Potential of Effective micro-organisms and *Eisenia fetida* in enhancing vermi-degradation and nutrient release of fly ash incorporated into cow dung – paper waste mixture

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Abstract

The interactions between earthworms and microorganisms activity has prompted several researchers to evaluate the potential of artificially inoculating vermicomposts with additional specific microbes, with the intention of enhancing the vermicomposting process. This study evaluated the potential of inoculating fly ash (F)-cow dung-paper waste (CP) mixture (F-CP) with a specialized microbial cocktail called Effective micro-organisms (EM) during vermicomposting using *Eisenia fetida* earthworms. Inoculation with EM alone did not result in significantly ($P > 0.05$) different changes in C/N ratio and dissolved organic matter (DOC) compared to the control with no EM and *E. fetida*. A significant interaction between EM and *E. fetida* presence resulted in greater changes in C/N ratio and DOC, which were not statistically different from the *E. fetida* alone treatment. It was also noteworthy that the activity of β – Glucosidase was not influenced by the presence of EM, but was significantly influenced ($P = 0.0014$) by the presence of *E. fetida*. However, the EM + *E. fetida* treatment resulted in a rate of weekly Olsen P release of 54.32 mg kg^{-1} which was 12.3%, 89.2% and 228.0% more than the *E. fetida* alone, EM alone and control treatments, respectively. Similarly, though higher in the *E. fetida* plus EM treatment, the phosphate solubilizing bacteria counts were not significantly different ($P > 0.05$) from the *E. fetida* alone treatment. It is concluded that inoculation of F-CP composts with EM alone may not be beneficial, whilst combining EM with *E. fetida* results in faster compost maturity and significantly greater Olsen P release. It would be interesting to evaluate higher optimized rates of EM inoculation and fortifying EM cocktails with phosphate solubilizing bacteria (PSB) on F - CP vermicompost degradation and phosphorus mineralization.

Keywords: Extractable phosphorus, phosphate solubilizing bacteria, enzyme activity, microbial activity, dissolved organic carbon.

1. Introduction

Vermicomposting has in the recent years been adopted over traditional composting, as a faster and environmentally friendly method of creating organic fertilizer from organic waste (Lazcano et al., 2008). Traditional composting generally relies on naturally occurring microbes and thermophilic temperatures to decompose and stabilize organic waste. This greatly slows the stabilization and maturation process during composting together with loss of essential nitrogen as ammonia during the thermophilic stage, thus reducing the quality of the resultant organic fertilizer (Lazcano et al., 2009). The earthworm species *Eisenia fetida* has been observed to be most suited for vermicomposting due to its wide tolerance to variations in composting environment (Dominguez, 2011). During vermicomposting, earthworm species like *Eisenia fetida* have been shown to mechanically fragment waste material with their gizzards consequently increasing substrate surface area, thus positively altering biological activity during vermicomposting and enhancing bio-degradation (Hong et al., 2011; Gomez-Brandon et al., 2013). Also, the endo-symbiotic micro-organisms within the earthworm intestines produce hydrolytic enzymes that degrade cellulose and phenolic compounds that enhance the degradation of material ingested by earthworms (Dominguez, 2011). The ability of earthworms to enhance microbial activity, enzyme activity and mineralization in composts, has enabled them to be used to enhance mineralization of inorganic materials like rock phosphate and fly ash resulting in higher nutrient availability (Unuofin and Mnkeni, 2014; Bhattacharya and Chattopadhyay, 2002).

Due to the link between earthworm activity and microbe activity, several researchers have evaluated the potential of enhancing the composting - microbe community by enriching both composts and vermicomposts with additional microbes, with the intention of improving the

composting process (Mupondi et al., 2006; Busato et al., 2012). Kumar and Singh (2001) enriched a vermicompost made from chopped stalk and leaves of pearl millet and rock phosphate, with nitrogen fixing microbes (*Azotobacter chroococcum* and *Azospirillum lipoferum*) and phosphate solubilizing *Pseudomonas striata*. They reported an enhanced phosphate solubilization from the rock phosphate and enhanced nitrogen fixation in the vermicomposts. Kumar et al. (2010) treated a compost with a combination of *Pleurotus sajorcaju*, *Trichoderma viridae*, *Aspergillus niger* and *Pseudomonas striatum* 30 days prior to the vermicomposting process and observed an overall reduction in composting period by 20 days due to accelerated degradation together with a nutrient rich compost. Mupondi et al. (2006) enriched a pine bark – goat manure compost with a special cocktail of microbes called Effective micro-organisms (EM) hoping to improve the composting process. However, contrary to expectations, inoculation with EM alone did not significantly influence the composting of pine bark mixed with goat manure.

According to the Japanese inventors of the technology, EM is a cocktail of natural and beneficial microbial populations of lactic acid bacteria, yeasts, actinomycetes and photosynthetic bacteria, living symbiotically (Yamada and Xu, 2001). Anecdotal evidence suggests that EM is a cheap way for enhancing the compost biodegradation process, reducing odour and ammonia volatilization (Di-fa et al., 2011). At present, there is paucity of information on the interactions between *E. fetida* activity and EM on changes in vermicompost chemical and biological properties that determine compost maturity. It would be important to evaluate the potential of the microbial inoculants in enhancing the vermicomposting process of inorganic waste materials like coal fly ash incorporated into an organic base of cow dung and paper waste. This study therefore sought to evaluate the effects of (i) EM inoculation and *E. fetida* presence during F - CP vermicomposting on

vermicompost chemical and biological activity, and (ii) EM inoculation and *E. fetida* presence during F – CP vermicomposting on extractable phosphorus and nitrogen release.

2. Materials and methods

2.1. Materials for composting

This study was carried out at the University of Fort Hare Alice campus vermicomposting research unit, Eastern Cape Province; South Africa (32°46' S and 26°50'E). Cylindrical plastic vermireactors measuring 0.30 m (top diameter) × 0.23 m (height) and 0.21 m base (diameter) with a total volume of 0.015 m³, having perforations underneath were used for the vermicomposting process. Cow dung was collected from a mainly pasture based dairy farm in Keiskammahoek, located about 60 km away from Alice. The cow dung was crushed to remove large lumps, air dried, and stored under shed in a dry area. Shredded paper waste was obtained from the University of Fort Hare, Alice campus. The fly ash sample used for the study was obtained from Matla power station (26° 16' S and 29° 08'E), which is located in Mpumalanga Province in South Africa. Mature earthworms (*Eisenia fetida*) which had been feeding mainly on cow dung and paper waste collected from our local wormery at the University of Fort Hare were used. The selected chemical properties of the cow dung, fly ash and paper waste used in this study are shown in Table 1. Cow dung was mixed with paper waste to give an appropriate C/N ratio of 30 ± 1 for effective vermicomposting (Unuofin and Mnkeni, 2014). The fly ash was then incorporated into the cow dung – paper waste mixtures at a F/ CP ratio of 1: 2 (w/w basis) as recommended by Mupambwa and Mnkeni (2015). The EM was ordered from a local South African supplier (Lindoros Whole Earth Consultants; <http://lindros.co.za/effective-micro-organisms>).

[Insert Table 1 here]

2.2. Treatments and experimental design

The experimental treatments were factorial combinations of two factors i.e. EM presence (with or without) and *E. fetida* presence (with or without). This gave four treatment combinations (i.e. EM only; EM plus *E. fetida*; *E. fetida* only and control with no EM or *E. fetida*) which were applied to fly ash mixed with an optimized mixture of cow dung and paper waste at a F/ CP ratio of 1: 2 (w/w basis) found to be ideal for fly ash vermicomposting by Mupambwa and Mnkeni (2015). Prior to application of the treatments, the F - CP mixtures corresponding to the different treatments were placed in vermireactors as described in Mupambwa and Mnkeni (2015), moistened to $60 \pm 5\%$ and allowed to pre-compost for 1 week to eliminate volatile toxic gases which could be harmful to the earthworms (Mupambwa and Mnkeni, 2015). Following this 1 week pre-composting period, the earthworms and EM was then added to their respective vermi-reactors. . Earthworms were introduced into *E. fetida* presence vermireactors at a stocking density of 25 g-worm per kg dry weight of F – CP compost determined to be an ideal *E. fetida* stocking density for F – CP vermicomposting by Mupambwa (2015). For EM presence treatments, EM was inoculated at 8 L m^{-3} based on manufacturer's recommendations (Lindoros Whole Earth Consultants, South Africa); with a total of 4 kg (dry weight) material of F-CP being used. The treatments were replicated three times and laid out in a completely randomized design. The experiment was undertaken within an enclosed room with no direct sunshine whilst enabling free circulation of air. Samples were collected at 0, 1, 2, 3, 4, 6, 8 and 10 weeks during the composting period and analysed for various chemical and biological parameters as described in section 2.3.

2.3. Chemical properties

2.3.1. C and N determination

Total C and N was determined using the dry combustion method and analysed using a LECO TruSpec C/N auto analyzer (LECO Corporation, USA).

2.3.2. DOC

Dissolved organic carbon was determined on a 5 g (fresh weight) homogenous sample extracted with 50 mL of 0.5 M K_2SO_4 . The samples were shaken on a reciprocating shaker for 30 mins at 160 rpm and the suspension was then filtered using Whatman number 42 filter paper (Jones and Willet, 2006). The concentration of DOC was determined colorimetrically using a Skalar continuous flow analyser (San 2++ Skalar CFA, Skalar Analytical B.V. The Netherlands).

2.3.3. Olsen extractable P

A solution of 0.5 M Sodium bicarbonate which had been adjusted to pH of 8.5 using 1 M Sodium hydroxide was used for extraction. 2.5 g sample of compost was extracted with 50 mL of the 0.5 M $NaHCO_3$ solution on a horizontal shaker for 0.5 h (Schoenau and O'Halloran, 2006). After shaking, the suspensions were filtered through Whatman™ No. 42 filter paper and the filtrates were then adjusted to pH 5 with 2.5 M sulphuric acid before analysis. The orthophosphate in the extracts was automatically determined using a continuous flow analyser (San 2++ Skalar CFA, Skalar Analytical B.V. The Netherlands) employing the ammonium molybdate – antimony potassium tartrate – ascorbic acid method.

2.3.4. Exchangeable ammonium and nitrate and nitrite

Inorganic nitrogen NH_4 (N), NO_3 (N) and NO_2 (N) were extracted using 50 mL of 0.5 M potassium sulphate (1:10 w/v). 5 g homogenous sample was weighed into a 150 mL plastic bottle and shaken on a reciprocating shaker for 30 mins at 160 rpm. The suspension was then filtered using Whatman™ No. 42 filter paper (Maynard et al., 2006). The concentration of ammonium was determined based on the Berthelot reaction involving salicylate whilst the concentration of nitrate and nitrite was determined based on the cadmium reduction method; all done automatically on a continuous flow analyser (San 2++ Skalar CFA, Skalar Analytical B.V. The Netherlands).

2.4. Enzyme activity

2.4.1. β -Glucosidase

The β -Glucosidase activity was measured based on the colorimetric determination of *p*-nitrophenyl released when a compost is incubated with *p*-Nitrophenyl - β - D- glucoside (PNG) at pH 6 (Deng and Popova, 2011). 1 g (fresh weight) compost sample was mixed with 0.2 mL of toluene, 4 mL of buffer solution and 1 mL of PNG and then incubated for 1 hour. A control with no PNG was also included for each respective sample. After incubating for one hour, 1 mL of 0.5 M CaCl_2 and 4 mL of 0.1 M of tris (hydroxymethyl) aminomethane (THAM) was added to all samples, with the PNG solution being added to the control samples at this time. The samples were then filtered using Whatman No.2 filter paper and the yellow colour intensity of the filtrate was measured using a Helios Thermo Spectrophotometer (Thermo Fisher Scientific, England) at 405 nm. The activity of β -Glucosidase was determined based on the μmol of *p*-nitrophenol released by reference to a calibration curve

developed with standards containing *p*-nitrophenol. The β -Glucosidase activity (μmol of *p*-nitrophenol g-dry weight compost⁻¹ hour⁻¹) was calculated as shown in equation 1 below:

$$\beta - \text{Glucosidase} = \frac{S-C}{DM} \quad \text{Eq. (1)}$$

Where: **S** is mean concentration of *p*-nitrophenol in the sample; **C** is concentration of *p*-nitrophenol in the control; **DM** is the dry mass of the compost (determined based on moisture content).

2.4.2. Alkaline phosphatase

Alkaline phosphatase activity was determined based on the colorimetric estimation of the *p*-nitrophenol released by phosphatase activity when a substrate is incubated with buffered sodium *p*- nitrophenyl phosphate solution at pH 11 and toluene (Acosta-Martinez and Tabatabai, 2011). 1 g (fresh weight) compost sample was mixed with 0.2 L of toluene, 4 mL of Modified Universal Buffer (MUB) at pH 11 and 1 mL of *p*-nitrophenol phosphate (PNP) solution. A control with no PNP was also included for each respective sample. All the samples were then incubated at 37 °C for 1 hour, following which 1 mL of 0.5 M of CaCl₂ and 4 mL of 0.5 M of NaOH was added, with the PNP being added to the controls at this moment. The suspensions were then filtered through Whatman No.2 filter paper and the yellow intensity of the filtrate measured using a Helios Thermo Spectrophotometer (Thermo Fisher Scientific, England) at 410 nm. The alkaline phosphatase activity was expressed as μg

of p-nitrophenol released per g-dry weight compost per hour, determined based on a reference calibration curve and calculated as shown in equation 2 below.

$$\text{Alkaline phosphatase} = \frac{S-C}{DM} \quad \text{Eq. (2)}$$

Where: **S** is mean concentration of p-nitrophenol in the sample; whilst the other parameters are similar to Eq. 1.

2.5. Microbiological analysis

Samples collected during the vermicomposting process were analysed for microbiological counts of total bacteria, total fungi, *E. coli* and phosphate solubilising bacteria (PSB), using the serial dilution and standard spread plate counting method (Germida and Freitas, 2006). 1g (fresh weight) sample from each treatment was suspended in 10 mL of sterile distilled water and shaken for 1 min on a rotary shaker and serial dilutions were prepared up to 10^{-5} . All samples were assayed by dilution with three replicates of each suspension.

Nutrient Agar was used for total bacterial counts with a serial dilution of 10^{-5} by pour plating and these were incubated at 37 °C for 24 h. The membrane faecal coliform agar (m-FC agar) was used for *E. coli* counts, with a serial dilution of 10^{-3} and incubation temperature of 44 °C for 24 h. For PSB counts, the Pikovskaya's Agar was used, with a serial dilution of 10^{-4} and an incubation period of 3 – 5 days at 30 °C. Rose Bengal Chloramphenicol agar was used for the determination of total fungi with a serial dilution of 10^{-3} and incubation at 30 °C for a period of 3 – 5 days. Following the incubation period, counts were made for number of

colony forming units (CFU) and these were expressed on a dry weight basis (Germida and Freitas, 2006).

2.6. Statistical analysis

Sampling was not destructive and thus data were analysed using repeated measures analysis of variance (ANOVA). Where sphericity assumptions could not be met, the Greenhouse-Geisser correction of P was used. All data was analysed using JMP version 11.0.0 statistical software (SAS Institute, Inc., Cary, North Carolina, USA, 2010).

3. Results and discussion

3.1. Effects of *E. fetida* and EM inoculation on C/N ratio and DOC

In this study, following 10 weeks of vermicomposting, the C/N ratio generally decreased for all treatments including the control (Fig. 1A). However, the presence of *E. fetida* and EM resulted in a significant overall decrease in C/N ratio ($P < 0.0001$, Table 2), as shown by the low final average C/N value of 12.25 for the *E. fetida* only and *E. fetida* + EM treatments relative to 22.03 for the control treatment. The presence of *E. fetida* together with EM inoculation resulted in a significant decrease in C/N ratio compared to the EM alone treatment, as indicated by a significant *E. fetida* presence $\times$ EM presence interaction ($P = 0.0076$, Table 2). This observed interaction between *E. fetida* and EM enhanced microbial activity resulting in a significant decrease in the C/N ratio which indicated faster degradation of organic matter (Mupambwa and Mnkeni, 2015). A C/N ratio of less than 15 has been recommended as one of the major characteristics of a mature compost (Bernal et al., 2009). Generally, the treatment with *E. fetida* and EM showed non-significantly lower ($P > 0.05$)

C/N values compared to the *E. fetida* treatment with no EM. It was, however, noteworthy that inoculation with EM together with *E. fetida* presence resulted in much faster compost maturity, taking around 6 – 7 weeks, slightly faster than *E. fetida* only. Di-fa et al. (2011) reported similar results, with the interaction between EM and *E. fetida* enhancing organic matter losses and this was attributed to the potential of EM in enhancing the living environment for earthworms. The EM only treatment showed a non-significant but lower C/N values across the 4 to 10 week period of vermicomposting compared to the control. These results support the idea that although micro-organisms are the main agents responsible for organic matter decomposition, earthworms play a much more critical role and their contribution to decomposition may be more direct through their digestive processes (Aira et al., 2006). Dominguez (2011) also suggested the vermicomposting process involves the bio-oxidation and stabilization of organic matter through the joint action of earthworms and microorganisms to transform its physicochemical and biochemical properties. The significant change in C/N ratio with time for all treatments with *E. fetida* also resulted in a significant interaction between *E. fetida* $\times$ time ($P < 0.0001$, Table 2).

[Insert Table 2 here]

[Insert Figure 1 here]

For all treatments, DOC generally increased from 0 to 4 weeks and subsequently decreased until 10 weeks at different intensities ($P < 0.05$, Fig. 1B). The degradation of solid polymeric material at the beginning of the composting process leads to the formation of high quantities of soluble organic matter (Gomez-Brandon et al., 2008) which could explain the increase in

DOC concentration observed during the first 4 weeks. The subsequent decrease in DOC concentrations could be due to the depletion of the original solid polymeric materials in the compost due to degradation and has been observed to be highly correlated to changes in total carbon in composts (Gomez-Brandon et al., 2008). Similar to changes in C/N ratio, the effect of EM was highly dependent on the presence of *E. fetida* as shown by the significant interaction between *E. fetida* presence $\times$ EM presence ($P = 0.0034$, Table 2). The DOC was highest in the EM only treatment followed by the control treatment, whilst the *E. fetida* only and *E. fetida* + EM treatments had the lowest DOC concentration, particularly from 4 to 10 weeks ($P < 0.05$, Fig. 1B). After 10 weeks of vermicomposting, the treatments EM only; control; *E. fetida* + EM and *E. fetida* only; had final DOC concentrations of 5.73, 5.02, 1.82 and 1.67 mg kg⁻¹, respectively. Zmora-Nahum et al. (2005) reported that a final DOC concentration of < 4 mg g⁻¹ signified a mature compost; with this study revealing that the presence of *E. fetida* played a critical role in compost maturation with or without EM.

3.2. Effects of *E. fetida* and EM inoculation on changes in extractable phosphorus and nitrogen

Phosphorus is one of the most important nutrients in fly ash with potential to improve fertilizer value in composts (Mupambwa et al., 2015). In this study, both EM and *E. fetida* presence significantly influenced changes in Olsen extractable phosphorus ($P < 0.001$, Table 2). It was noteworthy that throughout the entire 10 weeks of composting, the *E. fetida* + EM treatment showed the highest significant ($P < 0.05$) ability to increase Olsen P in the F – CP compost (Fig. 2), also indicated by the significant interaction between *E. fetida* $\times$ EM presence ($P = 0.008$, Table 2). On average, the ability of the different treatments in increasing Olsen extractable P followed the order *E. fetida* + EM $>$ *E. fetida* only $>$ EM only $>$ control;

resulting in a respective increases of 43.8%, 33.0% and 16.3% in final Olsen P concentrations after 10 weeks of composting relative to the control ($P < 0.05$, Table 3). The influence of EM inoculation was more pronounced on the changes in Olsen P with or without *E. fetida*. It is plausible to attribute the higher Olsen P concentration in the composts relative to the control to the activity of phosphate solubilizing bacteria (PSB) together with earthworm gut microbe's activity as reported by Nair and Okamitsu (2010) and Gomez-Brandon and Dominguez (2014). Similarly, using cow dung-sunflower cake mixtures incorporated with rock phosphate, Busato et al. (2012) observed significantly higher levels of labile phosphorus forms where phosphate solubilizing bacteria (PSB) had been included compared to the control with no PSB.

[Insert Figure 2 here]

[Insert Table 3 here]

Though inorganic nitrogen is not an important nutrient to consider during F – CP composting, the presence of fly ash and *E. fetida* during composting have been observed to affect the rate of nitrogen mineralization (Mupambwa and Mnkeni, 2015). The incorporation of EM and *E. fetida* significantly influenced the rate of change in nitrate/nitrite concentration in the composts ($P < 0.01$, Table 2), with no interactions between *E. fetida* and EM presence. The increase in nitrate/nitrite concentration during composting in all treatments showed a significantly different and almost linear increase until 10 weeks, hence the significant influence of time observed ($P < 0.0001$, Table 2). Beyond 4 weeks, it was noted that the *E. fetida* + EM treatment showed a consistently higher though non-significant nitrate concentration compared to the *E. fetida* alone treatment, whilst the EM only treatment

showed a significantly higher nitrate concentration compared to the control (Fig. 3A). After 10 weeks of vermicomposting, the nitrate/nitrite concentration followed the order *E. fetida* + EM > *E. fetida* only > EM only > control with significant differences ($P < 0.05$) between the treatments. The treatments *E. fetida* + EM; *E. fetida* alone and EM alone, resulted in 5.3, 4.4 and 1.8 times, respectively, more nitrate/nitrite concentration compared to the control. The superiority of the treatments with *E. fetida* presence in enhancing nitrate/nitrite release was possibly due to the earthworm gut associated processes taking place as the study of Gomez-Brandon and Dominguez (2014) seem to suggest. The presence of *E. fetida* significantly ($P < 0.0001$, Table 2) influenced ammonium concentration within the composts, whilst EM presence did not show an impact on the concentration. Generally, the ammonium concentration increased in all treatments with no consistent trend being observed between treatments (Fig. 3B). Though positive, the benefits of EM inoculation on increasing the nitrate/nitrite and decreasing the ammonium concentration within the F – CP composts were not significantly different from those with no EM.

[Insert Figure 3 here]

3.3. Effects of *E. fetida* and EM inoculation on enzyme activity

During vermicomposting, it is well known that most biochemical reactions occurring like C, N and P mineralization are catalysed by both intracellular and extracellular enzymes. These enzymes are the main mediators of the different depredating processes during vermicomposting (Tiquia et al., 2002). In this study two of the most important enzymes, β -Glucosidase and alkaline phosphatase, directly involved in carbon and phosphorus turnover respectively, during composting (Benitez et al., 1999) were studied. Except for the *E. fetida* +

EM treatment, β – Glucosidase gradually increased until 6 weeks and decreased thereafter with varying intensities (Fig. 4A). The activity of β - Glucosidase showed a decreasing trend beyond 2 weeks for the *E. fetida* + EM treatment, representing faster decrease in organic matter as supported by the significant correlation between β - Glucosidase and changes in C/N ratio and DOC ($r > 0.5$, $P < 0.0001$). It was noteworthy that the activity of β – Glucosidase was not influenced by the presence of EM alone treatment, but was significantly influenced ($P = 0.0014$, Table 4) by the presence of *E. fetida*. However, inoculation with EM seemed to introduce more microbes, which significantly interacted with earthworm activity to result in the faster organic matter decomposition in the EM + *E. fetida* treatment. The activity of β - Glucosidase has been observed to be a sensitive indicator of organic matter decomposition (Kayikcioglu and Okur, 2011); thus as the organic matter decreased, the activity of this enzyme consequently decreased.

[Insert Table 4]

[Insert Figure 4]

Alkaline phosphatase activity showed a much more consistent trend with the control treatment showing significantly ($P < 0.05$) the lowest activity throughout the 10 weeks whilst the EM + *E. fetida* treatment showed the highest (Fig. 4B). The activity of alkaline phosphatase was significantly influenced ($P < 0.0001$, Table 4) by both *E. fetida* and EM presence, with the effects of EM inoculation being influenced by the activity of *E. fetida* as shown by the significant interaction ($P = 0.0031$, Table 4) between EM inoculation and *E. fetida* presence. The average alkaline phosphatase activity throughout the 10 weeks of composting was 1453.9, 1340.6, 1087.1 and 823.5 $\mu\text{g} - \text{PNP g-dry weight}^{-1} \text{ h}^{-1}$ for the *E.*

fetida + EM; *E. fetida* only, EM only and control treatments, respectively, representing an average 76.6%, 62.8% and 32.0% difference respectively, compared to the control. The decline which was observed in alkaline phosphatase with time has been speculated to be due to the feedback inhibition of the enzyme by inorganic phosphate (Gomez-Brandon et al., 2008). The alkaline phosphatase activity showed no correlation with Olsen P as expected and contrary to other researcher's reports (Busato et al., 2012; Benitez et al., 1999). This may indicate that the activity of this enzyme depends on many factors with both intra and extra-cellular located enzymes contributing to the measured activity (Aira et al., 2007).

3.4. Effects of *E. fetida* and EM inoculation on total microbial counts in fly ash compost

During the vermicomposting process, diverse microbes are involved in the stabilization and maturation of the compost (Hachicha et al., 2009). The total bacterial counts were significantly influenced ($P = 0.005$, Table 4) by the presence of *E. fetida* and EM inoculation. These treatments modified the total bacterial counts as evidenced by the significant interaction between *E. fetida* and EM ($P = 0.0004$, Table 4). Beyond 6 weeks, the total bacterial counts showed significant differences with the *E. fetida* and *E. fetida* + EM treatments showing a continued increasing values of total bacterial counts while the EM only and control showed an almost constant value until 10 weeks (Fig. 5A). The increase in the bacterial population could be due to a conducive environment provided by the earthworm digestive tract and the nutrient rich product (Ravindran et al., 2015).

[Insert Figure 5 here]

The presence of earthworms rather than EM resulted in a significant increase ($P < 0.0001$, Table 4) in phosphate solubilizing bacteria (PSB) as observed by the consistently high level in the F – CP vermicompost across 10 weeks. Generally, across all treatments, the PSB increased from 0 to 4 weeks, whilst beyond 4 weeks, only the *E. fetida* + EM and *E. fetida* alone treatments continued to significantly increase compared to the EM alone and control treatments.(Fig. 5B). On average, the PSB counts for the *E. fetida* + EM and *E. fetida* alone treatments showed a non-significantly different PSB counts of 6.6 and 6.4 log₁₀ CFU g- dry weight⁻¹; whilst the EM and control treatment had another non significantly different average total PSB counts of 5.79 and 5.80 log₁₀ CFU g- dry weight⁻¹. In a similar study but without EM inoculation, Bhattacharya and Chattopadhyay (2002) also reported very high counts of PSB in *E. fetida* treated composts compared to the without *E. fetida* treatment. Hong et al. (2011) attributed this strong interaction between earthworms and PSB to the symbiotic and synergistic relationship between microbes and earthworms. This non-significant influence of EM on the PSB levels in the vermicomposts can mean that EM is devoid of these important phosphorus mineralizing microbes, and presents an opportunity for improving these EM cocktails.

Total fungal counts were significantly influenced by *E. fetida* and EM inoculation ($P = 0.005$, Table 4). Generally, total fungi counts showed a consistent almost linear increase across the entire 10 weeks of vermicomposting, with the control treatment having significantly the lowest total fungal counts (Fig. 5C). It was noteworthy that beyond 4 weeks, the treatments with *E. fetida* inclusion showed the highest total fungal counts until the end of the 10 week study. In another study, Pramanik and Chung (2011), observed a similar increase in total fungal counts during vermicomposting of fly ash and vinasse using *E. fetida*. They suggested that not all microbes that pass through earthworm gut are killed, but rather fungi population

increases in the material ejected by the earthworms. This is supported by the lower fungal populations observed in this study in the control and EM only treatment where there was no earthworm activity.

According to Hassen et al. (2001), *E. coli* is one of the most representative bacterium in the group of faecal coliforms, which are potential pathogens when vermicompost is used in fresh produce production. The presence of *E. fetida* or EM in composts resulted in non-significant differences between treatments compared to the control (Table 4). The values of total *E. coli* counts in our study were in a range similar to that reported by Hassen et al. (2001). Throughout the 10 week period, the total *E. coli* counts showed a consistent decrease until 6 weeks and then stabilized thereafter, as indicated by the significant influence of time on total *E. coli* counts ($P < 0.0001$, Fig. 5D, Table 4). It was noteworthy in our study that inoculation of F – CP vermicomposts with EM does not result in an increase, but rather decrease in the potentially pathogenic *E. coli* during the composting process in agreement with observations by Chroni et al. (2009).

4. Conclusions

This study showed that inoculation with EM did not improve the biodegradation of fly ash – cow dung – paper waste vermicomposts. However, inoculation of EM coupled with *E. fetida* enhanced degradation of the F – CP mixture though not significantly so relative to *E. fetida* alone, suggesting that optimizing the EM inclusion level could result in improved vermicomposting of the F-CP mixture. Nevertheless, inoculation of EM together with *E. fetida* resulted in higher Olsen P release in fly ash vermicomposts, suggesting a synergistic relationship between EM microbes and *E. fetida* in phosphorus mineralization.

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Figure captions

Fig. 1. Changes in C/N ratio (A) and dissolved organic carbon (B) during composting of F – CP with or without *E. fetida* and effective micro-organisms (EM). Error bars represent standard deviation.

Fig. 2. Effects of *E. fetida* and effective micro-organism (EM) presence on changes in Olsen extractable phosphorus during composting of F -CP.

Fig. 3. Changes in inorganic nitrogen measured as nitrate/nitrite (A) and ammonium (B) during composting of F- CP with or without *E. fetida* and effective micro-organisms (EM). Error bars represent standard deviation.

Fig. 4. Effects of effective micro-organisms (EM) and *E. fetida* on changes in β - Glucosidase activity (A) and Alkaline Phosphatase activity (B) during vermicomposting of F - CP. Error bars indicate standard deviation.

Fig. 5. Changes in total Bacteria (A); total Phosphate Solubilizing Bacteria (B); total Fungi (C) and total *E. coli* (D) counts during vermicomposting of F - CP with or without effective micro-organisms (EM) and *E. fetida*. Error bars represent standard deviation.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Table 1

Selected chemical characteristics of cow dung, paper waste and fly ash used in the study.

Parameter	Cow dung	Waste paper	Fly ash
pH (H ₂ O)	7.23 ± 0.28	8.1 ± 0.1	12.58 ± 0.01
EC (dS m ⁻¹)	6.07 ± 0.14	0.12 ± 0.02	6.84 ± 0.38
Total C (%)	26.8 ± 0.1	37.96 ± 1.01	nd
Total N (%)	2.08 ± 0.07	0.038 ± 0.01	nd
C:N ratio	12.88 ± 0.51	998.94 ± 1.74	-
Olsen extractable P (mg kg ⁻¹)	1110.7 ± 5.86	nd	256.2 ± 5.24
Extractable NO ₂ /NO ₃ (mg kg ⁻¹)	269.67 ± 1.84	nd	nd
Extractable NH ₄ (mg kg ⁻¹)	364.0 ± 3.6	nd	nd

Parameters reported as Means ± Standard Deviation, n = 3; nd = not detected.

Table 2

Repeated measures ANOVA for changes in C/N; DOC; inorganic nitrogen and Olsen P following 10 weeks of F-CP vermicomposting.

Source of variation		C/N ratio ^a	Dissolved organic carbon (mg g ⁻¹)	NO ₃ /NO ₂ – inorganic nitrogen (mg kg ⁻¹)	NH ₄ – inorganic nitrogen (mg kg ⁻¹)	Olsen Phosphorus (mg kg ⁻¹)
<i>Between subjects</i>						
EM presence (EM)	<i>F</i> _{1, 71}	10.1	7.7	29.5	3.6	85.7
	<i>P</i>	0.0193	0.0326	0.0016	ns	< 0.0001
<i>E. fetida</i> presence (EP)	<i>F</i> _{1, 71}	285.1	306.8	1417.3	1708.0	297.5
	<i>P</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
EM × EP	<i>F</i> _{3, 71}	1.5	21.8	0.359	2212.0	39.6
	<i>P</i>	0.0076	0.0034	ns	< 0.0001	< 0.0008
<i>Within subjects</i>						
Time	<i>F</i> _{5, 71}	125.4	43.9	1713.5	13767.4	154.9
	<i>P</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Time × EM	<i>F</i> _{11, 71}	0.6	2.4	53.0	189.3	4.22
	<i>P</i>	ns	ns	< 0.0001	< 0.0001	0.0358
Time × EP	<i>F</i> _{11, 71}	21.1	34.9	661.3	1485.3	16.6
	<i>P</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0002
Time × EM × EP	<i>F</i> _{23, 71}	0.8	0.7	3.0	474.3	6.6
	<i>P</i>	ns	ns	ns	< 0.0001	0.0093

^a Greenhouse-Geisser adjusted values of *P* for within subject factors; ns = not significant at *P* < 0.05.

Table 3

Relationship between incorporation of EM and *E. fetida* presence on changes in Olsen P during vermicomposting of F-CP.

Treatment	Regression equation	R ²	Rate of Olsen P release [†] (mg P kg ⁻¹ week ⁻¹)	Predicted Olsen P at 10 weeks ^{††} (mg kg ⁻¹)	Observed Olsen P at 10 weeks ^{†††} (mg kg ⁻¹)	Net Olsen P increase after 10 weeks (%) ^{††††}
Control (no <i>E. fetida</i> or EM)	$-2.5651x^2 + 42.981x + 709.9$	0.977	16.56d	883.20	878.23d	23.18d
EM only	$1.6694x^2 + 15.505x + 720.75$	0.965	28.71c	1042.74	1021.11c	43.35c
<i>E. fetida</i> only	$0.9187x^2 + 39.136x + 689.8$	0.994	48.39b	1173.03	1168.19b	64.03b
EM + <i>E. fetida</i>	$-4.1268x^2 + 93.977x + 738.67$	0.981	54.32a	1265.76	1263.02a	77.20a

[†] Calculated as [Olsen P at 10 weeks – Olsen P at 0 weeks]/ 10 weeks.

^{††} Calculated based in regression equation.

^{†††} Determined as the actual Olsen P after 10 weeks of vermicomposting.

^{††††} Calculated as [Olsen P at 10 weeks – Olsen P at 0 weeks]/ Olsen P at 0 weeks × 100.

Table 4

Repeated measures ANOVA for changes in selected biochemical and microbiological parameters following 10 weeks of F-CP vermicomposting.

Source of variation		β -Glucosidase ($\mu\text{mol} - \text{PNP g-dry weight}^{-1} \text{ h}^{-1}$) ^a	Alkaline Phosphatase ($\mu\text{g} - \text{PNP/g-dry weight}^{-1} \text{ h}^{-1}$)	Bacteria ($\log_{10}\text{CFU g-dry weight}^{-1}$)	<i>E. coli</i> ($\log_{10}\text{CFU g-dry weight}^{-1}$)	Phosphate Solubilizing Bacteria ($\log_{10}\text{CFU g-dry weight}^{-1}$)	Fungi ($\log_{10}\text{CFU g-dry weight}^{-1}$)
<i>Between subjects</i>							
EM presence (EM)	$F_{1, 71}$	0.7	142.2	17.7	0.9	2.4	14.4
	<i>P</i>	ns	< 0.0001	0.0056	ns	ns	0.009
<i>E. fetida</i> presence (EP)	$F_{1, 71}$	31.5	781.5	31.6	0.2	216.3	15.9
	<i>P</i>	0.0014	< 0.0001	0.0014	ns	< 0.0001	0.0072
EM $\times$ EP	$F_{3, 71}$	0.7	22.61	51.1	6.5	2.8	2.6
	<i>P</i>	ns	0.0031	0.0004	0.0437	ns	ns
<i>Within subjects</i>							
Time	$F_{5, 71}$	55.7	384.53	38.6	228.5	61.9	133.2
	<i>P</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Time $\times$ EM	$F_{11, 71}$	1.8	22.4	5.6	38.6	2.3	4.5
	<i>P</i>	ns	< 0.0001	0.0032	< 0.0001	ns	0.0287
Time $\times$ EP	$F_{11, 71}$	6.3	82.1	17.7	6.5	4.7	14.8
	<i>P</i>	0.0289	< 0.0001	< 0.0001	0.0025	0.0211	0.0003
Time $\times$ EM $\times$ EP	$F_{23, 71}$	5.0	5.2	3.5	16.7	4.0	4.5
	<i>P</i>	0.0464	0.0179	0.024	< 0.0001	0.0345	0.0295

^a Greenhouse-Geisser adjusted values of *P* for within subject factors ; ns = not significant at $P < 0.05$.