

Chapter 5

Effects of earthworm stocking density on end product quality and microbial community structure during vermicomposting

Introduction

The global food waste generation, which is currently about 931 million tonnes per annum, has increased by more than two times in the last ten years [1]. About 24% of the municipal solid waste dumped on land is also constituted of wasted food [2]. In developing nations, vegetable and fruit waste is the most significant component of the household waste[3]. Although wasted food is a growing problem worldwide, it is also an unutilized resource. However, the nutrient elements in these wastes remain unavailable due to the predominance of recalcitrant lignocellulosic compounds that are less susceptible to microbial decay; thus, their use in raw forms is difficult for agricultural practices [4]. Hence, the conversion of these wastes into a valuable resource through eco-friendly valorization techniques is the need of time. Among different valorization techniques, composting and vermicomposting are highly suitable for nutrient recovery from recalcitrant biowastes owing to their simplicity and eco-friendly nature [5]. Vermicomposting has been reported to be more advantageous than composting due to its rapidity, efficient nutrient recovery potential, and pollutant remedial role [6].

Technological up gradation of vermicomposting systems for waste valorization is an attractive research domain[7]. The efficacy of vermicomposting technology has been comprehensively evaluated for several urban and industrial wastes[8,5,9]. These studies primarily focus on the initial feedstock composition, nutrient mineralization kinetics, toxic element removal, and feedstock maturity. In contrast, about 4-5 studies related to the effects of the initial stocking density of earthworms on the vermicomposting process revealed some information about the impacts of stocking density on earthworm feeding rate [9], compost maturity [10], and nutrient recovery [11] while dealing various solid wastes (e.g., wastewater sludge and fly ash), could only be retrieved after an extensive literature search. At the same time, optimizing the balance between mineralization and humification is the key to achieving high-quality compost in a short period, primarily regulated by microbial growth and activity[12]. Microbial growth and their community

structure greatly influence the mineralization-humification kinetics during vermicomposting; while microbial diversity may considerably vary depending on the earthworm species and their activity in the vermibeds [4,16]. However, to the best of available knowledge, there is no information about the impact of earthworm stocking density on microbial community structure in lignocellulosic waste-based vermicomposting systems. Moreover, most studies have been performed with *Eisenia fetida*; therefore, it would be novel and exciting to study the impact of stocking density of other epigeic earthworm species (e.g., *Eudrilus eugeniae*) on nutrient valorization and microbial diversity in lignocellulosic waste mediated vermicomposting systems.

The metal removal efficiency of earthworms has been utilized to sanitize contaminated biomass through vermicomposting [6]. Earthworms are known to remove metals via their unique metal chelating mechanism, and they also promote the immobilization of toxic metals by elevating the humification process [6]. However, little is known about the effects of the initial stocking density of earthworms on the metal removal efficiency of vermicomposting systems. Moreover, the linkage between metal removal and microbial diversity has to be comprehensively ascertained for the efficiency improvement of vermitechology.

Under these contexts, the present study was conducted to appreciate the role of the initial stocking population of two earthworm species (*Eisenia fetida* and *Eudrilus eugeniae*) in balancing the mineralization-humification dynamics during vermicomposting of kitchen vegetable waste (hereafter, KVW) and fallen banana stalk (hereafter, BS) in combination with cow dung. The effect of earthworm population density on microbial growth, activity, and community structure was also evaluated. The phospholipid fatty acid (PLFA) analysis was performed to understand the microbial community shift in response to the initial earthworm population in the vermibeds. As an integral unit of the microbial cell membrane, PLFAs intensely respond to environmental variations, there by regulating the signaling process of microbial growth and reproduction [14]. This technique thus could be utilized to understand the language of microbial behavior during vermicomposting [15]. In the present endeavor, an attempt was made to understand the relationship between microbial activity, their community shift, and the metal remediation efficiency of the vermicomposting system, probably for the first time.

Materials and methods

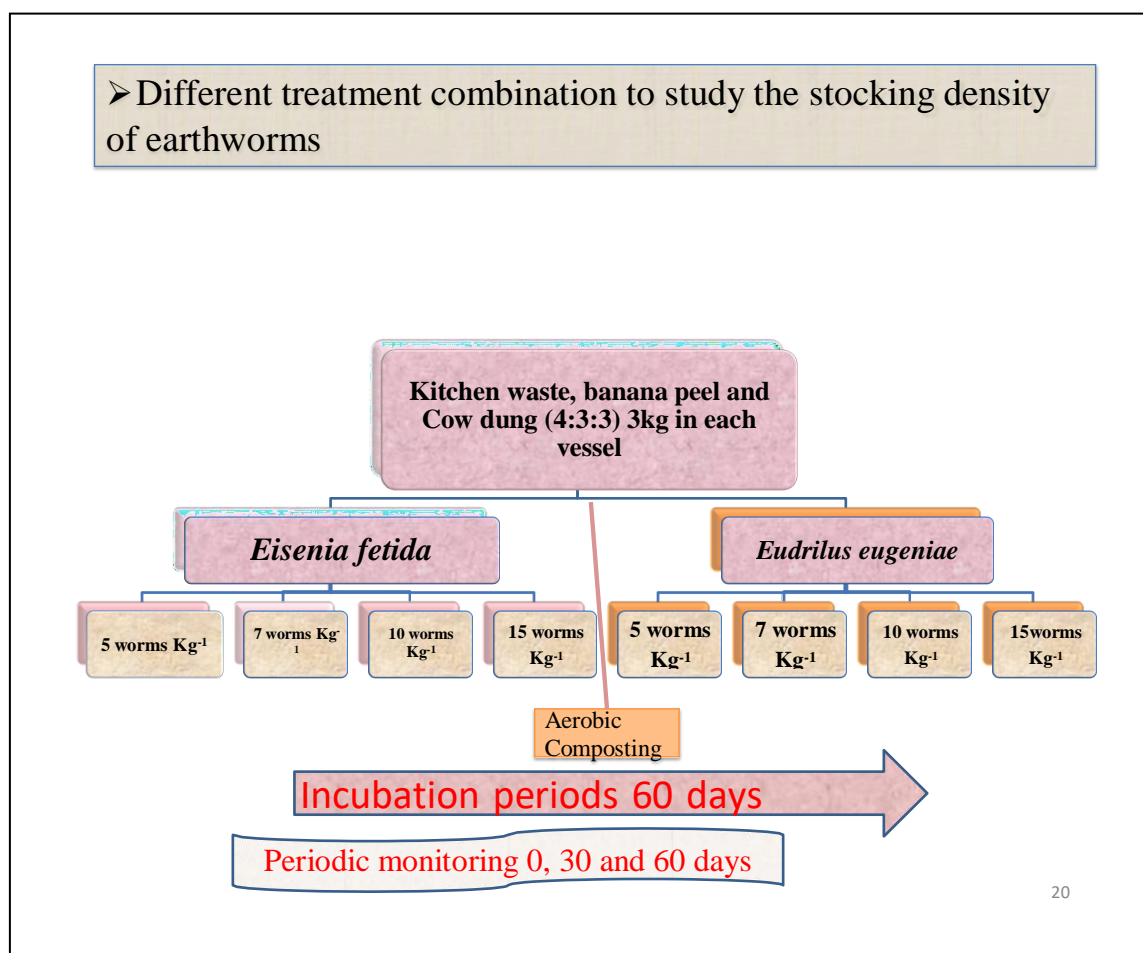
Collection of raw materials and feedstock formation

The lignocellulosic waste materials in the form of kitchen vegetable waste (lignocellulose waste) (KVV) and banana stalk (BS) were collected from the residential area of the Tezpur University campus. The KVV and BS samples were chopped into small pieces (~1 cm) to reduce the size. The urine-free cow dung (CD) was collected from the cattle yard of a neighboring village. Non-clitellated juvenile specimens of the two earthworm species (*Eisenia fetida* and *Eudrilus eugeniae*) were collected from the departmental vermiculture unit. Subsequently, the KVV, BS, and CD samples were pre-composted under a shed for 1-2 days. Then, a uniform feedstock composition was formed in bulk amounts (~35 kg) by making a homogenized mixture of the three components at a 4:3:3 ratio (i.e., KVV: BS: CD) for the experiment. Therefore, the uniformity in feedstock composition was maintained to nullify to specifically assess the impact of variable stocking density of earthworms on different chemical and microbial characteristics of the vermicompost. The initial chemical composition of the feed mixture was recorded as pH = 7.7±0.3; total organic C = 20.6±1.1%; total N = 4.2±0.2%; Av. P = 22.01±1.2 mg kg⁻¹; and Av. K = 64.8±2.4 mg kg⁻¹. The experiment was performed in truncated-cone-shaped and perforated earthen vessels of 5 kg capacity. The dimension of the reactors was: 0.50 m (depth) × 0.20 m (base radius) × 0.35 m (top radius). The vessels were washed and air-dried before use.

Experimental setup, maintenance condition, and sample collection

A two-month-long experiment was conducted at the vermicomposting yard of the Department of Environmental Science, Tezpur University, situated in Sonitpur District of Assam, India (26.7008N, 92.8303 E). Four different incubatory (i.e., initial) stocking densities (5, 7, 10, and 15 worms kg⁻¹ of feedstock) of the two earthworm species were used in this study. Eventually, 3 kg of the previously prepared feed mixture was uniformly poured into properly cleaned earthen vessels, and earthworms in various stocking densities were incorporated into designated containers. A series of aerobic composting (i.e., without earthworms) was also maintained for comparison. Hence, the following treatments were finally derived for the study:

	Vermicomposting	
	Uniform feedstock: KVW+BS+CD (4:3:3)	
	<i>Eisenia fetida</i> (Ef)	<i>Eudrilus eugeniae</i> (Eu)
Aerobic Composting	VEf5 - 5 worm kg-1	VEu5 - 5 worm kg-1
CP - KVW+BS+CD (4:3:3)	VEf7 - 7 wormkg-1	VEu7 - 7 worm kg-1
	VEf10 - 10 worm kg-1	VEu10 - 10 worm kg-1
	VEf15 - 15 worm kg-1	VEu15 - 15 worm kg-1



Each treatment was replicated thrice by maintaining identical reactor conditions. About 40-50% moisture content was kept for all the composting and vermicomposting reactors by regularly sprinkling water. Adequate aeration was ensured by turning the feedstocks (twice every three days) throughout the incubation period (i.e., 60 days). The samples were air-dried for a week, ground in a mortar pestle, and stored in polythene bags in a

freezer at 4 oC. This process was essential to resist the microbe-induced changes in the physicochemical properties of the feedstock and was performed based on standard prescription [16].

Growth and development of earthworm

The growth and development of the two earthworm species were assessed based on their population and body weight dynamics in the vermibeds. The population of the two earthworm species in different vermibeds (i.e., treatments) concerning their initial stocking densities was counted at periodic intervals (0, 30, and 60 d) by carefully spreading the feedstocks on a plastic sheet. The worms and feedstocks were eventually reconstituted in their respective vessels after counting counts on the 30th day. Necessary measures were also taken to avoid the loss of biomass. On the 60th day, the earthworms were separated from the vermicompost, counted, and released to the vermiculture unit. The body weight of the earthworms (both adult and juvenile) was also measured from each treatment.

Physicochemical analyses of compost and vermicompost samples

X-ray diffractometer (XRD)

The methodology for X-ray diffractometer (XRD) determination of sample has been described in the previous chapter 4 (see section 4.4.1)

pH

The methodology for pH determination of sample has been described in the previous chapter 4(see section 4.3.1)

Total organic C (TOC)

The methodology for Total organic C (TOC) determination of sample has been described in the previous chapter 4(see section 4.3.2)

Total Kjeldahl N (TKN)

The methodology for Total Kjeldahl N (TKN) determination of sample has been described in the previous chapter 4(see section 4.3.3)

Available P

The methodology for Available P determination of sample has been described in the previous chapter 4 (see section 4.3.4)

Available K

REAGENTS AND STANDARD CURVE:

1. **Ammonium acetate: 1N, pH 7:** To 700ml of distilled water, added 57ml 99% of glacial acetic acid (CH_3COOH) and then 69ml of conc. ammonium hydroxide (NH_4OH). Diluted a volume of 900ml and adjust pH to 7 by addition of more of 3N NH_4OH or 3N CH_3COOH make up to 1L. Alternatively dissolved 154g ammonium acetate in water and 1.8 L. Mix thoroughly. Adjust pH to 7 with dil. NH_4OH or HOAc as required and make to 2L.
2. **Potassium chloride stock solution:** Of 1000ppm standard K by dissolving 1.908g of AR grade potassium chloride in distilled water and diluting up to 1L. Prepare 100ppm standard by diluting 100ml of 1000ppm stock solution to 1L with the extracting solution.
3. **Standard Curve:** Pipette 0,5,10,15 and 20ml of 100ppm solution into 100ml volumetric flasks and bring the volume to mark with extracting solution. The solution contains 0,5,10,15 and 20 ppm K respectively.

PROCEDURE:

1. Taken 2g of sample in a 250ml conical flask.
2. Added 20 ml of 1N $\text{CH}_3\text{COONH}_4$ to it and shaken for 30 minutes on a mechanical shaker.
3. Then filtered the content and measure the available K in the filtrate by flame photometer

CALCULATION:

Total K = K concentration \times 10(dilution factor)

Humification factor (HF)

The extent of humification during vermicomposting and composting was estimated by enumerating the humification factor (HF) after [17]. Briefly, the compost and vermicompost samples were extracted with 1M NaOH in a 1:20 (W/V) ratio by shaking

for three hours in a mechanical shaker, followed by centrifugation at 3000 ×g for an hour. The optical density (i.e., absorbance) at 472 nm (signifies the predominance of aliphatic C compounds) and 660 nm (signifies the predominance of aromatic C compounds), respectively, was recorded in all the extracts in a UV-VIS spectrophotometer (Cary 60). Eventually, the HF was calculated using the formula (2):

$$\text{Humification factor (HF)} = \frac{\text{Aliphatic C (A}_{472}\text{)}}{\text{Aromatic C (A}_{660}\text{)}} \quad (2)$$

Microbial activity and community structure: phospholipid fatty acid analysis-based profiling

Microbial biomass C (MBC)

The methodology for Microbial biomass C (MBC) determination of sample has been described in the previous chapter 4 (see section 4.5.2)

Compost respiration (CR), microbial quotient (Mq), microbial metabolic quotient (qCO₂)

The methodology for Compost respiration (CR), microbial quotient (Mq), microbial metabolic quotient (qCO₂) determination of sample has been described in the previous chapter 4 (see section 4.5.3)

5.5.4. Phospholipid fatty acid (PLFA) analysis

The methodology for Phospholipid fatty acid (PLFA) analysis determination of sample has been described in the previous chapter 4 (see section 4.5.4)

Extraction and analyses of metals (Cd, Pb, Mn, Zn, and Cu)

Samples from each treatment were drawn at 60 d for estimation of different metals (Cd, Fe, Pb, Mn, Zn, and Cu) following the diethylenetriaminepentaacetic acid (DTPA) extraction method. DTPA was added to the samples as an extractant in the ratio of 1:2. The solutions were shaken mechanically for 2 hours at 250 rpm. The filtrates were used to estimate the presence of elements. Metal concentration in each extract was measured using an ICP-OES. The initial concentrations of the studied metals were also evaluated in the feedstock before adding the earthworms because a uniform feedstock composition was used in this study.

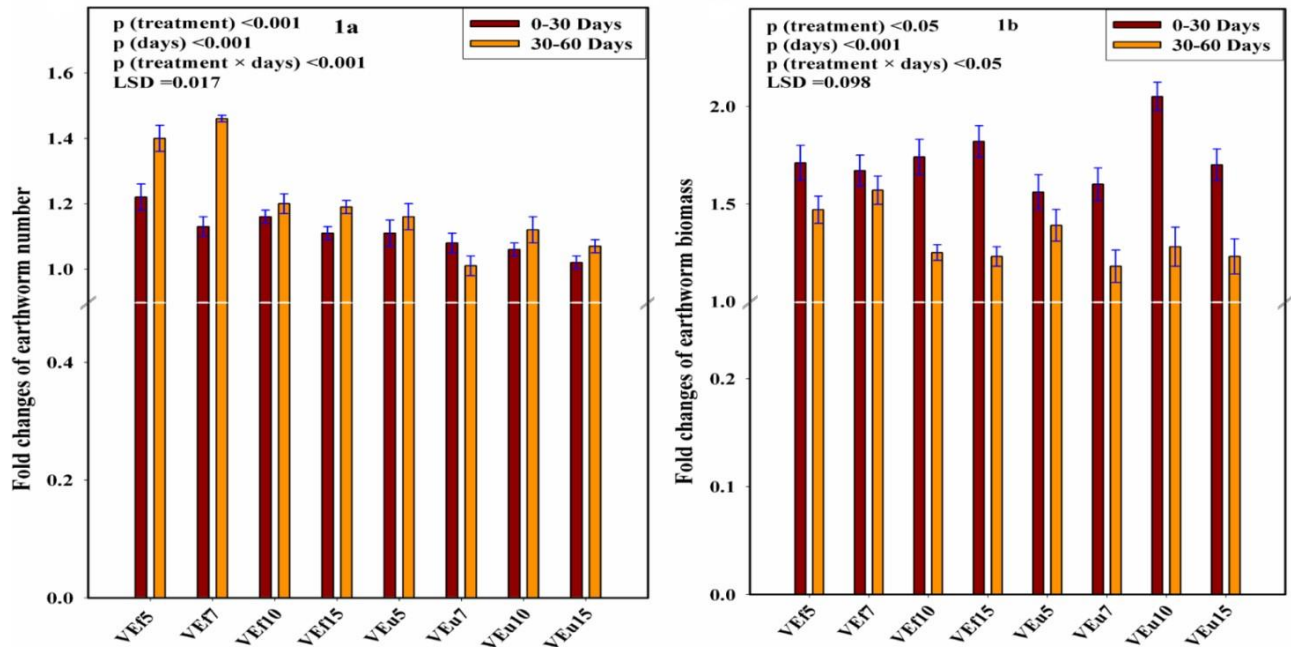
Statistical analyses

Two-way analysis of variance (ANOVA) was performed by taking treatments and days as the two non-random factors for all the estimated parameters (physicochemical and microbial) of compost and vermicompost beds to address the temporal dynamics. However, one-way ANOVA was conducted to assess the differences in metal concentrations in various treatments. The significance of variations regarding the effects of different treatments was evaluated by performing the least significant difference (LSD) posthoc test. In addition, Pearson's correlation statistic was utilized to identify the links between metal removal, nutrient mineralization, and microbial community shifts in the feedstocks. Before performing the correlation analysis, all data were normalized by log transformation (except pH).

Results and Discussion*Temporal dynamics of earthworm population and body weight*

A uniform feedstock composition (i.e., K VW + BS + CD) (4:3:3) was used for all stocking densities in this experiment to specifically appreciate the impact of the initial earthworm population on their fecundity and other parameters. The temporal dynamics of the earthworm population and the gross worm biomass have been presented as fold change in **Fig. 5.1a and 5.1b**. The count of *E. fetida* increased by 1.11-1.22 folds and by 1.19-1.46 folds between 0-30 days and 30-60 days, respectively. Although the population of *E. fetida* increased more vigorously than *E. eugeniae*; the rate of population increment was significantly higher in Vef5 and Vef7 vermibeds compared to the other treatments (Fig 1a; $P_{\text{treatment}} < 0.001$; $\text{LSD} = 0.017$). The population increment of earthworms is a vital sign of their adaptability in any substrate [18]. These data also suggest that the lignocellulosic feedstock was more palatable to *E. fetida* than *Eudrilus eugeniae*. As such, the high adaptability of *Eisenia fetida* in obstinate substrates has been reported in recent findings (Paul et al., 2020). In general, the total biomass (**Fig. 1b**) of both species significantly increased over time ($P_{\text{day}} < 0.001$), and the impact of initial stocking density on biomass gain of earthworms was explicitly significant ($P_{\text{treatment} \times \text{day}} < 0.001$). However, the rate of body weight increment was significantly higher in Vef15 and VEu10 between 0-30 days compared to other stocking densities. Although the rate of body weight gain was sharply retarded between 30-60 days for both species, the gross increment in biomass of *E. eugeniae* was considerably greater than *E. fetida* in the later

stage (i.e., 30 to 60 days). Toxic and aberrant substrate condition often induces body weight increment in earthworms, which is strongly related to their defense mechanism [20]. Hence, the current results specify that high initial population density (i.e., 10 and 15 worm kg⁻¹) might have generated substrate-induced stress in both earthworm species.



Abbreviation

VEf 5= *Eisenia fetida*- 5 worm Kg⁻¹
VEf 7= *Eisenia fetida*- 7 worm Kg⁻¹
VEf 10= *Eisenia fetida*- 10 worm Kg⁻¹
VEf 15= *Eisenia fetida*- 15 worm Kg⁻¹
VEu 5= *Eudrilus eugeniae*- 5 worm Kg⁻¹
VEu 7= *Eudrilus eugeniae*- 7 worm Kg⁻¹
VEu 10= *Eudrilus eugeniae*- 10 worm Kg⁻¹
VEu 15= *Eudrilus eugeniae*- 15 worm Kg⁻¹

Fig 5.1 (1a) and (1b) : Temporal variations in earthworm count (1a) and body weight (1b) in the lignocellulosic waste-based feedstock under different treatments (initial stocking densities of earthworms) during vermicomposting. Values represent mean ± standard deviation

Impact of earthworm stocking density on waste valorization

The valorization efficiency of the two earthworm species with different initial population densities was assessed based on variations of crystallinity index (CI), TOC, humification factor (HF), TKN, available P, and available K in the feedstocks (**Fig 5.2a-5.2g**). The CI was calculated from the XRD spectrum of 0, 30, and 60 d samples in the present study

(**Fig. 5.2a**). The initial CI values ranged between 22.43-22.82, indicating the uniform nature of the lignocellulosic feedstock. Interestingly, the CI sharply decreased in all treatments compared to the initial value; however, the extent of CI reduction was remarkably greater in VEu10 and VEu7, followed by Vef7, Vef5, and Vef15 ($P_{\text{treatment}} < 0.001$; $\text{LSD} = 0.27$). This data implies that a stocking density of seven worm kg^{-1} for *E. fetida* and ten worm kg^{-1} for *E. eugeniae* was favorable for the rapid breakdown of cellulose-rich feedstocks. It can also be presumed that the comminuting efficiency of earthworms substantially varies from species to species depending on their reproduction pattern. Thus, their initial stocking density could regulate vermicomposting systems. This interpretation was in good agreement with previous findings [23,12]

In general, the alkalinity of all the feedstocks under vermicomposting and composting increased between 0 to 30 days and then substantially reduced to neutral states (pH: ~ 7 -7.6; **Fig. 5.2b**). Alkalinity in lignocellulosic vermibeds indicates the predominance of ammoniacal species; which are the products of N-mineralization process [22]. The decrease in pH in the later stage of vermicomposting resulted from producing various organic acids (carboxylic, phenolic, and humic) during the humification process [7]. Interestingly, the pH-neutralizing efficiency of earthworms significantly varied depending on their initial stocking density (**Fig. 5.2b**). Such pH-neutralizing effect was more pronounced in VEu7, VEu15, VEu10, and Vef7, followed by Vef5 feedstocks ($P_{\text{treatment}} < 0.001$; $\text{LSD} = 0.04$). Hence, the pH balancing efficiency of *E. eugeniae* was greater than *E. fetida*, while 5 and 7 worm kg^{-1} stocking of *E. fetida* were also effective in such context (**Fig 5.2b**). A rapid decrease in feedstock pH indicates accelerated mineralization of the organically bound nutrient elements (Das et al., 2020). Consequently, a sharp reduction of TOC was evidenced in all the vermibeds (**Fig 5.2c**; $P_{\text{day}} < 0.001$), and there were significant treatment-time interactions regarding TOC profiles ($P_{\text{treatment} \times \text{day}} < 0.001$). Interestingly, the pattern of TOC reduction greatly varied with different stocking densities. For example, TOC decreased by 3.67 and 5.21 folds under Vef5 and Vef7 stockings, respectively. Similarly, about six folds reduction in TOC was recorded in VEu10 and VEu15 vermibeds. In the end, the TOC in different feedstock was in the order: VEu10 = VEu15 < Vef7 = VEu5 < Vef5 < Vef15 < VEu7 < Vef10 = CP ($\text{LSD} = 0.71$; **Fig 5.2c**). Hence, the data suggest that the C-mineralization potential of *E. eugeniae* with high stocking density (10 and 15 worm kg^{-1}) was most significant. While low stocking density (5 and 7 worm kg^{-1}) of *E. fetida* showed

promising C-mineralization efficiency. Generally, the rate of TOC reduction during composting increases by about two times in the presence of earthworms due to efficient modification of the substrate condition [11]. Therefore, the results of the present study imply that a lower stocking density of *E. fetida* probably facilitated the earthworm biophysical activity, thereby accelerating the substrate-induced microbial respiration in the vermibeds. On the other hand, increasing the stocking density of *E. eugeniae* promoted microbial proliferation, thereby resulting in an increment of C-mineralization vis-à-vis carbon loss in the substrates. This could be validated by the results of the PLFA assay described in 3.3. In contrast, humification was more pronounced than mineralization in *E. fetida*-mediated feedstocks. The balancing effect of *E. fetida* on humification-mineralization kinetics has been reported earlier [23]. This could be convincingly appreciated from the data on the humification factor (HF) (**Fig 5.2d**). HF is the ratio of aliphatic and aromatic compounds that indicates the maturity of the composting process. The HF of 30 days was generally higher in all the feedstocks, implying aliphatic compounds dominance over aromatics. The dominance of aliphatic compounds signifies active mineralization. On the other hand, a sharp decrease in HF between 30 to 60 days in all feedstocks implies that the formation of humified carbonaceous compounds was highly active (Das et al., 2020), implying maturation of the decomposition process ($P_{\text{day}} < 0.001$). Significantly low HF was recorded in VEu5, VEf5, and VEf10 at 60 days in the present study (**Fig 5.2d**; $P_{\text{treatment}} < 0.001$; $\text{LSD}=0.53$). In particular, the HF in VEu5 and VEf5 was significantly lower than the CP feedstock at the end (i.e., 60th day). The maturity of organic matter decomposition leads to the formation of stable obstinate humic substances [10]. Hence, low initial stocking density (i.e., 5 and 7 worm kg⁻¹) of both earthworms (*E. fetida* and *E. eugeniae*) could significantly escalate their decomposition and stabilization efficiency during vermicomposting the lignocellulosic feedstocks.

The profuse availability of essential nutrients (N, P, and K) is a vital feature of waste valorization. Hence, the temporal dynamics of N, P, and K availability were studied (**Fig 5.2e-5.2g**). The TKN levels were enhanced by 1.53-2.46 and 1.86-2.33 folds in *E. fetida*, and *E. eugeniae* mediated vermibeds, respectively (**Fig 5.2e**). The two-way ANOVA also revealed that the interaction effects of time (i.e., day) and treatment (i.e., stocking density \times time) were strongly significant under vermicomposting (P_{days} and $P_{\text{treatment}} < 0.001$). Interestingly, the extent of N-increment was remarkably higher in

VEf10, VEu10, VEf5, and VEu7 followed by VEu15, VEf15, VEu5, and VEf7 ($P_{\text{treatment}} < 0.001$; $LSD=0.58$). Yadav and Garg (2016) reported that N increment in vermibeds was directly proportional to earthworm stocking density. Contrarily, the present investigation's result connoted that lowering the stocking density of earthworms up to 5 or 7 worm kg^{-1} is a feasible option without comprising the N-enrichment potential of vermicomposting systems. Although microbe-associated enzyme activity plays a pivotal role in the enhancement of N-mineralization, excretory products and physical activity of earthworms also influence the N-mineralization dynamics in vermicomposting systems [24]. Moreover, N-mineralization during vermicomposting should not be significantly compromised unless microbial growth is severely affected due to the low initial stocking density of earthworms. The bioavailability of P and K also has dramatically increased under vermicomposting, with significant variations regarding the initial stocking density of earthworms (**Fig 5.2f and 5.2g**). The P availability increased by 8.36-13.52 folds and 9.62-15.09 folds in *E. fetida* and *E. eugeniae* mediated vermibeds, respectively. In the end, P availability was significantly high in VEu15, followed by VEf15, VEu10, VEf10, and VEu5 ($LSD= 13.76$). The K enrichment was, however, most spectacular in VEu15, followed by VEu10, VEu7, and VEf10 ($LSD=15.15$). These data imply that increasing stocking density might positively impact P and K escalation during vermicomposting. However, satisfactory impacts of low stocking density (i.e., 5 and 7 worm kg^{-1}) were also evidenced regarding P and K availability (**Fig 5.2f and 5.2g**). Generally, substrate pH, feedstock friability, microbial growth, and microbe-associated enzyme activity are the key regulating factors for P and K bioavailability in composting systems (Hussain et al., 2018). Therefore, low initial stocking density has not adversely affected the P and K mineralization process under vermicomposting systems.

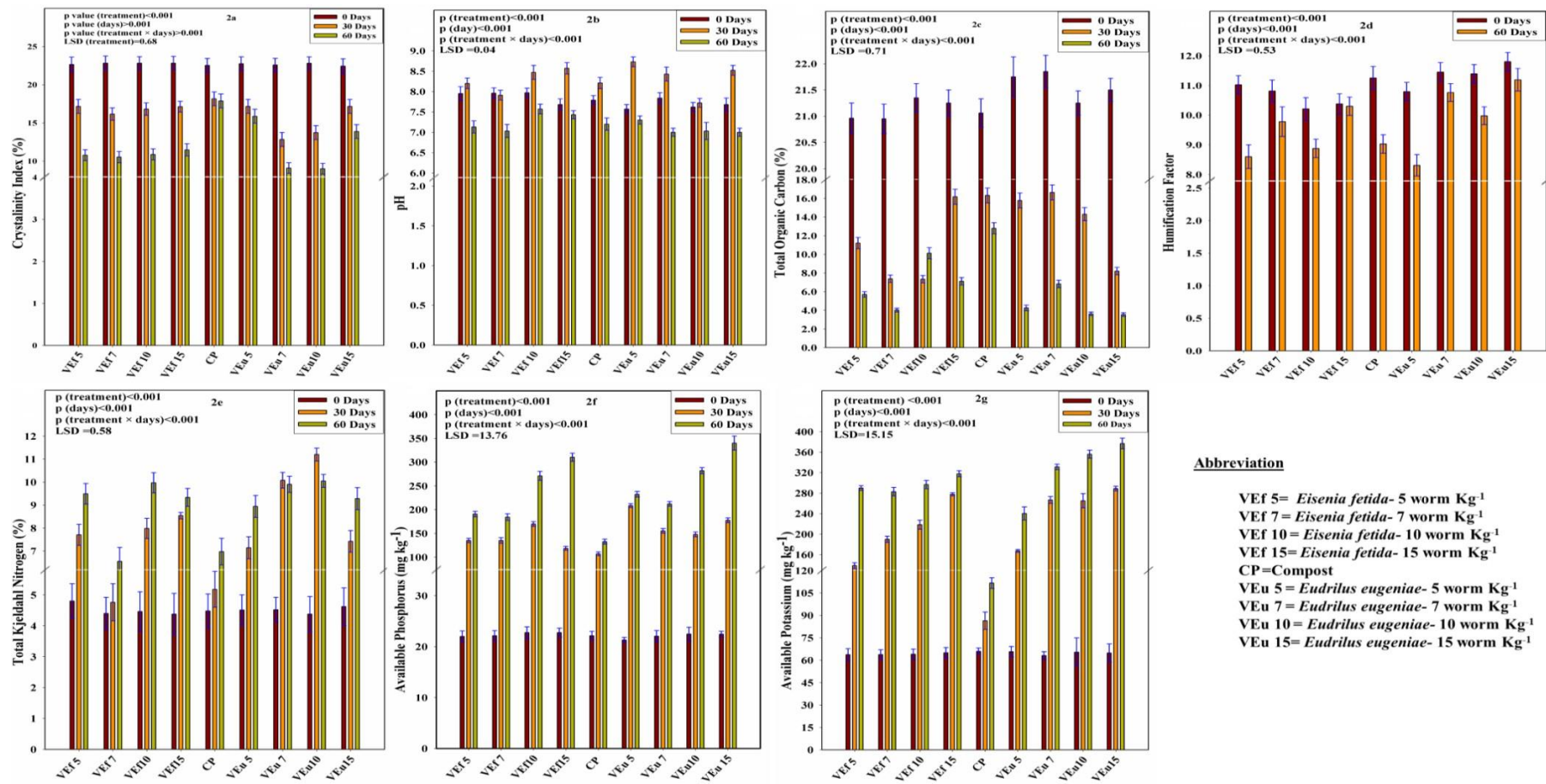


Fig 5.2: Changes in Crystallinity index (2a), pH (2b), Total organic carbon (2c), Humification factor (2d), Total Kjeldahl nitrogen (2e), Available Phosphorus (2f), and Potassium (2g) under different treatments (initial stocking densities of earthworms) during composting and vermicomposting of lignocellulosic waste-based feedstocks. Values represent mean ± standard deviation

Microbial metabolism and community structure: PLFA-based assessment

The microbial metabolic activity during vermicomposting was primarily studied based on microbial biomass Carbon (MBC), compost respiration (CR), and microbial quotient (MQ) (**Fig 5.3a-5.3c**). In general, the magnitude of increment in MBC was more spectacular under vermicomposting than aerobic composting (**Fig 5.3a**). Interestingly, the pattern of MBC increment in response to initial worm density was distinguishably different between the two earthworm species. For *E. fetida*, the MBC was highest for VEf5, followed by VEf10, VEf7, and VEf15 at 60 days; for *E. eugeniae*, the MBC at the final stage was in the order: VEu15>VEu10>VEu7>VEu5 (P_{treatment}<0.001; LSD=17.84; **Fig 5.3a**). Earthworms facilitate the augmentation of microbial biomass in the vermibeds via the release of their intestinal microorganisms and their excreta [25]. As such, an increase in earthworm density is expected to increase the microbial population [26]. However, the proliferation of gut-associated microflora also depends on the earthworm feeding rate, which may not be directly proportional to the stocking density [27]. Therefore, the results of the present investigation suggest that greater stocking density might have induced population stress on *E. fetida*, thereby inhibiting microbial growth. On the contrary, the enhanced stocking density of *E. eugeniae* was considerably promising for microbial enrichment. A similar kind of species-wise differential response regarding the defense mechanism of earthworms has been reported in a recent study [19]. The microbial vis-à-vis compost respiration (CR) in different feedstocks correspondingly increased, and the rate of increment was remarkably higher between 30 to 60 days compared to 0 to 30 days (**Fig 5. 3b**). The process of CR estimation followed in this study reflects the extent of basal respiration in the feedstocks. Hence, the increment in CR explains the elevated activity of autochthonous microorganisms that play a pivotal role in countering metal toxicity. In the end, the CR in different feedstocks was in the order:

VEu15=VEu10>VEf15>VEf7=VEu7=CP>VEf10>VEu5>VEf5 (P for treatment<0.001; LSD =451). These results imply that the highest stocking density of earthworm (i.e., 15 worm kg⁻¹) greatly facilitated the development of autochthonous microflora in the feedstocks (**Fig 5.3b**). However, the remarkable increase of MBC in VEf5 resulted in the highest MQ under this treatment among all the *E. fetida*-mediated vermibeds (**Fig 5.3c**). In contrast, the MQ, the ratio of MBC to TOC, was significantly higher in VEu7 followed by VEu10 at 60 days as compared to all treatments (P_{treatment}<0.001;

LSD=3.62; **Fig 5.3c**); despite the MBC increment was most significant in VEu15. This may be due to a sharp reduction in feedstock TOC under 7 and 15 worm kg-1 stocking density of *E. eugeniae*. These data imply that C-mineralization (i.e., TOC reduction) and microbial profiles in lignocellulosic biomass-based vermibeds significantly alter depending on the earthworm species and their stocking density. Therefore, the investigation felt the necessity of studying the microbial community profiles in vermibeds under varying stocking densities of earthworms.

The PLFA-derived active microbial biomass (i.e., total PLFA) in the vermibeds and composting beds was in the order: VEu15> VEf5>VEu10>VEu7>VEu5= VEf7> VEf10>VEf15> CP ($P<0.001$; LSD = 131.2; **Fig 5.3d**). The lowest microbial loadings in the composting beds suggest that the presence of earthworms greatly augmented microbial growth in the vermibeds. Interestingly, the total PLFA data showed a similar pattern of MBC loads in the vermibeds. The PLFA profile explains the impacts of change in the immediate environment on the microbial community shift [28]. Thus, the total PLFA data indicates that although the enhanced stocking density of *E. eugeniae* facilitated microbial growth, the reduced stocking density of *E. fetida* was rather favorable for microbial augmentation in the feedstocks. In general, the dominance of Gram-positive bacteria (**Fig 5.3e**) over Gram-negative bacteria (**Fig 5.3f**) was evident in the vermibeds. However, it was interesting to note that the Gram-positive and Gram-negative bacterial population was higher in the vermibeds than the composting beds, and the Gram-positive population was remarkably augmented in VEu5 followed by VEu15 and VEf15 (**Fig 5.3e**). In general, the Gram-negative bacterial population was significantly greater in *E. eugeniae* mediated vermibeds than in *E. fetida*, and it was significantly high in VEu7 and VEu15 (LSD = 32.4; **Fig 5.3f**). The predominance of the Gram-positive bacterial population in the vermibeds indicates that the feedstocks were conducive for both earthworm species. Yet, the feedstock-induced stress was probably greater in *E. eugeniae* mediated feedstocks than the *E. fetida* because the predominance of Gram-negative bacterial communities greatly proliferates under stressful conditions owing to their high tolerance limit [29]. The growth of eukaryotes was significantly higher in the composting bed than in all the vermibeds. Still, the VEu15, VEf10, and VEu10 stocking densities also promoted eukaryote growth compared to other stockings (**Fig 5.3g**). The predominance of eukaryotes signifies that the condition of the composting bed was probably less aerobic than the vermibeds, which might induce the growth of pathogenic fungal species in the composting beds [30]. The data indicate that

high initial stocking densities of earthworms in vermibeds might also perturb the aerobic conditions. The anaerobes PLFAs were significantly more predominant in Vef5, followed by Vef7 and VEu15 vermibeds, than the others (**Fig 5.3h**). This implies that *E. eugeniae* facilitated the diversity of microbial communities at 15 worm kg⁻¹ stocking density while stocking densities of 5 and 7 worm kg⁻¹ of *E. fetida* favored the augmentation of anaerobes and other communities. The substrate suitability for the growth of earthworms and their gut-associated microbes is reflected in the diversity of microbial communities [15]. The growth of actinomycetes PLFAs was in the order: VEu7 > VEu15 > VEu10 > Vef5 > Vef7 > Vef10 > Vef15 > VEu5 > CP (P < 0.001; LSD = 47.5; **Fig 5.3i**). This data indicates that 7 and 15 worm kg⁻¹ stocking density of *E. eugeniae* was more beneficial than the others for promoting the actinomycetes population in the vermibeds. However, 5 and 7 worm kg⁻¹ stocking density of *E. fetida* also performed satisfactorily in augmenting actinomycetes communities. Actinomycetes expedite recalcitrant organic matter decomposition and are involved in N-fixation and release plant growth-promoting biomolecules [31].

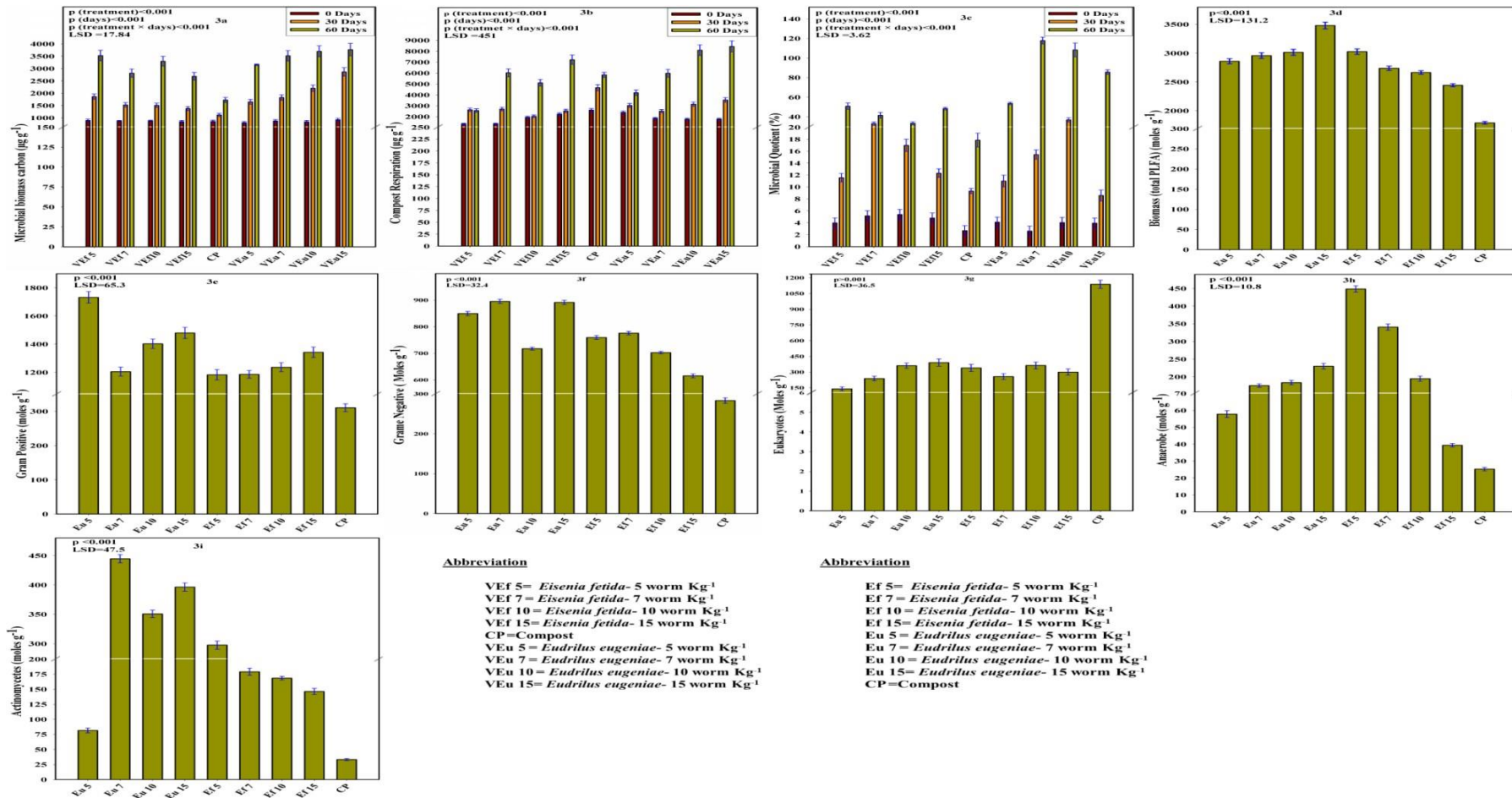


Fig 5.3: Variation in microbial community structure Microbial biomass carbon (3a), Compost respiration (3b), Microbial quotient (3c), Biomass (total PLFA) (3d), Gram-positive (3e), Gram-negative (3f), Eukaryotes (3g), Anaerobes (3h), and Actinomycetes (3i) in lignocellulosic waste-based feedstocks under different treatments (initial stocking densities of earthworms) during vermicomposting. Values represent mean ± standard deviation.

The profiles of fatty acids were also evaluated in the selected vermibeds (**Fig 5.4a-5.4g**). The relative abundance of straight-chain fatty acids was significantly high in VEu10, VEu15, VEu7, and VEf15 ($P < 0.001$; LSD = 4.3; **Fig 5.4a**); while the branched fatty acid PLFAs were remarkably higher in VEu5 followed by VEf15 compared to VEu7, VEf10, VEu15, VEf5, VEf15, and VEf7 ($P < 0.001$; LSD = 0.03; **Fig5. 4b**). A high abundance of straight-chain fatty acids indicates activation of in-situ (i.e., feedstock) bacterial communities [4]. On the other hand, the branched-chain fatty acids represent the sulfur-reducing anaerobic bacterial communities [32]. Hence, 15 worms kg⁻¹ stocking of both earthworms significantly induced the activity of the feedstock-borne microbial population. At the same time, the low abundance of branched-chain fatty acids in vermibeds reared with low stocking density (i.e., 5 and 7 worm kg⁻¹) of *E. fetida* signifies that aerobic condition was better ensured in such treatments as compared to others. Correspondingly, the relative abundance of mono-unsaturated fatty acids (MUFA) was higher in VEf7 and VEu7 vermibeds than in others (**Fig 5.4c**). The MUFAs are strong bioindicators of aerobic eukaryotes and prokaryotes [33]. Hence, low-density earthworm stockings could effectively maintain aerobic conditions in vermibeds. However, the abundance of polyunsaturated fatty acids (PUFAs), including the 18:1w9c, was more significant in *E. fetida*-mediated feedstocks than in *E. eugeniae* vermibeds (4d and 4e). Significantly high relative abundance of these PUFAs in VEf15, VEf10, VEf5, and VEf7 indicates that ectomycorrhizal fungal communities proliferated more in the presence of *E. fetida* than *E. eugeniae*. Interestingly, the occurrence of dimethylacetal fatty acids (DMA) was also significantly high in VEf5 and VEf7 vermibeds as compared to others ($P < 0.001$; LSD = 0.16; Fig 4f). The DMAs are components of the intestinal microflora of humans and some invertebrates [30]. Therefore, these fatty acids could be sourced from earthworm intestines. The relative occurrence of 10-Methyl branched fatty acids (BFAs) was considerably high in VEu7, followed by VEu15 and VEf5 vermibeds (Fig 4g). The BFAs are the signature biomolecules of actinobacteria and facilitate membrane stability [34]. Overall, the PLFA assay results suggest that the earthworm species and their varied stocking density strongly influence the microbial community structure and fatty acid profiles in vermicomposting systems.

Bioavailability patterns of potentially toxic elements (Mn, Zn, Cu, Pb, and Cd) in feedstocks

The data on the concentration of different studied metals are present in **Fig 5.5a-5.5e**. The Mn and Cd concentrations were considerably more significant than Zn, Pb, and Cu in the initial feedstocks. As such, Cd concentration in the initial feedstock was marginally higher than India's allowable limit for composted biomass [30]. Except for Zn, the levels of all the metals were substantially reduced in the feedstock due to composting and vermicomposting. The Zn bioavailability was inherently low in the feedstock, which may be the reason behind the increment of this element upon vermicomposting. The bioavailability of potentially toxic metals in the finished vermicomposts significantly alters depending on feedstock characteristics, initial concentrations, and earthworm activity [30]. At the end of the study period, Zn availability was significantly high in VEu10 and VEu15, followed by VEf15, CP, VEu5, and others ($P < 0.001$; LSD = 0.45; **Fig 5.5a**). Mn concentration was considerably high in the initial feedstock; which was significantly reduced in VEf5 (4.24 folds) and VEu5 (3.97 folds) feedstocks, followed by VEu15 (2.74 folds), VEu10 (2.01 folds), VEf15 (1.92 folds), and VEf10 (1.69 folds) ($P < 0.001$; LSD = 15.66; Fig 5b). The Cu, Cd, and Pb removal potential of *E. fetida* were more spectacular than *E. eugeniae*, irrespective of the initial stocking density (**Fig 5.5c-5.5e**). The Cd and Pb removal were highly significant in VEf5 (Cd: 12.6 folds; Pb: 12.75 folds), VEf15 (Cd: 581 folds; Pb: 102 folds), VEf10 (Cd: 3.8 folds; Pb: 306 folds), and VEf7 (Cd: 2.6 folds; Pb: 43.71 folds) as compared to the other vermibeds ($P < 0.001$; LSD: Cd = 0.23, Pb = 0.18). At the same time, Cu removal was remarkably high in VEf5 (31 folds), followed by VEu5 (10.3 folds). These results imply that removal efficiency for non-essential and highly toxic metals (Cd, Pb, and Cu) was prolific in vermibeds incubated with the lowest stocking density (5 worm kg⁻¹) of both earthworm species. The differential metal detoxification ability of *E. fetida* and *E. eugeniae* is a well-known phenomenon [35]. Specifically, *E. fetida* was more efficient in detoxifying toxic metals than *E. eugeniae* owing to their strong defense mechanism [19] and induced protein-mediated metal binding capacity in their body [19]. While *E. eugeniae* efficiently promotes the transformation of metals into insoluble forms by mobilizing the humification process in the substrates [6]. Therefore, the results of the current investigation implied that low initial stocking of *E. fetida* might have promoted their internal mechanism of metal detoxification. In contrast, the initial stocking density-dependent activation of metal removal efficacy of *E. eugeniae* was somewhat discrete.

Microbial profiles, community structure, C-dynamics, and metal bioavailability: Finding linkages using correlation statistics

The correlation analysis was performed with the finished vermicompost samples under selected treatments (**Table 5.1**). The exercise aimed to identify microbial activity's influence on metal bioavailability in the vermibeds treated with various stocking densities of two earthworm species. The MBC and CR had a strong positive correlation with Cu (MBC: $r = 0.489$, $P < 0.05$; CR: $r = 0.818$, $P < 0.01$) and Cd (MBC: $r = 0.498$, $P < 0.05$; CR: $r = 0.549$, $P < 0.05$). Strong positive correlation of MQ versus Zn ($r = 0.518$, $P < 0.05$), Cu ($r = 0.531$, $P < 0.05$), and Cd ($r = 0.553$, $P < 0.05$) was also evidence. Cu, Zn, and Cd concentrations considerably reduced due to vermicomposting, while microbial activity substantially increased over time. Therefore, such associations of MBC and CR with these potentially toxic elements imply that augmentation of microbial activity had a positive impact on metal reduction. MBC and CR strongly reflect the extent of metal-induced stress in the vermibeds [15]. However, Cd and Pb showed a strong negative correlation with TOC (Cd vs. TOC: $r = -0.574$, $P < 0.05$; Pb vs. TOC: $r = -0.535$, $P < 0.05$). TOC was also weakly but negatively correlated with Zn and Cu, while a strong positive correlation was recorded between TOC and Mn ($r = 0.703$, $P < 0.01$). Interestingly, a previous study showed that the amelioration of metal-induced stress is countered by the upregulation of microbial activity [15]. As a result, a robust reverse correlation of Cd and Pb with TOC was evidenced in the present investigation. However, Mn is comparatively less toxic than other trace elements, and oxide forms of Mn immobilize the non-essential harmful elements [36]. Therefore, the positive relation between TOC and Mn in the vermibeds was well understood. Interestingly, a strong reversed correlation of HF was observed with all the metals (r : HF vs. Pb = -0.550 , $P < 0.05$; HF vs. Mn = -0.684 ; HF vs. Zn = -0.634 ; HF vs. Cu = -0.632 ; HF vs. Cd = -0.669 , $P < 0.01$). The HF was substantially reduced in the vermibeds, implying enhancement of stable-humic compounds. Such compounds readily form complexes with the metals and reduce metal availability ([38]. Hence, the present data suggest that the elevation of aromatic compounds in the vermibeds was primarily responsible for the metal reduction. Correspondingly, a strong positive correlation of HF with total PLFA ($r = 0.604$, $P < 0.05$), Gram-negative ($r = 0.685$, $P < 0.01$), Gram-positive ($r = 0.563$, $P < 0.05$), Eukaryote ($r = 0.541$, $P < 0.05$), and anaerobes ($r = 0.497$, $P < 0.05$) suggests that proliferation of these microbial communities significantly promoted humification thereby facilitated immobilization of the toxic metals. Correspondingly, the strong negative correlation of

the total PLFA with Cd ($r = -0.664$, $P < 0.01$) was evidenced in the vermibeds. Moreover, the Gram-positive bacteria community was negatively correlated with all the studied metals (**Table 5.1**). The predominance of the Gram-positive bacteria community is a good indicator of sanitized composted products [4]. Thus, the correlation-based assessment revealed that microbial community structure and their activity significantly promoted humification of the feedstock in the presence of earthworms, facilitating metal immobilization vis-à-vis removal during vermicomposting.

Table 5.1: Pearson Correlation coefficients (r) and level of significance depicting the relationships among microbial attributes in vermibeds

Parameter	MBC	CR	TOC	HF	MQ	Microbial biomass (Total PLFA)	Gram Negative	Eukaryote	Gram Positive	Anaerobe	Actinomycetes
MBC	1										
CR	0.653**	1.000									
TOC	-0.362	-0.040	1.000								
HF	-0.449	-0.084	0.207	1.000							
MQ	0.565*	0.386	-0.889**	-0.321	1.000						
Microbial biomass (Total PLFA)	0.508*	0.143	-0.709**	0.604*	0.697**	1.000					
Gram Negative	0.212	0.037	0.517*	0.685**	0.551*	0.769**	1.000				
Eukaryote	0.095	0.369	-0.122	0.541*	0.140	0.465	-0.138	1.000			
Gram Positive	0.374	0.086	-0.699**	0.563*	0.756**	0.159	0.252	-0.415	1.000		
Anaerobe	-0.599**	-0.411	0.086	0.497*	-0.307	0.372	0.116	0.472*	-0.634**	1.000	
Actinomycetes	0.253	0.248	-0.099	-0.309	0.156	0.670**	0.554*	0.540*	-0.437	0.356	1.000
Lead	0.209	-0.271	-0.535*	-0.550*	0.321	0.051	0.088	-0.633**	-0.605**	-0.381	-0.236
Manganese	-0.034	0.326	0.703**	-0.684**	-0.522*	-0.307	0.055	-0.562*	-0.578*	0.042	0.223
Zinc	0.516*	0.764*	-0.256	-0.634**	0.518*	0.210	0.107	0.212	-0.574*	-0.543*	0.058
Copper	0.489*	* 0.818*	-0.158	-0.632**	0.531*	0.357	0.161	0.481*	-0.073	-0.542*	0.611*
Cadmium	0.498*	* 0.549*	-0.574*	-0.669**	0.553*	-0.664**	0.018	0.121	-0.597*	0.139	0.553*

*P < 0.05; **P < 0.01

Conclusions

Uniform vermibeds were used to specifically assess the impact of variable earthworm stocking density on microbial community structure. Low stocking density promoted earthworm population increment, which escalated their comminuting efficiency as the feedstock crystallinity significantly reduced under thinner stocking. The NPK-mineralization-C-humification balance was also optimized under thinner worm stocking. Interestingly, PLFA-derived microbial community profiling revealed that denser stocking of *Eudrilus* and thinner stocking of *Eisenia* was most effective in augmenting microbial diversity, which strongly regulated the metal remediation potency of vermicomposting systems. Overall, 5-7 worm kg⁻¹ stocking density of earthworms was optimum to produce nutrient and microbe-enriched sanitized vermicompost.

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