

Earthworm- Microbe Interactions: A key to sustainable nutrient exchange in soil ecosystems

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Abstract

Earthworms are important part of soil-macro fauna, bilaterally symmetrical, segmented soil dwelling invertebrates with a glistening dark brown covered with delicate cuticle. The gut consisted of mucus containing protein and polysaccharides, organic and mineral matter, amino acids and microbial symbionts viz., bacteria, protozoa and microfungi. They bring about pronounced changes in soil physical structure and can efficiently utilize energy from poor soils, hence can be used for soil improvements e.g. *M. posthuma*. The endogeic worms are known agents in boosting the nutritional status of the soil by altering its physico-chemical parameters by augmenting aeration, water holding capacity, porosity, organic matter, macro and micro nutrients and lowering pH, C:N ratio, moisture content and bulk density. I have taken fresh samples of habitat (soil) and castings of endogeic earthworms (*Metaphire posthuma* was collected separately from three different sites (I, II and III), in triplicate, from the campus area of Mahatma Jyotiba Phule Rohilkhand University, Bareilly. In these samples more WHC, organic matter and less saline pH than habitat which is more suitable for growth of plants. There are more number of bacteria, actinomycetes and fungi in the castings than soil but there are different varieties of species of microbes So it is concluded that there are sustainable nutrient exchange in soil by the earthworm which can be shown in castings so the earthworms are responsible for nutrient exchange which is good for soil health and ecosystem.

Key words: Earthworms, microbes, parameters, organic matter, sustainable

Introduction

Earthworms are important part of soil-macro fauna, bilaterally symmetrical, segmented soil dwelling invertebrates with a glistening dark brown covered with delicate cuticle. The gut consisted of mucus containing protein and polysaccharides, organic and mineral matter, amino acids and microbial symbionts viz., bacteria, protozoa and microfungi. Endogeic earthworms are small to large sized worms, with weakly pigmented body, life cycle of medium duration, moderately tolerant to disturbance, forms extensive horizontal burrows and they are geophagous feeding on particulate organic matter and soil. They bring about pronounced changes in soil physical structure and can efficiently utilize energy from poor soils, hence can be used for soil improvements e.g. *M. posthuma*. The endogeic worms are known agents in boosting the nutritional status of the soil by altering its physico-chemical parameters by augmenting aeration, water holding capacity, porosity, organic matter, macro and micro nutrients and lowering pH, C:N ratio, moisture

content and bulk density. One of such hidden factors may be worm's complex interrelationships with micro-organisms. The worms promote microbial activities in decaying organic matter by fragmenting and inoculating it with them, and disperse them widely through the soils. These micro-organisms constitute an important nutritional component of the earthworm's diet. Edwards and Fletcher (1988) summarized the experimental evidence that microorganisms provide a source of nutrients for earthworms. Earthworms mechanically mix mineral particles and organic matter through their digestive system which carries out disintegration, grinding and digestion of the ingested material, increasing or decreasing the activity and number of beneficial or pathogenic microorganisms (fungi, actinomycetes and bacteria) (Winding *et al.*, 1997). The participation of microorganisms within the digestive tracts of earthworms is of great importance and showed that these are involved in the degradation of organic matter (Byzov *et al.*, 2007). Microbes ingested by worms along with the soil might play a pivot role in decaying complex organic food present in the worm's gut. Most of the species of micro-organisms that occur in the alimentary canal of earthworms are the same as those in the soil in which the worms live. Satchell (1967) concluded that it was unlikely that earthworms have an indigenous gut micro flora. Stöckli (1928) reported that there was a great increase in the total numbers of bacteria and actinomycetes occurring in the earthworm's gut as compared with those in the surrounding soil, and other workers showed that their number increased exponentially from the anterior to the posterior portions of the earthworm gut (Parle, 1959, 1963a). Mariaglieti, (1979); Contreras, (1980); Szabo *et al.*, (1990); Hossein *et al.*, (1991); Křišťůfek *et al.*, (1993) have mentioned that particular group of microorganisms were stimulated selectively during passage through the earthworm gut. These include actinomycetes, *Nocardia*, *Oerskovia* and *Streptomyces sp.* and the bacteria, *Vibrio sp.* Interestingly, the species composition and relative abundance of actinomycetes in the hindgut was differed between different species of worms, at different times of the year and with respect to different types of food ingested (Křišťůfek *et al.*, 1990, 1993; Ravasz *et al.*, 1987a). This was probably true for bacteria and fungi too. Gram negative bacteria predominate in the gut (Ravasz *et al.*, 1986; Křišťůfek *et al.*, 1993). The micro-organisms, *Nocardia polychromogenes*, *Actinomyces sp.* and *Streptomyces coelicolor*, which have been isolated from the gut contents and castings of earthworms, to be particularly antagonistic towards the anaerobic spore forming bacteria (Ruschmann, 1953).

In the present paper microbial population was quantified for *M. posthuma* to evaluate the differential potentiality of microbial population of *M. posthuma* in different climatic condition and also to see their status in intensifying fertility status of the soil and the role of microbes inhabiting them.

Materials and methods

Collection of Samples

Habitat (soil samples for experimental endogeic worm)

Twenty g of fresh samples of habitat (soil) of endogeic earthworms (*Metaphire posthuma*) was collected separately from three different sites (I, II and III), in triplicate, from the campus area of Mahatma Jyotiba Phule Rohilkhand University, Bareilly.

Worm-castings

Castings of endogeic worms was collected from the same place from where the soil samples were collected.

A. Analysis of Physico-Chemical Parameters (PCPs)

A.1 Physical Parameters

These parameters were measured for moisture content, water holding capacity and bulk density of all the experimental samples.

I. % Moisture Content

It was measured by taking 1g fresh homogenized experimental samples as initial weight. The samples were dried in an electric oven at 105°C for 24 hours, cooled in a desiccator followed by taking its final weight. The percent moisture content was calculated using the following formula (Saxena, 1994):

$$\% \text{ Moisture content} = \frac{I - F}{F} \times 100$$

Where,

I = Initial weight of sample (g)

F = Final weight of dried sample (g)

II. % Water Holding Capacity (WHC)

Water holding capacity was assessed by taking fresh homogenized sample, dried in an oven at 105°C for 24 hours, placed a filter paper (Whatman No.1) inside the perforated bottom of circular soil boxes (5.6 cm diameter, 1.6 cm height, bottom perforated with holes of 0.75 mm diameter), and filled it with oven dried samples. These boxes were placed in petridish (10 cm diameter) containing water, for about 12 hours, so that water entered in the boxes and saturated the soil. After saturation, boxes were taken out of the water. The samples were kept for 30 minutes in the air for evaporation of excess of water. Now, the final weight of all the samples was recorded and percent water holding capacity was calculated by using following formula (Saxena, 1994):

$$\% \text{ WHC} = \frac{(W_2 - W_1) - (W_1 - W_0)}{W_1 - W_0} \times 100$$

Where,

WHC = Water holding capacity

W₀ = Weight of empty box (g)

W₁ = Weight of box with dried samples (g)

W₂ = Weight of box with water saturated samples (g)

III. Bulk Density

It was determined by taking fresh homogenized samples. Samples were dried in an oven at 105°C for 24 hours. A small amount of every dried experimental sample was transferred to measuring cylinder of 10 ml capacity and noted its volumes and its weight was recorded by using an electronic balance (Afcoset, ER-182A make). The bulk density was recorded by using the formula given below (Saxena, 1994):

$$\text{Bulk density (g/cm}^3\text{)} = \frac{\text{Weight of soil (g)}}{\text{Volume of soil (cm}^3\text{)}}$$

A.2 Chemical Parameters

Experimental samples were assessed for their pH and % organic matter.

I. pH

It was taken by using Systronics Digital pH Meter (make MK VI). Solutions of air dried samples were prepared in distilled water by mixing them in the ratio of 1:10 (w/v) in the separate test tubes. Mixtures were stirred thoroughly with glass rods and pH was recorded by dipping the electrode of the pH meter in the test tubes after standardization of the instrument (Saxena, 1994).

II. % Oxidizable organic matter

It was assayed by Walkley and Black (1947) method. Half g air-dried sample was taken in 500 ml Erlenmeyer flask and 10 ml of $K_2Cr_2O_7$ solution (reagent A) was added into it followed by pouring of 20 ml conc. H_2SO_4 . After half an hour, 200 ml of distilled water, 10 ml of 85% H_3PO_4 , 0.2 g of sodium fluoride and 1ml of diphenylamine indicator (reagent B) were mixed into it. The mixture was titrated against 0.5 N Ferrous Ammonium Sulphate solution and the end point was noted by the appearance of brilliant green colour. Side by side a blank titration was run, devoid of sample and **percent organic matter** was calculated as the formula given below:

$$\% \text{ Organic matter} = 6.791/W [1 - T_1/T_2]$$

Where,

W = weight of sample taken (g)

T₁ = volume of titrant used against sample (ml);

T₂ = volume of titrant used against distilled water blank (ml).

B. Microbiological analysis

Each sample of soil, cattle - dung and vermicompost and gut content were subjected to bacteriological and mycological analyses using standard techniques (Barrow and Feltham, 1993) and (Domsch *et al.*, 1980) respectively as given below:

B.1 Counting of total facultative anaerobic bacteria, actinomycetes and fungi was carried out.

B.2 Thereafter all the samples were subjected for isolation, purification and preservation of bacterial/actinomycetes/fungal organisms

B.2.1 (a) Identification of each of the purified bacterial/actinomycetes cultures by using above mentioned standard biochemical methods.

B.2.1 (b) Mycological analysis of the samples involved enumeration and isolation and characterization of fungi.

B.1 Enumeration (counting) of bacteria, actinomycetes and fungi

Bacteria and Actinomycetes

The number of facultative anaerobic bacteria of each soil sample, worm-castings, cattle-dung, vermicompost and gut content of sample was determined using serial dilution and spread plate techniques. All experiments were conducted under strict aseptic condition in a Class-I Biosafety cabinet.

- Nutrient Agar medium (HiMedia) was prepared and sterilized by autoclaving as per the instructions of the manufacturing unit as and when needed. The prepared medium was poured in pre sterilized glass Petri dishes (Corning) of 90 mm diameter. The plates were transferred and kept in incubator for 37°C overnight to test the sterility of the medium.
- A unit weight of each sample (1g or 0.5g) was aseptically suspended in 10 or 5ml of pre- sterilized 0.15N NaCl (Normal Saline Solution) and shaken vigorously for two minutes.
- A series of 10-fold dilutions of the suspension were prepared from 10^{-1} to 10^{-6} using pre- sterilized glass test tubes and pipettes of appropriate size.
- 100 μ l content of dilutions ranging from 10^{-2} to 10^{-5} were aseptically transferred to the pre-sterilized nutrient agar plate (in triplicate), and spreaded with the help of pre-sterilized spreader (HiMedia). Each plate was then incubated at 37° C for 15- 18 hrs.
- The dilutions showing 100-200 colonies after incubation were selected and total colony count was conducted.
- The bacterial load was determined by employing following formula:

$\text{Total bacterial count /g of sample} = \text{Average no. of colonies of the three plates for the dilution} \times \text{inverse of the dilution} \times 10$

Fungi

An agar overlay methodology (Warcup, 1950) was used for enumeration of fungi. A mycological medium, YpSs was used in place of nutrient agar for the purpose.

- 10 ml of 1 percent suspension of each sample was suspended in 100 ml sterilized NSS in a flask and shaken well for 2 minutes to make 1/10 (10^{-1}) stock solution. Further, the dilutions of 1/100 and 1/1000 were prepared from the stock solution.
- One ml of each dilution was mixed with 15 ml **YpSs medium** at 45° C, shaken carefully and poured into sterilized petridishes.
- Petri plates were incubated at 45° C for seven days regularly and inspected for appearance of thermophyllic fungal colonies daily. Number of colonies was counted.
- Single fungal colonies were isolated by using sterilized inoculation loop and transferred on to agar slants in pure forms and maintained them at 4°C.

B.2 Isolation, purification and preservation of bacterial and fungal isolates

ISOLATION

- 10% suspension of each sample was streaked on sterilized nutrient agar and YpSs plates separately and incubated at 37°C for 24 hrs nutrient agar and 45° C on YpSs for 4-5 days.

- Each single colony obtained on NA after incubation having distinct colonial characteristic was subjected to microscopic examination after Gram's staining and was sub cultured on fresh NA plate separately. All the plates were incubated at 37°C overnight.
- Each single colony of fungi was put on YpSs plates and incubated at 45°C for 24 hrs.

PURIFICATION

- The growths of the isolates obtained on each plate were subjected to microscopic purity of the bacterial or fungal isolate.
- Growth of each isolate was transferred to pre – sterilized NA or YpSs slant in duplicate, marked and incubated as above.

PRESERVATION

- Purity and morphological identity of each bacterial, actinomycetes and fungal isolate obtained on nutrient/YpSs agar slants was checked by conducting microscopic examination after staining.
- Slants of each isolate showing pure growth were sealed by paraffin wax and stored at 4°C until subjected for identification. One of the slants was used for all future experiments and the other was kept sealed.

B.2.1 Biochemical Identification of Bacterial / Fungal Isolates

All bacterial/actinomycetes isolates obtained from the samples were subjected to identification tests using recommended biochemical techniques of Barrow and Feltham (1993).

B.2.1 (a) Primary identification tests for bacteria and actinomycetes for Identification of Genus

Each bacterial isolate was subjected to the following primary identification tests that helped in determination of the genus of bacteria. These primary bio – chemical tests were carried out for:

I. Gram's staining characteristics, II. Electron Microscopy, III. Morphology, IV. Growth in air, V. Spore formation, VI. Motility, VII. Catalase, VIII. Oxidase, IX. Glucose (acid production), X. Oxidation – fermentation

I. Gram's Staining

Ready to use Gram's staining kit was obtained from HiMedia and kept at room temperature.

Method of Gram staining

Gram's staining was usually performed on young culture obtained immediately after incubation.

- A thin smear of film of the bacterial isolate was prepared.
- With the help of a platinum loop growth of bacterial culture from the NA slant or plate was transferred a drop of NSS on a grease free microscopic slide to prepare a thin film by quickly passing the slide through the flame of Bunsen burner several times. The thin film was allowed to dry in the air.
- The fixed slide was flooded with Gram's stain for 60 sec on a staining rack.
- The stain was poured off and the slide was flooded with iodine solution and was kept for 1- 2 minutes.

- The iodine was washed off in a stream of tap water until it was completely removed.
- Decolorizing solution was slowly poured on the film until the reagent showed no colour.
- After decolorization step the slide was washed in tap water and counter stain with saffranine for 60 sec.

The slide was washed in tap water; blot dried and observes under microscope using 100X objective lens (Olympus CH20i) organisms taking blue colour of crystal violet were identified by positive colony and counter stain by saffranine were identified as gram negative.

II. Scanning Electron Microscopy

It was carried out by using SEM (make –JEOL6610LV).

- Bacterial growth pellet was taken in Eppendorf tube.
- Sample was dipped in 2.5% glutaraldehyde solution for 8 hours.
- It was washed with 0.1M PBS buffer followed by further washing with distilled water.
- The sample was transferred into 30%, 50%, 70%, 90% and finally into absolute alcohol followed by centrifugation at 2000 – 2500 rpm for 10 minutes after every treatment in various grade of alcohols. All these processes of centrifugation were conducted at 4° C.
- After centrifugation, the sample were taken and fixed on stub and air dried followed by gold plating using gold coater (make - JFC1600) and photographs were taken.

III. Morphology of microbes

Morphological studies of bacteria and actinomycetes was carried out by using Olympus microscope (make – CH20i).

IV. Growth in air

The organisms that showed growth on NA after incubation in aerobic conditions were considered as aerobic. The present study was not conducted on microaerophilic or obligates anaerobic bacteria.

V. Spore formation.

- Each bacterial isolate was sub cultured on NA plate and incubated at 37° C for about seven days.
- The growth was stained with Gram's Method and observed under microscope.
- Presence of unstained areas within the vegetative cell of the bacteria or unstained empty spores without vegetative cell was considered as production of spores by the organism.

VI. Motility

- Each bacterial isolate was inoculated separately into peptone water and incubated at 37° C for 6-12 hrs.
- A wet film of each bacterium and actinomycetes grown in peptone water was prepared and observed by microscopic examination under 40 X objective lens.
- Bacteria showing active motility were considered as motile and bacteria showing no motility or Brownian movement were considered as non - motile.

VII. Catalase

- Each bacterial and actinomycetes isolate was transferred to a nutrient agar slant for conducting catalase test.
- Approximately 1ml of a 3% solution of hydrogen peroxide was poured over the growth on slant and the slant was tilted as that the solution covered the growth completely.
- A rapid ebullition of gas bubbles was considered as a positive catalase reaction.

- Negative catalase test was indicated by absence of gas bubbles.

VIII. Oxidase

Oxidase test was conducted to determine the production of cytochrome oxidase by the organism using oxidase reagent impregnated discs (HiMedia) and stored at 4° C.

- A loop full of the test organism was transferred to the oxidase disc and rubbed slightly on to its surface.
- Development of deep violet purple colour within 10 sec. was considered as a positive oxidase test.
- Development of no colour change was considered as oxidase negative.

IX. Glucose (Acid production)

This test was conducted in glucose medium. The test organism was transferred in the glucose broth medium with the help of platinum wire and incubated at 37° C for 24 hrs. An additional tube without any bacteria was also incubated along with the inoculated tube as control. Change of the colour of medium from blue to yellow was considered as positive glucose test provided the inoculated tube showed no colour change.

X. Oxidation-Fermentation Test

- This test is used to determine whether the organism splits sugar (glucose) by fermentation or oxidation paired tubes of a medium known as Hulse and Leifson's Medium is used to conduct the test.
- H-L medium was procured from HiMedia and tubes were prepared and sterilized by autoclaving as per the manufacturer's instructions.
- With the help of straight platinum wire each bacterial isolate was stabbed inoculated in two tubes one of which were aseptically sealed with sterilized liquid paraffin (HiMedia). Both tubes were incubated at 37° C overnight.
- The metabolic reaction of the test isolate was determined according to change in indicator colour of the indicator (from blue to yellow) in both the tubes.
- The bacteria that showed acid production only in open tube was considered as oxidative (O).
- The bacteria that showed acid production in both open and covered tube was designated as fermentative (F).
- The bacteria that showed no colour in either tube was considered to be metabolically inactive towards glucose.

B.2.1 (b) Secondary Biochemical tests for bacteria and actinomycetes for determination of species

The genus of each bacterial isolate was determined as per the results of primary biochemical tests. The species of individual genus was determined by applying secondary biochemical tests as per the recommendations of Barrow and Feltham (1993) for the genus.

I. Growth on MacConkey Agar

- This is a selective cum differential medium that is used for selective isolation and differentiation of media belonging to the family *Enterobacteriaceae*.
- Readymade MacConkey agar medium was procured from HiMedia and prepared in petridishes in accordance with the manufacturer's instruction.
- Each test isolate was streaked on separate plate and incubated at 37°C overnight.

- Presence and growth of colony determine the bacterial characteristic. Pink and red colour colonies were considered as lactose fermenters where as white or buffy colonies were considered as non- lactose fermenters.

II. Utilization of Sugars (Acid production)

Test medium

i. Basal Medium

The basal medium was sterilized and stored in 10 ml quantities in separate sterilized test tubes.

ii. Sugars

- 10% w/v solutions of glucose maltose, resorcinol, fructose, sucrose, rhamnose and mannitol were prepared in suitable quantities
- Different sugar solutions were filtered by using 0.45 μ syringe filters and stored aseptically at - 20°C.

Test medium

- About 10 ml of basal medium was added aseptically in 1 ml of the sterilized sugar medium and dispensed in pre sterilized sugar tubes.
- The sterility of all the test tubes was tested by keeping them at 37°C overnight.

Test procedure

- The sugar fermentation profile of the test organism was determined by inoculating the organism in each of the seven sugar medium tubes and incubating at 37°C overnight.
- Change of colour from blue to yellow was considered as acid production in the sugar.

iii. Methyl –Red and Voges Proskauer (MRVP Test)

The MRVP test was conducted using readymade MRVP broth medium from HiMedia. The tubes of the medium were prepared as per manufacturer's instructions.

Procedure

5 ml broth medium was inoculated in the test medium and incubated at 37° C for 5 days.

MR Test

- It was carried out for 5 days old culture of the organism by adding about 5 drops of methyl red solution was added.
- A positive reaction was indicated by distinct red colour whereas appearance of yellow colour was considered as negative.

VP Test

- About 1 ml of the 5 days incubated culture was transferred to a separate tube and about 0.6 ml of α - naphthol solution was added.

- After gentle shaking, 0.2 ml of KOH solution was added. The mixture was gently shaken and left for 5 – 10 minutes.
- Appearance of bright orange red colour indicated a positive VP test with the production of acetyl methyl carbinol whereas negative test was remained red.

IV. Urease Test

- The Urease test was carried out by Maslen's Urea broth.
- The test culture of bacteria and actinomycetes was inoculated into the medium using a platinum wire and incubated at 37° C for 24 hrs. Appearance of red colour showed the expression of urease enzyme by the organism.

V. Phosphatase Test

- Phenolphthalein phosphate agar was prepared and dispensed in sterile petridishes.
- Each of the test culture was inoculated on to a separate Petridish containing sterility tested medium and incubated at 37° C for about 18 – 24 hrs.
- The petridishes were then exposed to ammonia vapours for 10 -15secs by carefully holding the plate above an open bottle of ammonia solution.
- The colonies of organisms that produces Phosphatase enzyme showed a colour change to bright pink.

VI. Starch Hydrolysis Test

- This test was conducted to determine the capacity of bacteria and action to produce amylase enzyme i.e. responsible for hydrolysis of starch into simple sugar.
- The medium for the test was prepared and dispensed in petridishes as per the methodology.
- Heavy inoculums of each test culture were streaked on to the surface of the medium and incubated at least for 24 hrs.
- Each plate was flooded with Lugol's iodine solution for about 5 minutes and excessive fluid was discarded aseptically into a container with a aseptic solution.
- Appearance of clear colourless zone around the colonies indicated hydrolysis of starch by the organism if the organism did not produce the enzyme, no colourless zones were present around the colonies.

VII. Gelatin Hydrolysis Test

- The test was carried out to determine whether the test organism hydrolyse gelatin by production of gelatinase.
- The gelatin agar medium was prepared, autoclaved and dispensed in sterile petridishes.
- Heavy inoculums of each test isolate were inoculated onto a gelatin agar and incubated at 37° C for about 3- 5 days.
- After incubation the agar surface of each plate was flooded with 5 – 10 ml mercuric chloride solution.
- Gelatin hydrolysis was indicated by presence of clear zone around the colonies.

C. Statistical Analysis

A one-way ANOVA (analysis of variance) (XL software package, version 5; SAS Institute Inc., 2002) was used to evaluate statistical significance of cfu members, microbial activity between substrates and castings of all the three species of experimental earthworms.

Result

A. Analysis of Physico-chemical parameters (PCPs)

To understand the relationship of gut associated bacteria, fungi and actinomycetes of *M. posthuma* earthworm and their relation with worms' habitat, the physico-chemical parameters of worms' habitat of respective soils in this worm and their surface fresh castings.

A.1 PCPs of the habitat (Soil) and castings of *M. posthuma*

A.1 (a) *Physical Parameters*

I. % Moisture Content

It was quantified less in the fresh castings of all the sites than that of habitats (soil-samples) of *M. posthuma*. It was recorded the maximum (1.07 times more) in the castings of site-III than that of site-I; while it was 1.09 times more in the habitat of site-III than that of site-I and 1.13 times less in the castings than that of the habitat (Table-3).

II. % Water Holding Capacity

It was found more in the fresh castings of all the sites than that of different habitats of the worms and found the maximum (1.11 times more) in the castings of site-III than that of site-I; while 1.05 times more in the habitat of site-III than that of site-I and 1.09 times increment of WHC in the castings than its respective habitat (Table-3).

III. Bulk Density

Bulk density was noted less in the castings of all the sites than that of the respective habitats of *M. posthuma*. It was the minimum (1.14 times less) in the castings of site-III and maximum in site-I; while it was 1.30 times less in the habitat of site-III than that of site I and 1.09 times less in the castings of site III than that of habitat (Table-3).

A.1 (b) *Chemical Parameters*

I. pH

It was low in the castings of all the sites than that of all the three habitats of worms. It was the minimum (1.17 times less) in the castings of site-III and the maximum (1.17 times more) in the soil of site-I. At the same time, it was recorded 1.11 times less in the habitat of site-III than that of site-I and 1.08 times decreased in the castings of site III than that of respective habitat soil (Table-3).

II. % Organic Matter

The % organic matter was found more in the castings of all the sites than that of all the three experimental habitats of worms. It was the maximum (1.35 times more) in the castings of site-III and minimum in site-I; while it was 1.16 times more in the habitat of site-III than that of site-I and 1.26 times more in the castings of site-III than that of habitat soil (Table-3).

Table-1: Physico-chemical parameters (PCPs) of habitat (soils) and castings of *Metaphire posthuma*

PCPs of soils			Parameters	PCPs of worm's castings		
Site-I	Site-II	Site-III		Site-I	Site-II	Site-III
26.35 ±0.86	27.84 ±0.98	28.85 ±1.01	% MC	23.65 ±0.97	24.75 ±1.02	25.46 ±1.03
45.58 ±1.92	47.50 ±1.48	48.10 ±1.27	% WHC	47.52 ±1.62	50.66 ±1.08	52.82 ±1.38
1.08 ±0.08	1.02 ±0.09	0.83 ±0.13	BD (g/cm ³)	0.87 ±0.09	0.85 ±0.14	0.76 ±0.09
8.91 ±0.36	8.08 ±0.52	7.96 ±0.21	pH	8.65 ±0.30	7.89 ±0.19	7.35 ±0.21
3.98 ±0.10	4.35 ±0.19	4.65 ±0.21	% OM	4.35 ±0.28	5.05 ±0.17	5.89 ±0.15

*WHC = Water Holding Capacity, BD = Bulk Density, MC = Moisture Content and OM = Organic Matter.

B.1 Population density of facultative anaerobic microbes (Bacteria and Actinomycetes)

1.1 Habitat (soil)

The facultative anaerobic microbial population in the habitat (soil) of *M. posthuma*, collected from three different sites respectively, are presented in Table-6. The mean viable count was found to be within range of 10^5 to 10^7 organisms/g of the soil.

B.2 Population density of Fungi

2.1 Habitat (soil)

The fungal population in the habitat of *M. posthuma*, collected from three different sites has been presented in Table-7. The count was found to be within 10^5 organisms/g of the soil.

Table-2: Population density of microbes (bacteria and actinomycetes) in the habitat and castings of earthworm species

Substrate	Mean of population density of microbes (cfu/g)
Soil	$1.4 \times 10^6 \pm 0.88$
Worm-castings	$2.7 \times 10^6 \pm 1.23$

Table-3: Population density of fungi in the habitat and castings of *M. posthuma*

Earthworm Species	Substrate	Mean of population density of fungi (cfu/g)
<i>M. posthuma</i>	Soil	$6.00 \times 10^5 \pm 0.33$
	Worm-castings	$7.94 \times 10^5 \pm 0.54$

C.1 Biochemical identification of microbes associated with earthworm habitat and castings

A total of 30 facultative anaerobic organisms have been isolated and purified from 18 habitats and worm-castings samples. The genus of all the isolates was first identified by biochemical tests. The results of biochemical tests conducted on these isolates along with their genus identification have been presented in Table-4. The species of all the 30 bacterial isolates belonging to various genera were identified by secondary biochemical tests. The results of the secondary tests conducted on each genus have been documented in Table-5.

C.1.1 Bacterial species associated with habitat (soil)

M. posthuma

The pre dominant bacterial species in the habitat of *M. posthuma* was found to be *Bacillus lentus*. Simultaneously, *Rothia dentocariosa* was isolated from all the three sites. The results have been presented in Table-6, Plate-1 and 2.

Table-4: Primary biochemical tests of isolates from habitat and castings of earthworm species

*R =	Name of site	Sample	Isolate code	Gram staining	Shape	Spores	Motility	Growth (in aerobic condition)	Growth (in anaerobic condition)	Catalase	Oxidase	Glucose	O/F	Genus identified	Rods
	Site-II	Soil	Mp ₂ S ₂	+ve	R, chain	+ve	+ve	+ve	NR	+ve	+ve	+ve	F	<i>Bacillus</i>	
	Site-II	Castings	Mp ₂ C ₂	+ve	Cocci	-ve	-ve	+ve	NR	-ve	-ve	+ve	F	<i>Streptococcus</i>	
	Site-II	Castings	Mp ₂ C ₃	-ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Aeromonas</i>	
	Site-III	Castings	Mp ₃ C ₁	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Bacillus</i>	
	Site-III	Castings	Mp ₃ C ₂	-ve	R	-ve	-ve	+ve	NR	+ve	-ve	+ve	F	<i>Escherichia</i>	
	Site-I	Soil	Mp ₁ S ₁	+ve	R, chain	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	
	Site-I	Soil	MP ₁ S ₂	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	
	Site-I	Soil	Mp ₂ S ₁	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	
	Site-III	Castings	Mp ₃ S ₁	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	
	Site-I	Castings	MP ₁ C ₁	+ve	R, chain	-ve	-ve	+ve	NR	+ve	+ve	+ve	O	<i>Nocardia</i>	
	Site-II	Castings	Mp ₂ C ₁	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	
	Site-III	Castings	Mp ₃ C ₂	+ve	R	-ve	-ve	+ve	NR	+ve	+ve	+ve	F	<i>Rothia</i>	

*MpS = *M. posthuma* Soil MpC = *M. posthuma* Castings

Table-5: Secondary biochemical tests of microbes isolated from habitat and castings of earthworm species

S.No	Genus	Growth of MacConkey	Utilization of sugars						MR	VP	Urease	Phosphatase	Nitrate reduction	Starch hydrolysis	Gelatin hydrolysis	Species identified
			Maltose	Resorcinol	Fructose	Sucrose	Rhamnose	Mannitol								
1.	<i>Bacillus</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	<i>Bacillus lentus</i>
2.	<i>Streptococcus</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	<i>Streptococcus porcinus</i>
3.	<i>Aeromonas</i>	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	<i>Aeromonas salmonicid</i>
4.	<i>Bacillus</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	<i>Bacillus lentus</i>
5.	<i>Escherichia</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	<i>Escherichia coli</i>
6.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
7.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
8.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
9.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
10.	<i>Nocardia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Nocardia asteroides</i>

11.	 <i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	 <i>Rothia dentocariosa</i>
12.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
13.	<i>Serratia</i>	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	<i>Serratia marcescens</i>
14.	<i>Acinetobacter</i>	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Acinetobacter calcoaceticus</i>
15.	<i>Achromobacter</i>	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	<i>Achromobacter xylosoxidans</i>
16.	<i>Cardiobacterium</i>	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Cardiobacterium hominis</i>
17.	<i>Enterobacter</i>	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	<i>Enterobacter gergoviae</i>
18.	<i>Bacillus</i>	+ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Bacillus pumilus</i>
19.	<i>Bacillus</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	<i>Bacillus lentus</i>
20.	<i>Rothia</i>	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	<i>Rothia dentocariosa</i>
21.	<i>Acinetobacter</i>	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Acinetobacter calcoaceticus</i>

22.	<i>Enterobacter</i>	-ve	-ve	+ve	+ve	+e	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	<i>Enterobacter gergoviae</i>
23.	<i>Aeromonas</i>	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	<i>Aeromonas salmonicida</i>
24.	<i>Enterobacter</i>	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	<i>Enterobacter gergoviae</i>
25.	<i>Aeromonas</i>	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	<i>Aeromonas salmonicida</i>
26.	<i>Acinetobacter</i>	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Acinetobacter calcoaceticus</i>
27.	<i>Escherichia</i>	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	<i>Escherichia coli</i>
28.	<i>Enterobacter</i>	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	<i>Enterobacter gergoviae</i>
29.	<i>Enterobacter</i>	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	<i>Enterobacter gergoviae</i>
30.	<i>Acinetobacter</i>	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	<i>Acinetobacter calcoaceticus</i>

Table-6: Bacteria and actinomycetes associated with habitat of earthworm species

Name of earthworm	Name of habitat	Type of microbes	Name of microbes
<i>M. posthuma</i>	Soil	Bacteria	<i>Bacillus lentus</i>
		Actinomycetes	<i>Rothia dentocariosa</i>



Plate-1: Petriplate showing growth of pure colony of actinomycetes (*Rothia dentocariosa*) in the habitat (soil) of earthworm, *M. posthuma*



Plate-1: Petriplate showing growth of pure colony of Bacteria (*Bacillus lentus*) in the habitat (soil) of earthworm, *M. posthuma*

Table-7: Microbes (bacteria and actinomycetes) associated with castings of earthworm species

Name of earthworm	Type of microbes	Name of microbes
<i>M. posthuma</i>	Bacteria	<i>Streptococcus porcinus</i> , <i>Aeromonas salmonicida</i> , <i>E. coli</i> , <i>Bacillus lentus</i>
	Actinomycetes	<i>Nocardia aesteroides</i> , <i>Rothia dentocariosa</i>



Plate-3: Petriplate showing growth of pure colony of bacteria (*Streptococcus porcinus*) in the castings of *M. posthuma*.



Plate-4: Petriplate showing growth of pure colony of actinomycetes (*Nocardia asteroides*) in the castings of *M. posthuma*.

D. Fungal colonies associated with habitat (soil) and castings

A total number of fungal isolates were purified from the habitat samples and their respective castings identified by using microscope (make-Olympus CH20i).

D.1 In habitat (soil)

M. posthuma

Aspergillus terreus and *Thermoascus aurantiacus* were found in habitat (soil) of *M. posthuma*. The results have been presented in Table-8 and Plate-5.

D.2 In castings

M. posthuma

Two *Aspergillus* species were found associated with worm castings of *M. posthuma*. The results have been presented in Table-9 and Plate-6

Table-8: Fungi associated with the habitat (soil) of *M.posthuma*

Earthworm species	Name of Habitat	Name of fungi
<i>M. posthuma</i>	Soil	<i>Aspergillus terreus</i> , <i>Thermoascus aurantiacus</i>

Table 9: Fungi associated with the castings of *M.posthuma*

Earthworm species	Name of fungi
<i>M. posthuma</i>	<i>Aspergillus fumigatus</i> , <i>Aspergillus terreus</i> ,

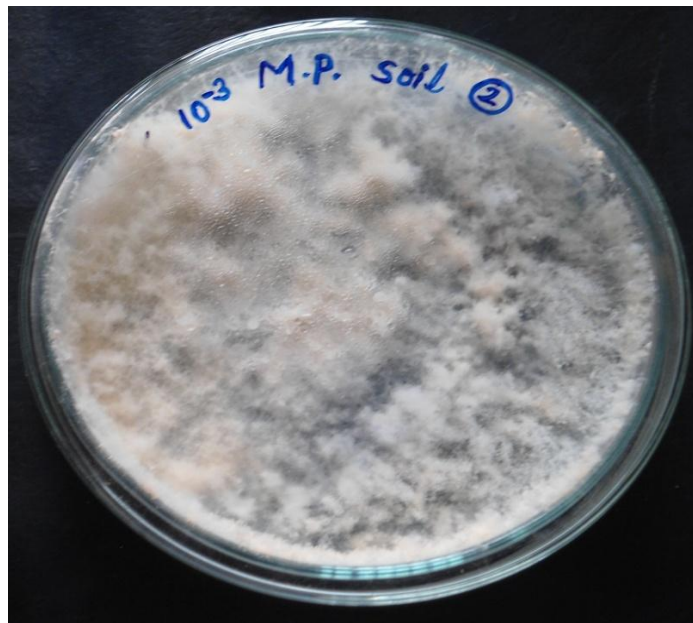


Plate-5: Petriplate showing growth of pure colony of fungi (*Thermoascus aurantiacus*) in the habitat (soil) of *M. posthuma*.



Plate-6: Petriplate showing growth of pure colony of fungi (*A.terreus*) in the castings of *M. posthuma*.

Discussion

Earthworms are one of the major macro fauna of the soil and are considered as natural bioreactors as they redesign the physical structure and chemical makeup of the soil environment by ingesting soil particles along with litter and by depositing castings on the soil surface (Ansari, 2011; Pederson and Hendrickson, 1993; Verma and Shweta, 2011). Worm's activities profoundly affect the physical, chemical and biological properties of soil. Most of them are omnivorous and polyphagous creatures as they fed upon a wide variety of organic materials (Kale and Krishnamoorthy, 1981). They exercise considerable level of selection while feeding. Surface dwelling earthworms take their food – materials more selectively (Dash *et al.*, 1979, 80) than their deep soil – dwelling associates, which usually ingest soil while burrowing (Lavelle *et al.*, 1980). These have many complex interrelations with micro – organisms. They depend upon micro – organisms as their major source of nutrients, promote microbial activity in decaying organic matter by fragmenting it and inoculating it with micro – organisms, and disperse micro – organisms widely through soils.

Generally, nutrition of earthworms is affected by the density and diversity of microbial population in the habitat. These microbes play a substantial role by helping them in the breakdown of organic matter. Several workers have studied microbial density in the worm – castings and microbial dynamics in the soil where they live (Hornor and Mitchell, 1981; Lattaud *et al.*, 1999 and Prabha *et al.*, 2007).

Besides, worms are potentially important vectors of microbial populations too as these live in the upper part of the soil profile which is generally rich in microbial diversity and pass large amount of soil, approximately 200-400 t ha⁻¹ year⁻¹ through their gut as castings (Lavelle *et al.*, 1983). It has been reported by several workers that worm's castings are much more microbiologically richer in microflora than that of their surrounding soils (Parthasarathi *et al.*, 2007; Scheu, 1987).

In the present studies, an attempt has been made to analyze the physico – chemical parameters (PCPs) of three different habitats of three common earthworms with respect to their castings to evaluate worm-soil-castings relationships. The study was further extended by conducting investigations to evaluate complex interrelationship of worms with microorganisms as these essentially promote decaying of organic matter thereby enhancing nutritional status of the soil in case of both the endogeic worms (*M. posthuma*). In these studies, the moisture content and pH were less in the castings of endogeic earthworm (*M. posthuma*) than that of their habitats. However, Bahl (1933 – 34) has done some work on habit and habitat of *M. posthuma* and also the moisture content in their castings but he had not concluded the importance of the findings. Gupta (1975) studied the effect of pH and temperature on the intestinal amylase of these worms. Some work on the pH of soils and castings of *M. posthuma* has also been carried out by Varma and Chauhan (1979).

%WHC was more and bulk density was less in the castings than that of their habitat. Such changes in these two physical parameters have also been noted by Gupta and Sakal (1966 – 67) while doing comparative studies of the physico – chemical composition of earthworm castings of the garden and cultivated soils. They have reported that castings always have more WHC than that of their respective soils where the worms live; while castings have less bulk density than that of the parent soil.

It has been noticed that the % organic matter was more in the castings of experimental endogeic earthworm than their habitat. This is in support of findings of Martin (1991). He had reported that castings of tropical earthworm, *M. anomala* had much less coarse organic matter than the surrounding soil, indicating that the longer particles of organic matter were fragmented during passage through the earthworm's gut.

Several workers did chemical analysis of vermicompost and reported that it had lower pH, electrical conductivity (EC) and organic matter (OM) (Nardi *et al.*, 1983, Albanell *et al.*, 1988 and Mitchell, 1997), supporting the work of this paper.

In such diversified and multiferous state of three experimental soils, the microbial population (bacteria, actinomycetes and fungi) might be different and also due to varied nutritional status of the soil. In present findings, the population of bacteria and actinomycetes in the castings of endogeic species of earthworm, *M. posthuma* was recorded 1.92 times more than that of its habitat (soil). It may be added that the population of fungal colonies were also found 1.06 times more in the fresh castings of *M. posthuma*. These findings showed that the castings of *M. posthuma* have more suitable physico – chemical parameters for microbes to be associated than that of its habitat.

Many workers have reported that there were greater numbers of microorganisms in earthworm casts than in the bulk soil (Stöckli, 1928; Ghilarov, 1963). Teotia *et al.*, (1950), Kollmannsperger, (1952, 1956), Ruschman, (1953) and Schultz and Felber (1956) all have reported greatly increased microbial populations in earthworm casts as compared to the surrounding soil.

Several workers have also mentioned that the microbial population of cast soil is much larger than that of the surrounding soil (Bassalik, 1913; Zrazhevskii, 1957; Went, 1963; Shaw and Pawluk, 1986a; Tiwari *et al.*, 1989). Comparisons of field collected earthworm casts with the surrounding soil has revealed that the casts usually have greater populations of fungi, actinomycetes and bacteria and higher enzyme activity than the surrounding soil (Dkhar and Mishra, 1986; Tiwari *et al.*, 1989; Tiwari and Mishra, 1993). Tiwari and Mishra (1993) sampled earthworm casts and adjacent soil at 30 different sites in India and reported that the casts usually contained larger fungal populations and a greater number of fungal species than did in the soil. Casts are usually rich in ammonia and partially organic matter and thus provide a good substrate for growth of micro – organisms. Some of the intestinal mucus secreted during passage through the earthworm gut is egested with the casts, where it continues to stimulate microbial activity and growth (Barois and Lavelle, 1986; Scheu, 1991). Earthworm's casts have been reported to be much more microbiologically active and richer in micro flora than their surrounding uningested soils (Scheu, 1987, Daniel and Anderson, 1992). Several workers have compared the mineral composition of the earthworm's food substrate with those of its excreted casts and reported that the earthworm's activities significantly increase the nitrogen mineralization in the soil (Sharon *et al.*, 2002). On the basis of present experimental findings, it may be concluded that *M. posthuma* plays a better role in enriching microbial population in the soil.

This variation in the organic content in the worm-castings might be due to variations in the nutritional status and their physico-chemical parameters. Other reasons for such upgrading of the organic content would be due to different intestinal secretions and body secretions of the earthworms that may be added into the castings during their passage; It is further noted that there was more microbial population in the castings of *M. posthuma* than that of the habitat soil. It has been noticed there were four varieties of bacterial species in the castings; while there only one bacterial species was isolated from the ingested soil by the worm. Their castings also have two species of actinomycetes and only one in the ingested soil; while the number of fungal species remained the same. Only two fungal species have been isolated in the castings and soil. Although, except *A. terreus*, other two species of the castings and ingested soil were found different. The microbial species diversity might be due to their presence in the form of spores in the soil and when such soil was ingested by the worm, *M. posthuma*, the worm's gut may provide suitable conditions for them for further development. This causes bursting of spores and release of newly developing microbes (bacteria, actinomycetes and fungi) in the castings of *M. posthuma* or be due to action of various gut enzymes on the walls of spores that cause its disintegration and release of new species of microbes in the gut and thereby in the castings. In case of fungal species, two species have been recorded from the soil and two from the castings. However, only one species *T. aurantiacus* was found in the soil and castings both but another species (*A.*

niger) which was isolated from the soil was found missing in the castings and the species (*A. fumigatus*) that was missing in the soil was found in the castings

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