maturity stage is higher than those of initial stage (Table 1).

The influence of additional biochar into the composting mixture at the initial phase (Fig. 1a and c) is observed by higher peak of second wave in biochar blended composts (PM + B, CM + B), which are described in dotted lines. This has resulted from that biochar originated from hard-wood mostly consists of recalcitrant compounds, which are combusted at W_2 range (350–550 °C) in an oxidant atmosphere of air. Consequently, W_2/W_1 ratio at initial time (Table 1) increased in biochar blended piles (PM + B, CM + B) from the piles of non-biochar addition (PM, CM). After maturation stage (Table 1), W_2/W_1 ratio markedly increased in the biochar blended composts (PM + B, CM + B, 2.3, and 1.6, respectively), reflecting the high relative proportion of recalcitrant biochar.

3.2 Study of the PNP retention on biochar blended compost

The colorimetric determination of PNP was influenced by the degree of stability of the composting mixtures, which affected the relative proportion of biochar in the mixture. The biochar blended composts showed more retention of PNP, especially in the case of maturity stage (Figs. 2 and 3). The amount of PNP retained by the biochar blended composting mixtures (CM + B and PM + B) varied from 41 % in the starting composting

5 mixtures up to 74% in mature composts. This result might have attributed to gained dominance of biochar amount inside composting mixtures which was gradually increased during the composting process. The recalcitrance of biochar character was remained until the maturation stage, while labile organic materials in the composting piles were lost due to the selective degradation, as already shown by TGS measurement (Fig. 1). Therefore the effect of the physico-chemical properties of biochar on the compost structure is expected to be more dominant in the mature stage than at the initial stage.

10 The PNP retention by biochar also depends on pH status of the buffer solution, used by each specific enzymatic activity. At high pH condition (pH 11), representing alkaline phosphatase assay, the PNP retention is observed in the range between 15 and 30% of the added PNP (Table 2). However, the same spiking assays performed a low pH (pH 6.5 and 5 from acid phosphatase and β -glucosidase activities, respectively) exhibited high PNP retention from 30% (acid phosphatase determination in PM + B) up to 70% which is the case of the β -glucosidase determination in CM + B. These results are in agreement with the pH dependence of phenol adsorption efficiency by activated carbon reported by several authors (Ayranci and Duman, 2005; Tang et al., 2007), concluding that the absorption efficiency of activated carbon is lower in alkaline solution than neutral or acid solution. An increase in the amount of OH ions in alkaline solution reduces the diffusion of phenol ions due to an electrostatic repulsion of negatively charged site of the sorbent and phenolic ions. As the pH increases, the surface charge of pyrogenic materials became negative and decreases its sorption capacity (Beker et al., 2010). Furthermore, other authors (Zhang et al., 2010) reported that, regarding the mobility of biochar particle, the lower pH solution, the lesser transport of the biochar particle.

25 Sorption affinity of pyrogenic material is also influenced by physical properties such as microporosity and surface area, as well as chemical properties such as hydrophobicity in relation with O/C content (Al-Asheh et al., 2004; Ko et al., 2007; Tsui and

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Juang, 2010). All these biochar properties are dominantly defined by feedstock and the pyrolysis conditions used for the preparation of the biochar (Uchimiya et al., 2010).

The PNP retention by the organic matter of the composting mixtures prepared without biochar (PM and CM) was also affected by the pH gradient. Table 2 shows that at low pH solution (pH 5) the initial stage of composting, CM has 69 % of PNP recovery, meaning 31 % of PNP was retained. This methodological problem in the determination of the enzymatic activities is well-known in clay mineral soils or soils enriched with organic matter (Tabatabai and Bremer, 1971; Trasar-Cepeda et al., 1988). The organic material containing large amount of humic substances are known to easily absorb PNP molecules (Chen et al., 2009).

In conclusion, the presence of biochar limited the validity of enzymatic essays for the colorimetric determination of PNP since PNP was strongly retained in biochar blended compost. It is challengeable to improve the colorimetric methods of PNP determination for biochar interaction, and clear-cut solution has not been found until present day. More other factors are necessary to be considerable for understanding the biochar interaction with enzymatic activity assay.

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Table 1. Physical and chemical properties in poultry manure compost (PM), poultry manure blended with biochar (PM + B), cow manure (CM), and com blended with biochar (CM + B).

Origin	Initial stage					Maturity stage				
	C (%)	N (%)	O.M. ¹ (%)	pH	W_2/W_1^2	C (%)	N (%)	O.M. (%)	pH	W_2/W_1
PM	36.9	1.7	79.9	5.8	0.5	27.9	2.9	55.2	8.2	0.8
PM + B	39.9	1.7	84.9	5.8	1.0	33.6	2.0	73.0	7.8	2.3
CM	35.6	1.7	75.9	6.6	0.4	29.8	2.4	59.7	7.7	0.8
CM + B	37.1	1.3	79.7	7.0	0.9	31.5	1.8	68.8	7.6	1.6

¹ O.M. = Organic matter.

² W_2/W_1 = The main weight losses occurred in the 110 to 350 °C (W_1) and 350 to 550 °C (W_2) ranges.

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Table 2. Percentage of PNP recovery calculated by the linear fitting of the measured amount of PNP (PNP_{exp}) and the amount of PNP added (PNP_{add}), according to the equation ($\text{PNP}_{\text{exp}} = k \times \text{PNP}_{\text{add}}$). The percentage of PNP recovery is expressed as $100 \cdot k$. Poultry manure compost (PM), poultry manure blended with biochar (PM + B), cow manure (CM), and cow manure blended with biochar (CM + B).

Enzymatic assay	Poultry Manure				Cow Manure			
	Initial Stage		Maturity Stage		Initial Phase		Maturity Stage	
	PM	PM + B	PM	PM + B	CM	CM + B	CM	CM + B
Alkaline Phosphatase (pH 11)	88.9 (3.9) ^a	78.6 (5.2)	96.03 (3.4)	80.8 (2.9)	91.3 (3.4)	84.8 (3.4)	90.2 (3.5)	70.5 (4.6)
Acid Phosphatase (pH 6.5)	84.6 (4.9)	71.3 (2.8)	80.8 (5.1)	43.5 (0.7)	112.9 (6.5)	62.2 (3.5)	86.3 (2.9)	55.9 (1.8)
β -glucosidase (pH 5)	72.7 (5.6)	59.0 (1.9)	89.8 (3.5)	39.2 (2.9)	61.2 (1.3)	60.3 (1.0)	63.8 (2.1)	32.7 (1.9)

^a Standard error in parenthesis.

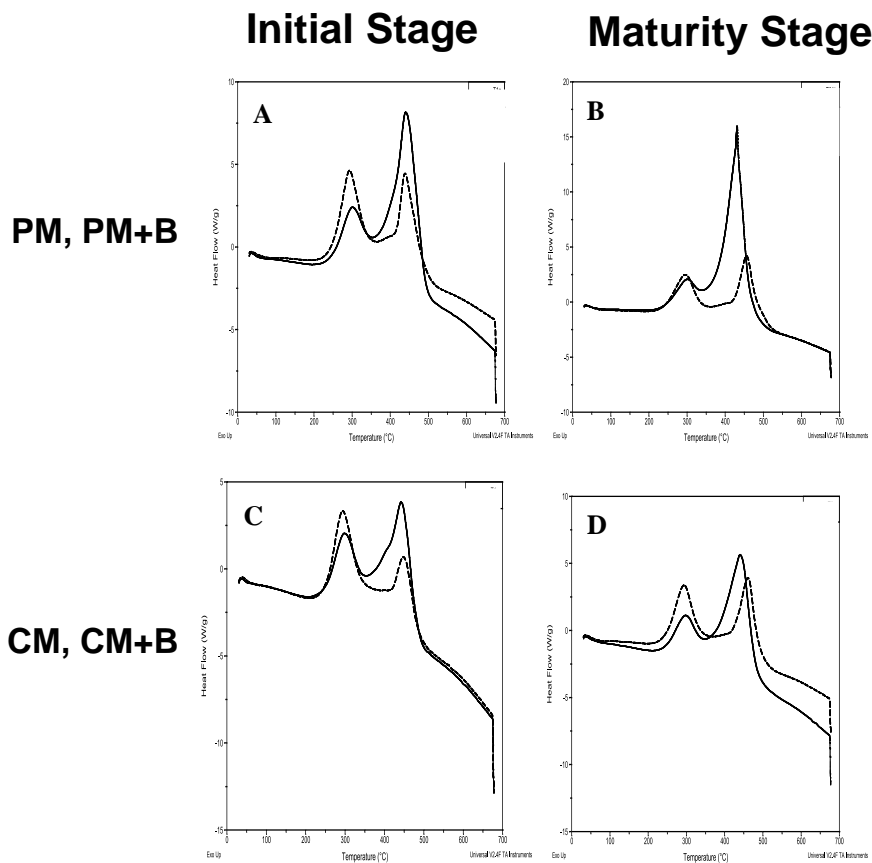


Fig. 1. The TGS-DSC analysis of poultry manure PM, poultry manure + biochar (PM + B), cow manure (CM), cow manure + biochar (CM + B). Dotted lines exhibit the compost without biochar, while continuous lines exhibit the compost with biochar. **(A)** Initial stage of PM + B and PM; **(B)** maturity stage of PM + B and PM; **(C)** initial stage of CM + B and PM; **(D)** maturity stage of CM + B and CM.

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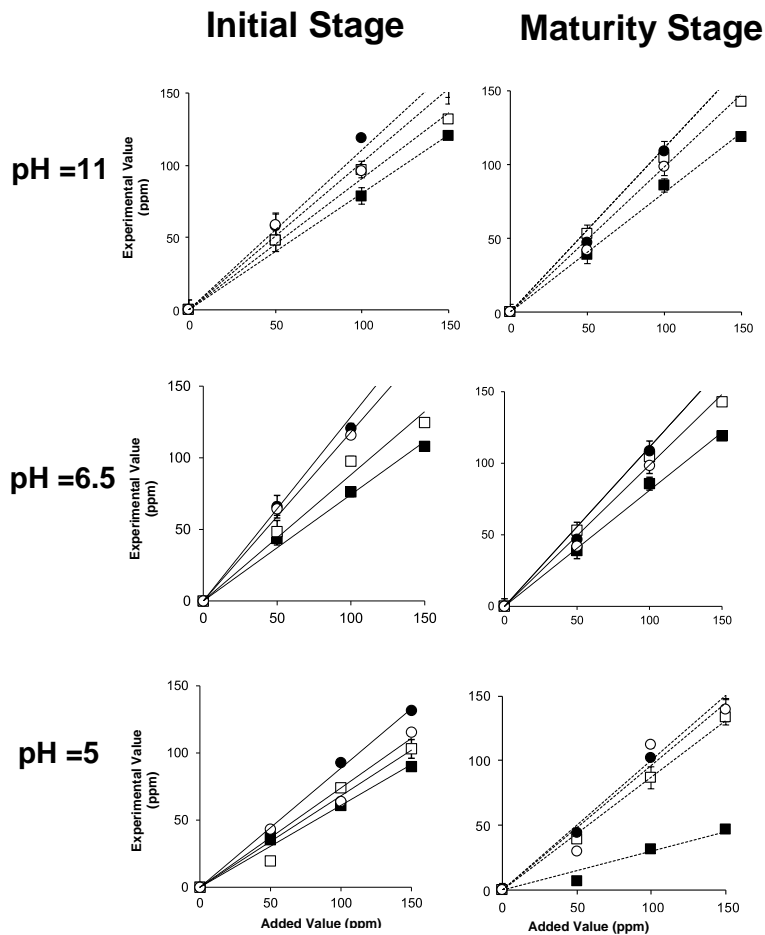


Fig. 2. Retention of PNP with different pH solution in the compost samples of poultry manure PM (□), poultry manure + biochar PM + B (■), poultry manure control PMC (○), and poultry manure + biochar control PMB-C (●) during the composting process.

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Interactive Discussion



Initial Stage

Maturity Stage

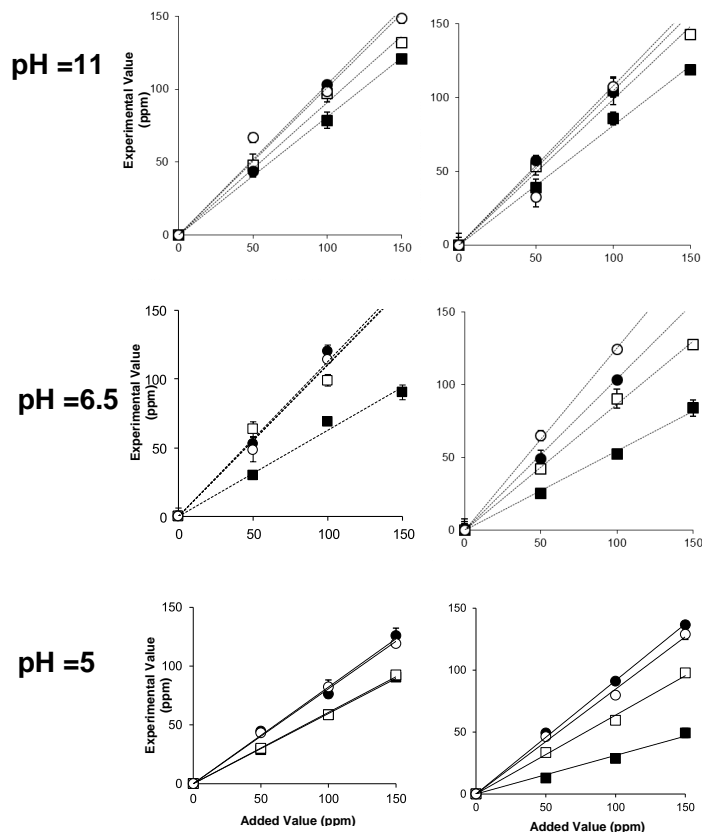


Fig. 3. Retention of PNP with different pH solution in the compost samples of cow manure CM (□), cow manure + biochar CM + B (■), cow manure control CM-C (○), and cow manure + biochar control CMB-C (●) during the composting process.