Applied and Environmental Soil Science

Status, Trends, and Advances in Earthworm Research and Vermitechnology

Guest Editors: Natchimuthu Karmegam, Radha D. Kale, Thilagavathy Daniel, M. Nurul Alam, and Martín Gerardo Rodríguez

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Editorial Board

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Editorial

Special Issue on Status, Trends, and Advances in Earthworm Research and Vermitechnology

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The articles in this special issue reflect the developments in the fields of earthworm research and vermitechnology. Charles Darwin's observation on earthworms is a milestone in understanding the soil biology and enormous contribution to some aspects of the genesis of humus and of its role in soils. Earthworms are the best known soil inhabiting animals commonly called "*friends of farmers*" due to the beneficial role they play in soil. The research on earthworms has gained importance in India as well as in other countries. In the year 1981, an international symposium entitled Earthworm Ecology: Darwin to Vermiculture was held at Cumbria, UK, to commemorate the centenary celebration of Darwin's book *The Formation of Vegetable Mould through the Action of Worms, with Observations on Their Habits* that was published in 1881 by Murray, London, UK. In the year 2000, Vermillenium—an international workshop and symposium—was held at Kalamazoo, USA, to realize the progress achieved in this field after a decade (since 1991). Recently, Ninth International Symposium on Earthworm Ecology (ISEE-9) that was held at Xalapa, Mexico, during the 5th to 10th of September 2010 clearly proved the importance of earthworms and vermitechnology by the participation of scientists from different countries. About 300 papers were received from the researchers across the world.

Considerable research is in progress with regard to significant role of earthworms and vermitechnology. This issue addresses the existing situations by providing complete, collective, and up-to-date knowledge and recent trends. It is a compilation of research articles on ecology, behavior, and functional role of earthworms at organismic levels. There are also papers to highlight the cellular and molecular studies.

The first paper by U. Kutschera and J. M. Elliott points out the origin of earthworm research from the time of Darwin (1881) that was responsible for the recent developments in biogeographical studies. A very interesting point is about the lineage of the earthworms described in this paper.

K. R. Butt and N. Grigoropoulou have given a detailed description on the tools and methods to be adopted for studying the earthworms at field level. Their contribution supports a number of suitable ecological methods and access to various tools to support earthworm research.

The paper by R. D. Kale and N. Karmegam highlights the research carried out by different scientists in India on aspects of earthworm population dynamics and species diversity associated with other soil fauna and microflora. The paper also deals with the importance of earthworm activity on physicochemical properties of soil with reference to India and other tropical countries. They laid stress

on the earthworm plant association and importance of the secretions of earthworms as plant growth stimulators and their role as bioindicators. Several other papers on earthworm ecology are also included. The review article on the role of earthworms in soil fertility maintenance through the production of biogenic structures explains the effect of farming practices on earthworm population (T. Bhadauria and K. G. Saxena). P. Bescansa et al. have reported the casting activity of an anecic earthworm, *Scherotheca gigas* in no tillage Mediterranean soils (Ebro Valley in Navarre, NE, Spain) and its role in organic matter incorporation. Influence of the earthworm on aridity factors is also discussed. This study gives an evidence for incorporation of organic matter and in particular the most labile fractions to the soil by *S. gigas*. M. Birkas and coauthors have reported that the earthworm density in Hungary is directly linked to physical characteristics and soil mulch. The same may hold good even for other geographical regions.

Nitrous oxide (N_2O) emission has been the threat for climatic changes. N. K. Evers and his coworkers have shown the direct correlation between density of earthworm (*Lumbricus terrestris* L.) population and soil moisture content to N_2O emissions in a controlled greenhouse experiments. They are of the opinion that the benefits that are normally seen from earthworms in agricultural systems may be masked by their influence on facilitating the production of N_2O and in turn climate change. But various other factors like the associated microorganisms, and the levels of organic matter, soil porosity for oxygen supply to soil layers have to be studied in soils with and without earthworms to derive at any conclusion. There is sufficient scope to further the research in this regard.

The influence of earthworms on plant growth may vary depending on soil structure. K.-R. Laossi and coauthors in their review article have suggested the experimental approaches to be developed to assess the influence of soil type on response of plants in the presence of earthworms. This line of study is very essential to relate the animal, plant, and edaphic factors.

Soil community is a complex food web. Earthworms, being the major macro fauna, contribute to modification of the structure and functions of different decomposers and predators. The research paper of C. Villenave et al. depicts the changes that occur in the abundance of bacterial and fungal feeding nematodes in presence of the earthworm, *Pontoscolex corethrurus.*

The agrochemicals are known to have adverse effect at different levels on life and activities of soil organisms including earthworms. Two papers that appear in this issue describe the effect of pesticides and herbicide on growth and reproduction of earthworms (S. Yasmin and D. D'Souza) on population growth and on histology and reproduction of *Eisenia fetida*, respectively (M. Gobi and P. Gunasekaran).

Probably, earthworms are the most promising sources to combat various physiological and pathological disorders that are disturbing the human life in the near future. One such product from earthworms is the protease enzyme. In this regard, the extensive review on earthworm protease compiled by R. Pan et al. gives an impetus to further research on biomolecules that have significance in pharmacological applications.

Finally, the molecular approach at DNA level is an important contribution to use earthworms as the tool to understand the possible damage that can be caused by different metallic pollutants. These are entering into the food chains of higher animals including humans. Many of these pollutants at different levels may cause damage to DNA which may be cancerous. It is inspirable from the study of T. Hirano and K. Tamae that the earthworms can also be used as model organisms for studying the carcinogenesis.

Though more articles were expected on vermicomposting and related studies, only one paper on nutrient status of vermicompost derived from urban green waste was submitted for publication. It is a comparative study to identify the most efficient epigeic earthworm (S. Pattnaik and M. V. Reddy).

The organization of the papers in this special issue represents the landmark of earthworm and vermitechnology research. Altogether the articles presented provide the reader with descriptions of earthworm ecology and different approaches to study their role in ecosystems, physiology at cellular level, and finally the vermicomposting. We hope that this issue would provide the resources necessary to understand and to promote advances in this important field.

We hope that the readers and the research workers will find this as a useful source of information. We would like to thank the reviewers who helped us in reviewing the articles and timely recommendations. We also would like to thank the Chief-Editor, Dr. Siobhán Staunton, Director of Research from Ecologie Fonctionnelle et Biogéochimie des Sols (Montpellier Cedex, France), and other staff of the Editorial Section of *Applied and Environmental Soil Science* (AESS), who had faith in us and cooperated at all stages of compilation. Special thanks are due to Dr. Nada Ahmed who had coordinated all communications at all stages of compilation of this special issue.

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Review Article

Charles Darwin's Observations on the Behaviour of Earthworms and the Evolutionary History of a Giant Endemic Species from Germany, *Lumbricus badensis* (Oligochaeta: Lumbricidae)

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The British naturalist Charles Darwin (1809–1882) began and ended his almost 45-year-long career with observations, experiments, and theories related to earthworms. About six months before his death, Darwin published his book on *The Formation of Vegetable Mould, through the Actions of Worms, With Observations on their Habits* (1881). Here we describe the origin, content, and impact of Darwin's last publication on earthworms (subclass Oligochaeta, family Lumbricidae) and the role of these annelids as global "ecosystem reworkers" (concept of bioturbation). In addition, we summarize our current knowledge on the reproductive behaviour of the common European species *Lumbricus terrestris*. In the second part of our account we describe the biology and evolution of the giant endemic species *L. badensis* from south western Germany with reference to the principle of niche construction. Biogeographic studies have shown that the last common ancestor of *L. badensis*, and the much smaller sister-taxon, the Atlantic-Mediterranean *L. friendi*, lived less than 10 000 years ago. Allopatric speciation occurred via geographically isolated founder populations that were separated by the river Rhine so that today two earthworm species exist in different areas.

1. Introduction

In his *Autobiography*, Charles Darwin (1809–1882) briefly commented on his last major publication in the following words: "I have now (May 1, 1881) sent to the printers the MS. of a little book on *The Formation of Vegetable Mould through the Actions of Worms*. This is a subject of but small importance; and I know not whether it will interest any readers, but it has interested me. It is the completion of a short paper read before the Geological Society more than forty years ago, and has revived old geological thoughts" [1, page 136]. In a foot-note on the same page, Darwin's son Francis (1848–1925), who edited the letters as well as other documents after his father's death, remarked that "between November 1881 and February 1884, 8,500 copies were sold." Charles Darwin's "little book" [2] later gave rise to the scientific concept of "bioturbation", which can be defined as "the biological reworking of soils and sediments by all kinds of organisms, including microbes, rooting plants and burrowing animals" [3]. These ongoing activities of different soil (or sediment) organisms, which leads to a modification of geochemical gradients and the redistribution of organic substances, can be viewed as a kind of "ecosystem engineering." Moreover, it is obvious that soil texture is modified and different soil particles are dispersed [3].

In this article, we review the history and current status of Darwin's "earthworm research-agenda" (Figures 1 and 2), summarize the significance of his classical monograph [2] with respect to modern soil biology, and describe the ecology and biogeography of a rare, endemic species, *Lumbricus badensis*. The last section of our account is in part based on unpublished observations on this enigmatic annelid.

Figure 1: Charles Darwin as an earthworm scientist: caricature from the journal *Punch*, published in the year 1882.

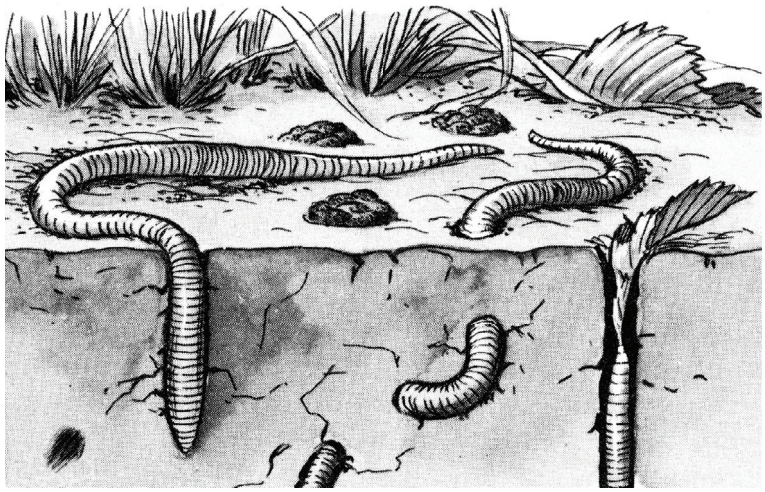

Figure 2: Schematic drawing of common European earthworms (*Lumbricus terrestris*) in their natural habitat. The annelids live in self-made burrows and forage by night on rotten leaves.

2. A Trivial Gardening Matter and Darwin's Speech to the Geological Society of London

Darwin first indicated the importance of earthworms in a lecture "On the formation of mould" to the Geological Society of London on 1 November 1837. This was published in the following year [4] and does not appear to have had a great impact on his colleagues [5]. Darwin probably realised this because he repeated his ideas in three following publications (published in 1840, 1844, 1869) [6–8], the last two being aimed at gardeners. The terms "vegetable mould" or "plant earth" were used by the Victorians to refer to what is called today "humus-rich topsoil" or the "A horizon" (Figure 3) or "mollic epipedon". It was his 1881 book [2] that had the greatest impact on those who had often regarded earthworms as pests that disfigured well-manicured Victorian lawns with their casts. They were thought to be useful only as fish bait or food for hens, but Darwin gave them a noble and useful character and even, more controversially, considered that they had intelligence.

Although the book was a great success at the time and was verified by many studies soon after its publication, it has become neglected in some areas of biology, especially soil science. A detailed review by Johnson [9] shows how biomechanical processes were largely ignored in models of landscape evolution, and how soil science became dominated by chemical and hydrological processes. However, this review also demonstrates how more recent models of dynamic denudation incorporate bioturbation on equal terms with other major archaeogenic, geomorphogenic, and pedogenic processes. Finally, it should be noted that although Darwin's book on earthworms became neglected by the earth sciences, it has continued to be quoted in zoological texts [10–12].

3. Earthworms as Living Ploughs: Darwin's Major Conclusions

As there are already some lengthy reviews of the scientific findings in Darwin's book on earthworms (e.g., [10, 13]), only the more important conclusions are summarised here. Darwin was probably the first scientist to examine a soil profile and suggest factors responsible for the structure of the various layers (Figures 2 and 3). This vertical soil section with a depth of about 13 cm was taken in October 1837 from a field near the family home of Darwin's uncle, the famous English potter, Josiah Wedgewood. The field had been drained, ploughed, harrowed, and covered extensively with burnt marl and cinders 15 years earlier. Darwin observed that this layer (Figure 3(c)) was now well below the surface and concluded that this was due to the actions of earthworms, a process described as bioturbation [3]. He was the first naturalist to point out the importance of earthworms in the formation of the layer of humus-rich topsoil that covers the land surface in every moderately humid country of the Earth.

Darwin first recognised this importance of earthworms in soil formation (pedogenesis) by them acting as agents of physical and chemical decomposition (weathering of rocks), by promoting humus formation, and by improving soil texture. More recent work has shown that they also influence soil pH and enrich the soil [11]. The processes of physical and chemical decomposition occur in the earthworm gut, chiefly in the gizzard and crop. Darwin concluded that the ingestion of the topsoil, and its mixing, grinding, and digestion in the gut, continually exposed rock particles to chemical alteration, increasing the amount of soil. This process, and the addition of faecal casts from the worms, builds up the humus-rich topsoil (Figure 3(a)) and buries various materials originally on the surface (e.g., seeds, pebbles, archaeological artifacts) down to depths of 2 m, depending upon the depth of the earthworm burrows. Darwin estimated rates of topsoil deposition in the range of 0.20–0.56 cm per year, and the mean amount of soil brought upwards by the worms as 17–40 t per ha per year. More recent studies in Britain, France, Switzerland, and Germany have produced similar values for grass-dominated vegetation in a temperate climate [13].

Earthworms are predominately saprophages and feed chiefly on organic detritus, usually the decomposing leaves and stems of plants together with smaller amounts of roots, seeds, algae, fungi, and testate Protozoa. They prefer materials rich in nitrogen and sugar, but low in polyphenols [14]. Variable amounts of mineral soil can be ingested together with organic material, and the mineral fraction reflects that of the external medium. Darwin observed that an "astonishing number of half-decayed leaves" were drawn down by the worms into their burrows (Figure 2). Here they were stored until they were sufficiently decomposed to be eaten. He thought that the mixture of partially digested leaves and mineral soil in the faecal casts was responsible for the characteristic dark colour of humus-rich topsoil (Figure 3(a)). It is now known that the darkening is a much slower process, involving primarily chemical reactions and microbial activity [15]. However, the earlier processing of the material in the earthworm gut may facilitate this process.

Darwin was also the first scientist to state that earthworms improved the quality of soil by improving soil texture. Earthworm activity facilitates the physical comminution of organic particles, the amelioration of soil pH, the enhancement of microbial activity, and the mixing of soil from different strata in the profile. They promote the formation of organomineral complexes and, by delivering faecal casts at the surface, they bring organo-mineral crumbs from the deeper parts of the profile to the surface. Earthworms also facilitate the transport of certain elements to the soil surface so that their faecal casts have concentrations of calcium, sodium, potassium, magnesium, available phosphorus, and molybdenum that are higher than in the surrounding soil. Therefore, earthworms not only improve the soil texture but also enrich the soil [3, 9, 10, 15].

Darwin suggested that earthworms may change the chemical composition of materials that pass through their gut. However, there is still little evidence that they can accelerate the alteration of parent materials or the breakdown of larger soil particles [11]. Some work with the large *Octodrilus* sp. in the Romanian Carpathians suggests that these worms are able to affect the clay mineralogy and the formation of illite in their habitat, a process that usually

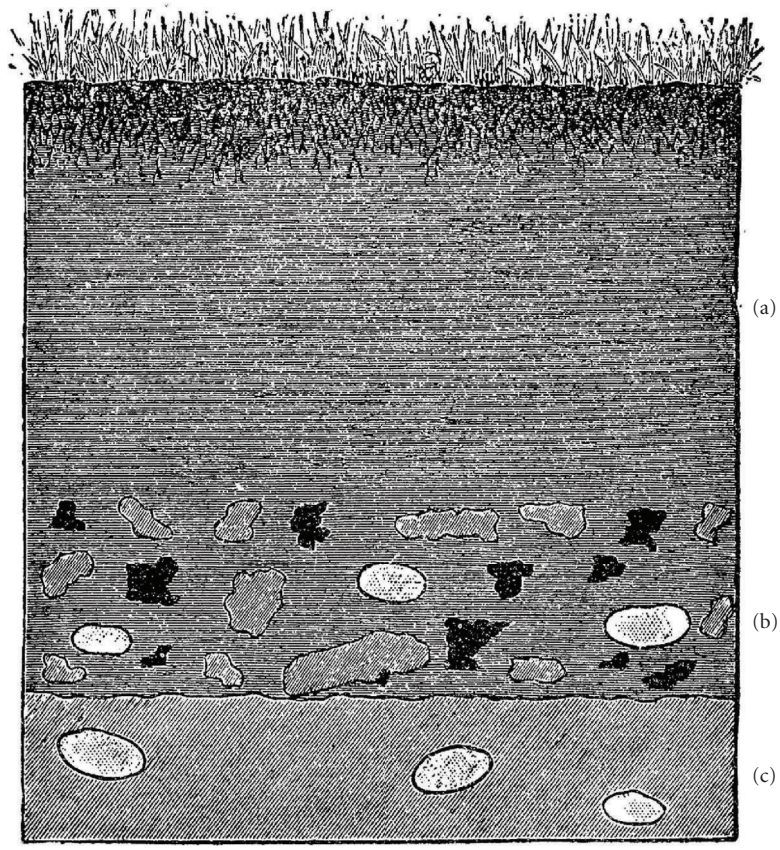

Figure 3: Cross section of the vegetable mould in a field, drained and reclaimed fifteen years previously. (a) Vegetable mould, without stones, (b) Mould with fragments of burnt marl, coal-cinders and quartz pebbles, (c) Subsoil of black, peaty sand with quartz pebbles (adapted from Darwin 1881 [2]).

takes many years [16]. If other earthworms are able to do this, then it is an important finding because sorption of radiocaesium on illitic-type clay minerals served to reduce the amount of radiocaesium entering terrestrial and freshwater food chains after the Chernobyl accident of 26 April 1986 [17]. Most of the radiocaesium became fixed in the interlayers between the platelets of the illite minerals. Thus, when the Chernobyl plume passed over Northwest England and it rained, the effects of the radiocaesium fallout varied considerably among lakes in the area and fish in the lakes [18–21]. In lakes with illitic minerals in their catchment, levels of radiocaesium decreased rapidly in the water, sediments, and fish, presumably because most of the radiocaesium was trapped in the catchment. In contrast, levels remained high in the water, sediments, and fish of lakes surrounded by acid moorland.

The most controversial section of Darwin's book dealt with earthworm behaviour and if they could be described as intelligent. This section was chiefly responsible for the popularity of the book. The poor worms were subjected to various tests, including response to touch and vibrations, strong breath and odours, a wide range of foods (e.g., fat, raw meat, onions, starch, beads, paper, leaves of various plants), and light and temperature gradients. He found that they were sensitive to touch and vibrations but not to sounds, also to odours with a "selective sense of smell", and to light, preferring darkness or very low irradiation, except when they were mating. He also concluded that they had favourite foods. Darwin observed that earthworms plug the mouth of their burrows with leaves, leaf stalks, or twigs and considered that an intelligent animal would draw such irregular-shaped objects into a cylindrical hole by their narrowest part (Figure 2). Therefore, he placed around the burrow entrance leaves of various native and foreign plants and triangular pieces of paper of various sizes. In the majority of trials, these objects were drawn into the burrows by or near their narrow apex. The only exception was pine needles that were drawn in, by, or near their base. He concluded that worms, although standing low in the scale of organisation, possess "some degree of intelligence instead of a mere blind instinctive impulse" [2, page 312].

Before considering this conclusion further, it is useful to compare earthworms with their cousins, the leeches. Both belong to the phylum Annelida and both are hermaphrodite with some segments near the middle of the body modified in mature animals to form a clitellum that secretes a cocoon for the eggs (see Figure 4). Hence, they are regarded as subclasses Oligochaeta and Hirudinea of the class Clitellata [11, 22]. Unlike earthworms, leeches are active predators [22–25]. Some species suck the blood of their prey whilst other species

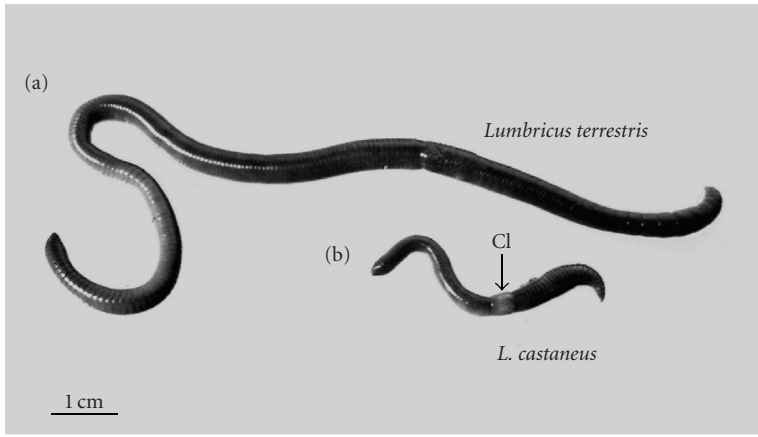

Figure 4: Photograph of two German earthworm species that inhabit grasslands, but occupy different ecological niches. *Lumbricus terrestris* (a), a burrowing (anecic) species, and *L. castaneus* (b), an epigeic earthworm. The swollen clitellum (Cl) of the sexually mature *L. castaneus* is indicated.

suck in their whole prey or devour pieces of moribund or dead animals. They have fewer segments than earthworms and a more compact, muscular, body with anterior and posterior suckers. Leeches are good swimmers but also travel by looping, using their suckers. Their sense organs are well developed so that they can detect movements of potential prey and chemicals released by injured prey. Most species have more than two pairs of eyes that can detect changes in light intensity and direction. The medicinal leech can also detect the warmest parts of its mammalian prey where it sucks its blood meal [26]. With such a range of senses, it is not surprising that leeches have a well-developed brain consisting of a fusion of ganglia in the anterior segments of the body. Leeches can therefore react rapidly to a wide range of stimuli but it would be wrong to regard them as intelligent; their behaviour is instinctive [23, 24]. Leeches can be regarded as "worms with character". The so-called "brain" in earthworms is much smaller than that of leeches, which is not surprising in an animal that is adapted to a subterranean life and is usually nocturnal. There are no definite eyes, but light-sensitive cells occur on the dorsal surface, especially at the anterior and posterior ends of the body, the regions most frequently exposed to light [11]. As noted above, they must have sense organs that are sensitive to chemicals, changes in temperature, and especially touch and vibration transmitted through solid objects. Like leeches, their behaviour is instinctive and it is wrong to describe them as intelligent animals.

4. Darwin and the Humble Earthworms: The Immediate Impact of His Book

Darwin's last monograph was published in October 1881 [2]. This book was distributed one year later in the United States of America via the publisher *D. Appleton and Company*, New York. The US-company advertised this last publication of the famous British naturalist, using a selection of sentences from book reviews that were published during the previous year (November/December 1881). We will quote here from some of these articles in order to document the immediate impact of Darwin's little "earthworm book".

In the journal *The Academy, London*, it was pointed out that "Mr. Darwin's powers of work are inexhaustible, and not less remarkable than his genius. Here is another delightful book from his pen, … One of the charms of the present work is, that it is extremely easy to read … it will delight everyone, every page being full of interest". In the *Sunday Review* we read that "Mr. Darwin's little volume on the habits and instincts of earth-worms is no less marked than the earlier or more elaborate efforts of his genius by freshness of observation, unfailing power of interpreting and correlating facts, and logical vigor in generalizing upon them. … All lovers of nature will unite in thanking Mr. Darwin for the new and interesting light he has thrown upon a subject so long overlooked, yet so full of interest and instruction, as the structure and the labors of the earth-worm".

In the *New York Graphic*, a similar, very positive evaluation was published: "The result of the author's observations is the production of proof that the small and apparently insignificant earth-worm is the cause of mighty changes in the surface of the earth, seeing that each of them, on the average, passes about twenty ounces of earth through its body every year, which earth it brings often from a depth of eight or ten feet below the surface to deposit it on the mould at the top, thus doing the work of a plow. What the result of this must be will be evident when it is known that an average of 30,000 such plows are at work in every acre of common arable land, and the worms must, therefore, work over about ten tons of earth per acre every year".

The review published in the *Brooklyn Times* emphasized the novelty of Darwin's observations and conclusions: "Darwin confers upon the despised and humble earth-worm an interest it never possessed before, and introduces it as a factor of, perhaps, unsuspected importance in agriculture. Portions of his book read almost like a romance, for there is much in his revelations of surprising strangeness and novelty. So much is seen that might be patent of the dullest eye that it

seems remarkable that so little should have been known of earth-worms before".

In the *New York World*, it was pointed out that Darwin's book is "Curious and interesting throughout". Finally, in the *Boston Adviser*, the role earthworms that have played over thousands of years are described in the following words: "Respecting worms as among the most useful portions of animate nature, Dr. Darwin relates, in this remarkable book, their structure and habits, the part they have played in the burial of ancient buildings and the denudation of the land, in the disintegration of rocks, the preparation of soil for the growth of plants, and in the natural history of the world".

These statements on Darwin's last publication and his general conclusions concerning soil biology and so forth. document that the "little book on a subject of small importance" had a large, immediate impact (Figure 1). The monograph sold so well that on 5 November 1881, less than four weeks after the book became available, a clerk of the British publisher John Murray (London) wrote to Darwin: "We have now sold 3500 worms !!!" [13]. Only five months later, on 19 April 1882, Charles Darwin died. In the following years, his "worm book" was translated into several foreign languages, but this monograph never became so well known as his work on the species problem published in 1859 [27, 28].

5. Biodiversity and Reproductive Behaviour of European Earthworms

In his most famous book *On the Origin of Species* [27], Charles Darwin (1859) did not define what species are and how they can be distinguished from varieties [29]. Decades later, Theodosius Dobzhansky (1900–1975) and Ernst Mayr (1904–2005), two of the "architects" of the synthetic theory of biological evolution of the 1950s, introduced the biological species concept that defines species as "populations of interbreeding organisms that are reproductively isolated from other such groups" [30]. Darwin's relaxed opinion concerning species definitions may have been the reason why he did not identify the species of earthworms he was investigating [28]. It is likely that Darwin (1881) [2] studied the most abundant burrowing (anecic) earthworms of Britain, *Lumbricus terrestris* (widespread), *L. friendi* (rare), *Aporrectodea longa*, and *A. nocturna* (both widespread) [11, 15, 29]. However, it is well known that in southern English grasslands, 8 to 10 earthworm species occur [29]. Hence, more than the four taxa listed above may have contributed to the "physical soil engineering" or "bioturbation" described by Darwin [2].

The common earthworm *L. terrestris* lives solitarily in vertical, aerated burrows that are 1 to 2 m deep. The oligochaetes forage and mate on the surface at night (Figure 2). After heavy rainfall and inundation of the soil, the oxygen-dependent (aerobic) invertebrates escape from their anoxic burrows and creep over the moist soil. During these forced excursions, most of the free-living earthworms are eaten by predators (birds, etc.) or die as a result of intense radiation and heat caused by the reemerging sun. These well-known, dramatic "mass destructions" of earthworms after the submergence of their burrows were not mentioned by Darwin [2]. Representative specimens of two common earthworm species that were captured after a heavy rainfall in Germany (*L. terrestris* and *L. castaneus*) are depicted in Figure 4.

As summarized above, Darwin [2] analyzed the behaviour of earthworms with reference to their sensory capacities, the construction of their burrows, nutrition, and their supposed "intelligence" in burying of leaves. However, he only briefly mentioned the reproductive biology of these terrestrial oligochaetes.

Four decades later, a detailed description of the reproductive biology of the common species *L. terrestris* was published by Grove (1925) [31]. Oligochaetes (earthworms) and hirudineans (leeches) (class Clitellata) are simultaneous (or protandrous) hermaphrodites with reciprocal insemination [23, 24]. In other words, in contrast to gonochorists, hermaphrodites function as males and as females. The mating process of *L. terrestris*, as described by Grove [31] and supplemented by more recent studies [32, 33], is depicted in Figure 5. During these nocturnal episodes, which last from one to 3 hours, the partners remain anchored in their home burrow with their tail end, which permits a rapid retreat in case of an attack of a predator. During copulation, both worms establish a contact to the clitellar region of the partner (see Figure 4). Thereafter, both earthworms bend their anterior segments away from the partner's body, which results in an "s-like" position. During this tight body contact, both partners exchange sperm and hence function as males. After reciprocal insemination is finished, the worms separate from each other, a mechanical process that can cause severe body damage due to the partner's sharp copulatory bristles (setae).

According to Michiels et al. [33], the lunar cycle affects mating activity, since the relatively high copulation frequency during dark nights (once every 7 to 11 days) is very low during the full moon. Moreover, the authors have discovered that sometimes smaller individuals are pulled out of their burrows by the larger partner after a "tug-of-war" that ends a mating episode. As pointed out by Nuutinen and Butt [32], *L. terrestris* is the only earthworm species for which mating on the soil surface has been documented. In general, the mating process in *L. terrestris* (Figure 5) is reminiscent to that of aquatic leeches of the genus *Erpobdella* and that of the European land leech *Xerobdella lecomtei* [24, 25]. Several days after copulation, the earthworms act as females and produce lemon-shaped capsules (cocoons) that contain 5 to 8 fertilized eggs via their clitellum, a process that resembles that of worm-leeches of the genus *Erpobdella* [23, 24].

6. The Discovery of the Giant Endemic Earthworm *Lumbricus badensis*

In contrast to his son Francis, who supported his father in his researches on the movements of plants, as well as the earthworm studies described here, and later became a professional plant physiologist, the geologist/biologist Charles Darwin never visited Germany. The older Darwin would have been

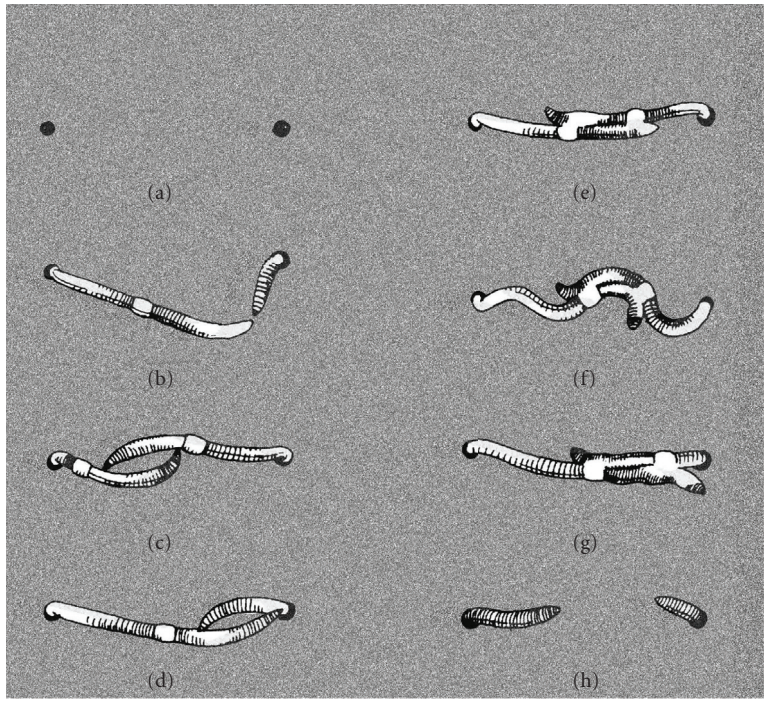

Figure 5: Precopulatory behaviour and mating in the common earthworm (*Lumbricus terrestris*), observed during dark summer nights with special equipment. Both partners remain anchored in their burrows (distance of the holes ca. 7 to 8 cm) (a) and evaluate each other in an extensive courtship process (b), (c), (d). Copulation occurs via the reciprocal attachment of the clitella (see Figure 4) and results in the exchange of sperm (e), (f), (g). After 1 to 3 hours the worms separate and retreat into their burrows (h) (adapted from [32]).

pleased to study the geology and biology (fauna, flora) of the Black Forest (Schwarzwald), a wooded mountain range in southwest Germany (Federal State Baden-Württemberg) that consists of a cover of sandstone on top of a core of gneiss. During the Würm glaciation, which ended ca. 10 000 years ago, the Black Forest was covered with glaciers. The six highest mountains are the Feldberg (1493 m), Herzogenhorn (1415 m), Belchen (1414 m), Spieshorn (1349 m), Schauinsland (1248 m), and the Kandel (1241 m above sea level). The dense forests consist mostly of pines (*Pinus sylvestris*) and Norway spruce (*Picea abies*), which are grown in many places as commercial monocultures. In addition, beech (*Fagus sylvaticus*) forests form integral parts of the lower regions of this unique landscape in Southern Germany.

More than a century ago, the German annelid specialist W. Michaelsen investigated the soil of the southern part of the Black Forest, but no common earthworms (*L. terrestris*) were found in this habitat. However, he discovered a single individual of an unidentifiable earthworm that he later described as *L. papillosus* (Syn. *fiendi*) var. *badensis* [34]. Hence, Michaelsen interpreted his giant earthworm from the Federal State Baden-Württemberg as a local (geographic) variety of the common Atlantic-Mediterranean taxon *L. friendi*. Later, it was discovered that this large earthworm (Figure 6) represents a separate biospecies that is not closely related to the widespread *L. terrestris* but is a sister taxon of *L. friendi* [35], a relatively small species (length ca. 12 cm) that Darwin [2] may have studied in Great Britain [10, 13, 29].

In contrast to the common earthworm (*L. terrestris*), which can reach a body length of 15 cm (diameter at rest ca. 0.6 cm) (Figure 4) and does not cooccur with *L. badensis*, adult Black Forest-worms (Figure 6) are up to 34 cm long with diameters of 1.2 to 1.6 cm (body mass: 25 to 40 g). When fully extended, adult *L. badensis* individuals can reach a length of up to 60 cm [35] and hence are on average as large as the common limbless burrowing reptile *Anguis fragilis*. This vertebrate is also known under the name "slow worm" and has been confused with *Lumbricus badensis*. In Figure 7, an adult *L. badensis* that was isolated from its burrow and an *A. fragilis* of average length are juxtaposed. Both animals were collected in the same habitat. The enormous body size of the giant Black Forest earthworm becomes apparent [35].

In forests with large litter layers, the usually deep-digging (anecic) species *L. terrestris* can live epigeic, without the construction of a burrow [37]. Darwin [2] would have been surprised to hear that one of his "common worms" has, in certain European habitats, adapted to such an alternative way of life. Detailed studies have shown that the Black Forest earthworm (Figures 6 and 7) displays a switch from an epigeic to an anecic (burrowing) way of life during its ontogeny. This aspect of the life cycle of *L. badensis* is described in the next section.

7. Biogeography, Evolutionary Origin, and Ecology of *Lumbricus badensis*

After decades of research it is now definitively clear that the giant earthworm *L. badensis* is a neoendemic species that inhabits exclusively the acid soils in a relatively restricted

Figure 6: Photograph of a giant Black Forest earthworm (adult individual of *Lumbricus badensis*) in its natural habitat. Note that the worm is anchored with its anterior body part in its burrow (adapted from [36]).

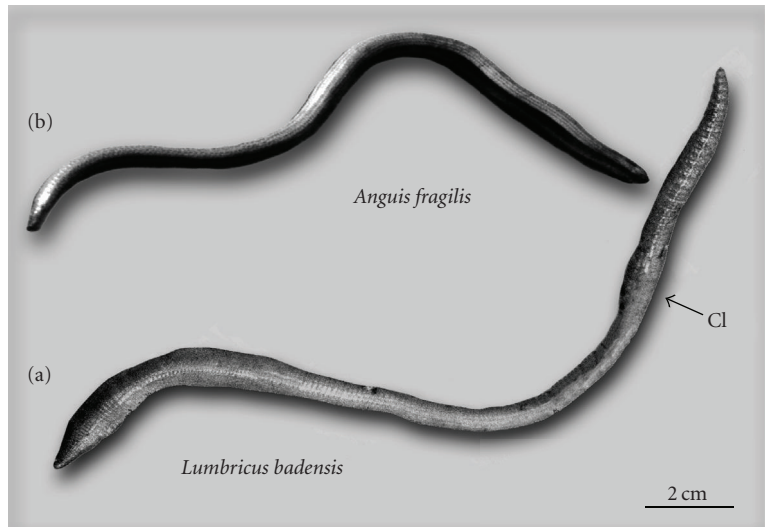

Figure 7: Photographs of adult individuals of the giant earthworm (*Lumbricus badensis*) (a) and a limbless reptile (*Anguis fragilis*), the "slow worm" (b), collected in the same habitat in the Black Forest (Schauinsland, Southern Germany, ca. 1200 m above sea level). Note that the worm and the reptile are about the same size. The heads of both animals point to the right side. Cl=Clitellum.

area of the Black Forest, a region where no other *Lumbricus*-species occur [36, 37, 39]. Detailed biogeographic studies on the occurrence and habitats of the sister taxa *L. friendi* (length ca. 12 cm) and *L. badensis* (length up to 34 cm) revealed that, after the end of the last ice age (ca. 10 000 years ago) founder populations of the smaller and more widespread ancestor (a species closely related to extant *L. friendi*), were separated via the river Rhine and hence became geographically isolated [37]. The young founder populations of ancient *L. friendi*, which may have originated during a time period between 8000 and 6000 years ago, rapidly occupied the new habitat in the Black Forest in regions from 300 to 1400 m above sea level, where presumably no other competing earthworm species lived. As mentioned above, the common species *L. terrestris* does not cooccor with *L. badensis,* possibly due to the high acid content of the soil that the Black Forest worms inhabit. In this specific habitat, which represented a vacant ecological niche at that time, the geographically separated Atlantic earthworms established a new, reproductively isolated biospecies. Due to the large difference in body size and hence the dimension of the corresponding clitellum, no copulations are possible between extant *L. friendi* and *L. badensis*. Zoogeographic studies along the narrow regions in Southwest Germany, where both species co-occur, have never found hybrids [35, 39]. It follows that Ernst Mayr's model of allopatric speciation accounts for the evolutionary origin of the endemic Black Forest-earthworm *L. badensis* [30, 40]. However, the question why *L. badensis* evolved such an enormous body size within only a few thousand years is not yet answered. It is likely that these neoendemic earthworms rapidly adapted to the new habitat where predators were abundant and hence larger individuals in the variable founder populations had a better change of survival, but more work is required to further corroborate this hypothesis. As an alternative, it has been postulated that specific environmental conditions, such as the composition and structure of the soil, were factors that caused the selection and survival of larger individuals

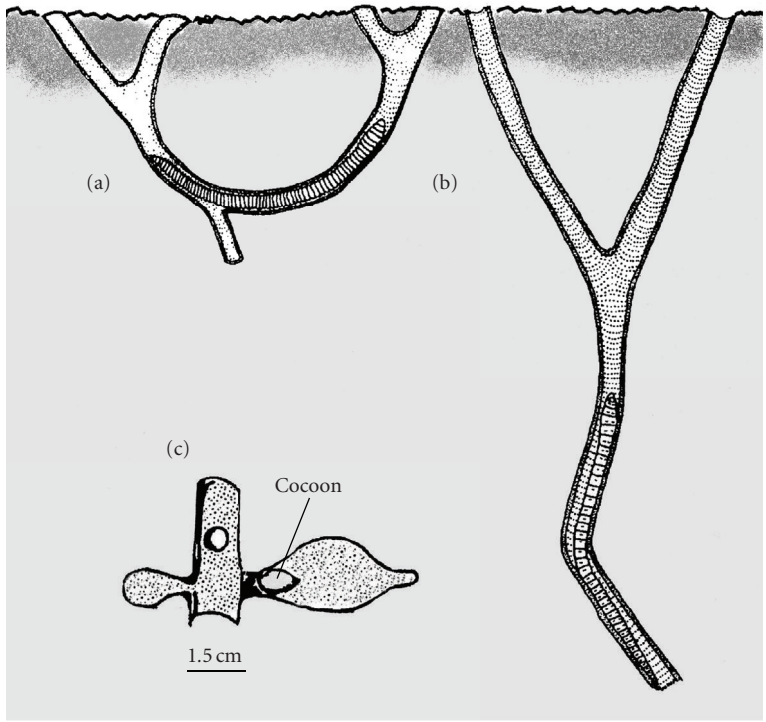

Figure 8: Schematic drawings of the burrows of juvenile (a) and adult (b) individuals of *Lumbricus badensis*. At depths of 40 to 150 cm below the soil surface, along burrows of adult *L. badensis* individuals, cocoon chambers with egg-capsules have been observed and documented (c). This indicates that parental investment is part of the reproductive strategy in this endemic earthworm species (adapted from [37]).

over thousands of subsequent generations [35], but no direct evidence supports this idea. It should be noted that, according to Wikelski [41], a number of hypotheses have been proposed to account for the evolution of body size in animals. Unfortunately, no consensus has yet emerged as to a general explanation for this phenomenon.

Twenty five years ago, the burrows of juvenile and adult *L. badensis* were investigated in fir-beech forests located in the southern part of the Black Forest about 1000 m above sea level [35, 37]. After hatching from the cocoons, which are deposited in chambers located 40 to 150 cm below the soil surface (Figure 8(c)), *L. badensis*-individuals are 5 to 7 cm long (body mass: 0.4 to 0.6 g). It should be noted that nothing is known about the mating behaviour in this earthworm species. The juvenile worms crawl upwards until they reach the soil surface. Most of the newly hatched individuals, which are found during the spring, build horizontal tubes with their casts, usually between the soil surface and pieces of bark, and so forth. One to 2-year-old earthworms (body mass: 1.5 to 2.5 g) construct U-shaped burrows (Figure 8(a)) that are similar to those of adult *L. terrestris* (see Figure 2) [42]. Older juveniles with body masses of more than 2.5 g construct deep, V-shaped burrows (depth ca. 2.5 m) that are indistinguishable from those of the adults. A characteristic feature of all of the *L. badensis* burrows investigated in the Black Forest is that the tube splits into several (2 to 6) outlets near the soil surface (Figure 8(b)). In the burrows of adult earthworms, several cocoon chambers along the main tube were found in the region at 40 to 150 cm below the soil surface (Figure 8(c)). As in semiaquatic leeches of the genera *Hirudo* and *Haemopis* [24, 25], the giant earthworm constructs brood chambers for the next generation. Hence, parental investment has evolved as a survival strategy of the populations in this endemic Black Forest earthworm. It is not known whether such a sophisticated mode of parental investment occurs in any other earthworm species.

At any rate, Charles Darwin, who explicitly pointed out that his metaphorical "struggle for life" does not only mean the competition for limited resources but also includes the care for young by adults, and hence nonselfish, cooperative behaviour [43], would have been pleased if he had known that one European "worm species" had evolved such an "intelligent" mode of reproduction. In addition, it is obvious that the earthworm burrows are a striking example for "niche construction", that is, the active modification of the habitat of organisms with positive consequences for survival and mode of reproduction [12, 44].

8. Conclusions

In 1837, one year after his return from the voyage of the *Beagle*, Charles Darwin started his career as an independent scientist with observations and a subsequent speech on earthworms that was published in 1838 [4]. Almost 45 years

Figure 9: Cartoon by E. L. Sambourne, published in the *Punch* in 1882 with the sentence "Man is but a worm". This parody of Charles Darwin's concepts on the origin of humanity has been corroborated by recent molecular data on the phylogenetic relationships of annelids and vertebrates (adapted from [38]).

later, he ended his life with the publication of a little book on "worms" [2]. This became so popular that a famous cartoon connected ancient annelids, via intermediate forms, with the human species, represented by Charles Darwin (Figure 9). It should be noted that Darwin's monograph rapidly modified the perception of earthworms by society. Up to then, earthworms were considered by gardeners, agriculturists, and so forth, as soil pests that have to be eliminated—Darwin's work changed this belief forever and finally led to the concept of bioturbation as well as the discipline of soil biology [3, 10, 13].

It is likely that Charles Darwin [2] was referring to the common species *L. terrestris* when he pointed out that earthworm burrows are "... not mere excavations, but may rather be compared with tunnels lined with cement" [2, page 112]. Hence, according to the British naturalist, earthworms actively construct their home according to their needs. This is one of the earliest examples for the concept of "niche construction" we could find in the scientific literature on the evolution of macro-organisms on Earth [12, 44].

We conclude that Darwin's monograph on the biology of earthworms was not simply a "curious little book of small importance" [1], but a significant work that is still cited today in a variety of scientific disciplines [3, 28]. Finally, we want to point out that modern "earthworm research", which originated with Darwin [2], yielded the insight that geographic separation of founder populations can result in the "creation" of new *Lumbricus* species within a time period of less than 10 000 years [35]. In his masterpiece *On the Origin of Species* [27], Charles Darwin argued that speciation events are too slow to be observed (or reconstructed) within the lifetime of one human being. He would have been pleased to read that, 150 years later, earthworm researchers have elucidated a rapid speciation event that occurred after the end of the last Ice Age in a restricted area of the south western part of Germany.

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Review Article

Basic Research Tools for Earthworm Ecology

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Earthworms are responsible for soil development, recycling organic matter and form a vital component within many food webs. For these and other reasons earthworms are worthy of investigation. Many technologically-enhanced approaches have been used within earthworm-focused research. These have their place, may be a development of existing practices or bring techniques from other fields. Nevertheless, let us not overlook the fact that much can still be learned through utilisation of more basic approaches which have been used for some time. New does not always equate to better. Information on community composition within an area and specific population densities can be learned using simple collection techniques, and burrowing behaviour can be determined from pits, resin-insertion or simple mesocosms. Life history studies can be achieved through maintenance of relatively simple cultures. Behavioural observations can be undertaken by direct observation or with low cost webcam usage. Applied aspects of earthworm research can also be achieved through use of simple techniques to enhance population development and even population dynamics can be directly addressed with use of relatively inexpensive, effective marking techniques. This paper seeks to demonstrate that good quality research in this sphere can result from appropriate application of relatively simple research tools.

1. Introduction

There is no need to make a case for studying earthworms, as their role within the soil has been recognized for more than a century [1]. Collectively, these organisms are able to pass vast quantities of soil through their guts and by doing so bring about the creation of an improved crumb structure which incorporates mineral and organic elements and can become a seedbed for plant growth [2]. In addition, earthworms may aerate soils and increase water infiltration, hence reducing soil erosion, by burrow creation [3]. On top of all this some species are more highly regarded as they are attributed with ecosystem engineering capabilities; that is, they are able to directly influence the environment around themselves and the availability of resources to other organisms [4].

Many avenues of research are available and this article could very easily seek to review and critique some of the more advanced techniques currently in use within the sphere of earthworm ecology. These might include DNA-related work examining the genome of selected species [12]; ecotoxicology, following the accumulation of, for example, heavy metals in the tissues of earthworms on contaminated land [13]; or, for example, isotopic work, looking at the transfer of radio-labelled elements through earthworm-linked food chains [14]. However, such relatively high-tech methods will not be the focus of this work, which seeks to generally avoid reliance upon potentially costly and high-maintenance equipment. This article actually aims at doing one thing; it seeks to show that the use of low-technology methods is still able to gain insights into fundamental questions relating to earthworms. Much is still to be fully understood about this group, and although many advances have recently been made using sophisticated, expensive equipment/techniques, there is still room for the under-resourced professional or educated amateur to make a serious contribution. To demonstrate this, the article focuses on the following: a description of simple collection techniques, which can assist in revealing a great deal of earthworm community structure, followed by investigation of a major earthworm activity—burrowing and then a close inspection of earthworm life history and behaviours. Each aspect will hopefully show that basic techniques exist within earthworm ecology that can reveal previously unknown information and assist in building a more comprehensive picture of this important animal group.

Table 1: Recent British examples of earthworm density/biomass/community structure from sampling with the same techniques (digging and application of a mustard vermifuge).

Location	Habitat	Sampling date	Earthworm density (No m^{-2})	Earthworm biomass (gm^{-2})	Earthworm species	Dominant species	Reference
Aughton Woods, Lancs	Deciduous woodland	Oct 2006	37	29.8	Ach; Dr; Lt; Oc	Oc	[5]
Aughton Woods, Lancs	Pasture	Oct 2006	183	110.9	Ach; Ac; Al; Ar; Lr; Lt; Oc	Ac	[5]
Meresands Wood, Lancs	Dry Heathland	Oct 2001	167	75.0	Ach; Ac; Al; Ar; Et; Lc; Lr; Lt; Oc	Ac	[6]
Wistman's Wood, Devon	Upland oak woodland	May 1999	13	9.3	Le; Lr; Dr	Dr	[7]
Down House, Kent	Pasture	March 2004	310	149.6	Ach; Al; Ar; Lr; Ot; Sm	Ot	[8]
Down House, Kent	Kitchen Garden	March 2004	715	261.0	Ach; Ac; Al; Ar	Ach	[8]
Isle of Rum, Scotland	Upland moorland	May 2000	9	3.0	Do; Dr; Lr	Dr	[9]
Malham Tarn, Yorkshire	Pasture	May 1998	291	86.1	Ach; Ac; Al; Ar; Do; Lc; Lt; Oc	Ach	[10]
Newton Rigg Farm	Winter Barley	April 2006	18	6.0	Ach; Al	Al	[11]
Newton Rigg Farm	Conservation Headland	April 2006	118	76.2	Ach; Ac; Al; Ar; Do; Lr; Oc: Sm	Al	[11]

Key: Ach: Allolobophora clorotica; Ac: Aporrectodea caliginosa; Al: Aporrectodea longa; Ar: Aporrectodea rosea; Do: Dendrobaena octaedra; Dr: Dendrodrilus rubidus; Et: Eiseniella tetraedra; Lc: Lumbricus castaneus; Le: Lumbricus eiseni; Lr: Lumbricus rubellus; Lt: Lumbricus terrestris; Oc: Octolasion cyaneum; Ot: Octolasion tyrtaeum; Sm: Satchellius mammalis.

2. Collection Techniques (First Catch Your Earthworm)

It is often desirable to quantify earthworm number or biomass in a given habitat and/or seek to collect them. A few species show their presence by surface casting (e.g., *Aporrectodea longa*) or creation of middens (e.g., *Lumbricus terrestris*) but most require some form of intervention to locate them, due to their totally subterranean existence. To this end, various techniques have been developed to enable earthworm collection. Digging is the simplest, as it requires only a spade and perhaps a quadrat for density calculations but may detect only near surface (epigeic) earthworms and horizontal burrowing (endogeic) species. Adults of deeper burrowing (anecic) species may be missed unless the researcher is prepared to dig a hole to a depth of several metres!

An alternative to digging is the application of a vermifuge (expellent), which when poured on to the soil drives earthworms to the surface as it acts as a skin irritant when contacted in their burrows (direct application, e.g., via a syringe to *L. terrestris* burrows may be very effective). Various chemicals have been used, with a dilute solution of formaldehyde (formalin) currently recognized as a standard [15], but as this has been reported as carcinogenic, further options have been sought. It is also suggested [16] that there are severe negative effects to other soil fauna, soil respiration, and vegetation cover if formaldehyde is applied. A suspension of table mustard in water has been used [17], but tests [10] have shown that a suspension of mustard powder (e.g., 50 g in 10 litres water) is both cheaper and more effective. More recently use of "hot" mustard has been used to give a more consistent index of earthworm abundance across a range of soil types [18]. As the type of mustard may also affect results, an extract derived from mustard seed Allyl isothiocyanate (AITC) has been used for earthworm collection [19]. AITC has recently been shown as a reliable and promising chemical expellant whether or not used in combination with hand-sorting [20]. Many researchers now advocate that the most effective collection technique is indeed a combination of digging and hand-sorting of soil (deposited e.g., on a plastic sheet in the field) followed by application of a vermifuge to the hole created [10, 20]. Different techniques have in the past given rise to differential collection of species and provided results which are not directly comparable. By contrast, Table 1 provides recent examples of data relating to earthworm density, biomass, and community structure from a variety of British habitats using the same combination of digging and mustard application for collection.

Another collection method is application of an electrical current to the soil. This method is attractive as little or no damage is done to the area sampled and only fallen leaves and overgrown vegetation need be removed prior to sampling to assist earthworm detection. To date only limited work has been undertaken with this method, specifically in agricultural soils [21] possibly because equipment is expensive as an extraction unit to sample 0.2 m^2 at a time will cost (at 2009 prices) in excess of $3000.

Figure 1: Earthworm enclosures (1 m^2) used for manipulating density of *Lumbricus terrestris* in managed woodland (earthworms added to enclosure in foreground).

Figure 2: Plan view of a tunnel trap showing a mature *L. terrestris* exiting a 20-litre mesocosm into the attached 1-litre capture pot (lids removed). Insert shows a lateral view of the whole setup.

Having determined which earthworms are present in a given habitat, if desired, it is then possible to experimentally manipulate the earthworms themselves or resources, such as food, in the habitat. Several studies have used field enclosures to investigate the effects of earthworms on soil properties and plants [22, 23]. Such enclosures can be formed with PVC walls, buried in slit trenches to a depth of up to 45 cm and a height of 15 cm above the soil surface. These have been shown to act as effective barriers to lateral earthworm movements. Results have suggested that both earthworm removal and addition of field-collected earthworms within enclosures can be an effective and useful approach for assessing the influence of earthworms on ecosystem processes (see Figure 1).

Associated with earthworm enclosures is a novel method ("tunnel" trapping) that can be used to observe and record emigration of earthworms. Trap units can be combined with earthworm fencing in the field [24], or with mesocosms in laboratory experiments allowing examination of emigration rates, while manipulating biotic and abiotic factors (e.g., population density, community structure, predation, resources availability, temperature, precipitation).

Tunnel traps can be prepared using 1 litre plastic pots with mounted needle-perforated lids. Holes ($r = 6$ mm) drilled in these smaller "capture pots" just below the lid allow insertion of PVC tubing (10 mm ID, 5 cm long) to connect to either earthworm fencing in field enclosures or larger soil-filled mesocosms. Surface migrating species can move from enclosures/mesocosms into traps via the tubing that is aligned at the soil surface (Figure 2). Movement of captured individuals back into containers is prevented by filling capture pots with soil or other suitable medium to half of their total volume. Providing acceptable conditions (e.g., soil and food) in capture pots can allow earthworms to survive for long periods therefore permitting relatively infrequent examination. Tunnel traps have been successfully used in both field and laboratory experiments which aimed to examine dispersal of the anecic *L. terrestris* as affected by population density and resources availability [24].

The types of simple investigation associated with earthworm sampling should allow some of the following questions to be answered.

(i) Which species of earthworms are present within the community in the given habitat?

(ii) At what densities (number m^{-2}) and biomasses (gm^{-2}) are these animals present?

(iii) What proves to be the most efficient method for collection of given earthworm species?

(iv) Can populations be experimentally manipulated to test density-related hypotheses (using addition/removal, fencing, and trapping)?

3. Burrowing and Burrow Morphology

As with unearthing which species are present, as previously described, working out which species are active and at what depths is not so simple. Again, it usually requires some form of intervention as many earthworms are relatively small and generally live below the surface of the soil. However, some species do proclaim their presence by depositing their casts (faeces) on the soil surface. This is particularly true of larger species which may be digging burrows and have relatively large amounts of earth to dispose of and others which are almost constantly "head down" and "bottom up" producing surface casts. In temperate soils a good example of this is *Aporrectodea longa* (the black-headed or long worm). When present at high densities, this species is capable of almost totally covering the grass surface of a pasture with casts. It has been suggested that the amount of casting could even be used as a proxy for the density of (known casting) species present in an area [25]. Where the spread of *A. longa* was being followed, after introduction to an unpopulated site, casting activity was used to follow dispersal of this species through the soil over many years [26, 27]. Another deep burrowing earthworm which provides signs of its presence on the soil surface is *L. terrestris*. This species constructs "middens" and these structures are normally engineered above the opening of the near vertical burrow used by this animal. Scientists have been aware of such structures since Darwin's day, but the precise function is still uncertain. Middens consist of organic (e.g., leaf) and inorganic (e.g., pebble) materials gathered together by the

resident earthworm and often cemented together with casts. Regulation of burrow temperature and moisture content may be an obvious function, but protection from predators and provision of a food store (a minicompost heap) may be others [28]. Whichever way, the midden and associated burrow forms an integral part of the life of this relatively sedentary earthworm. Recent work [29] has also revealed that many other earthworm species are associated with *L. terrestris* middens compared with adjacent nonmidden soil; so middens may play a major part in determining distribution of other earthworms at a microscale.

Nevertheless, most earthworms are mainly active below the soil surface; so most investigations need to proceed within the soil. Using burrows that open at the surface, such as those of *L. terrestris,* is one way. Observations have shown that large burrows (often referred to as macropores; diameter 8–10 mm) may have the capacity to accept relatively large volumes of rainwater and assist with prevention of surface soil erosion. Testing of this type of water entry into the soil is easily undertaken. The simplest method is to create a water-tight, isolated area at the soil surface (an infiltration "ring") covering a known surface area and then add a known volume of water to that area and record the time taken for all water to enter the soil. Comparing different areas within a given habitat/field can be very revealing, particularly when coupled with earthworm collection from the same areas. A slight elaboration on this technique is to use a vertical column of water (Marriot device) which can be fed directly into a single burrow. Such work investigated the burrow systems of *L. terrestris* in agricultural systems [30]. Infiltration of water into burrows was examined with the resident earthworm present or after its removal (with a vermifuge)—the earthworm itself forming something of a plug. To further quantify and equate water ingress with burrow morphology, efforts were made to assess the volume of individual burrows. This was finally achieved by the use of a polyurethane resin, poured down the burrow and allowed to set hard [30]. Subsequently the solid representation of the burrow void was dug out by excavation of a pit alongside. Use of coloured pigment within the resin makes visual inspection in situ and after extraction much easier [31] (see Figure 3). A simpler technique than use of resin is use of coloured dyes. Dyes such as methylene blue in water can be poured into burrows or cracks in the soil [3] and then the area around excavated to see the extent of burrow systems present.

If access to a large digging machine is possible, then excavation of a pit in any soil can be very revealing. As mentioned "resin-cast" burrows can be revealed, but unadulterated burrows, if large enough, may also be seen. For example, during an investigation undertaken during a period of frost depth to 0.5 m, [32] it was possible to follow burrows down to a depth of 1 m by "picking away" at the exposed soil profile with knives. This investigation, more interestingly, revealed much on the behaviour of *L. terrestris* and the (usually) shallow working *Aporrectodea caliginosa* during relatively cold periods. However, should it prove impossible to create a large soil pit, then it is possible to consider the activities of earthworms under more controlled conditions in a nonfield setting.

Figure 3: A burrow of *Lumbricus terrestris* filled with white-coloured resin and exposed in the soil profile to its terminal depth at 1 m.

A soil pit exposes a cut surface through the soil profile, which is in essence a 2-dimensional view. This can be recreated by production of what might be viewed as a "wormery"—a structure comprising 2 sheets of glass separated by a very small distance, for example, 5–8 mm. Such structures not only have in the past been sold for domestic use (by children) to observe earthworms but also have a more research-focussed application. Early work [33] allowed use of such structures to observe the burrow formation of earthworms, and more recently these "Evans' boxes"—also referred to as 2D mesocosms—have been used [34] for similar aims but more specific objectives (see Figure 4). These workers examined the burrowing of *L. terrestris* but were specifically interested in the interactions between the various life stages of this species and found, until then, previously unrecorded aspects of cocoon deposition in side chambers and encasement of these cocoons with castings (see Figure 5). Such findings clearly demonstrate that observations of this type can reveal burrow-related behaviours which may have some significance in the life of these animals and not have been recognised before, even though this is a very well-studied species [28]. Table 2 shows some of the experimental data also gathered from this investigation.

Other ways of tracking earthworm burrows and assessing burrowing behaviours under controlled conditions are available and might be thought more appropriate as they do not occur in two dimensions. Soil cores can be extracted from the field (within suitable housing such as plastic cylinders), for example, by driving these into the soil from above and then maintaining them for the desired purpose. This may be to examine earthworm communities within and how they may assist other ecosystem process, for example, by comparing intact cores with those frozen to remove earthworms. Relatively recently, use of X-ray tomography

Table 2: Details of cocoons and hatchlings of *Lumbricus terrestris* (mean ± SD) produced under a number of adult manipulations in Evans' boxes, kept at 17 °C in darkness (CTRL: no manipulation; CLtRm: earthworm removed and reintroduced; LtRp: earthworm removed and replaced by another; LtRm: earthworm removed—adapted from [34]).

Treatment	CTRL	CLtRm	LtRp	LtRm
Total cocoons	27.8 ± 2.2	24.8 ± 9.0	26.2 ± 7.3	12.6 ± 2.7
No spent cocoons	2.0 ± 2.2	3.3 ± 2.6	5.4 ± 3.0	1.8 ± 2.5
Hatchlings	1.8 ± 2.9	2.5 ± 3.1	2.2 ± 1.6	1.6 ± 1.8
Hatchling survival (%)	67 ± 29	71 ± 34	47 ± 33	67 ± 24
Hatchling mass (g)	0.19 ± 0.22	0.09 ± 0.06	0.10 ± 0.04	0.40 ± 0.38

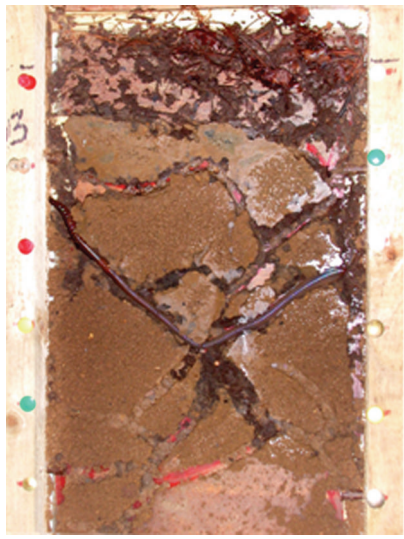


Figure 4: Upper 30 cm showing view through the glass side of an 80 cm deep Evans' box used to examine burrowing behaviour of a single mature *L. terrestris* (the adult can be seen across the centre).

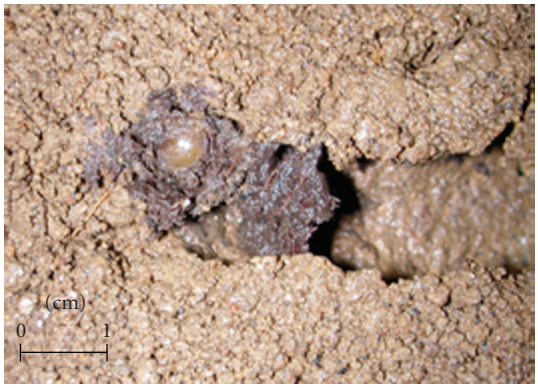


Figure 5: Detail of a side burrow with *L. terrestris* cocoon encased in parental casting seen in an Evans' box with one glass side removed (to permit better photography).

[35] has been used to determine burrow configurations in such cores. Whilst this may be a useful tool; it is one which required access to hospital-grade equipment so it cannot be considered basic. However soil cores can be utilised to study relatively simple "ecosystems" with earthworms as a component. These may allow examination of different animal species present and also plants growing at the soil surface, if kept in glasshouses. Inputs and out flows could also then be measured in simple terms. Taken to extreme lengths, researchers have developed systems such as the "Ecotron" [36] which has incorporated earthworms into its experimental systems but this facility was produced at a cost of $1.5 million. Despite this cost and sophisticated equipment for measuring in and out flows of gases and liquids, the choice of earthworm species, as a part of a biodiversity and ecosystem behaviour experiment [37], may not have been appropriate to the given mesocosms. Once again, a situation, where most expensive and modern, does not necessarily mean most appropriate and insightful. Much more simple investigations in sealed mesocosms (pots) may not give rise to the bigger ecosystem "picture" but may provide good data on earthworm life histories (see below).

Surface-related and burrow-associated investigations might enable some of the following questions to be addressed.

(i) Which species are present at which horizons/depth in the soil profile?

(ii) What can be learned from earthworm activities at the soil surface?

(iii) Do burrows assist water infiltration?

(iv) How can earthworm burrow extent and volume be measured?

(v) Can the field (cores) be brought into and utilised in a controlled setting?

(vi) Can mesocosms be used to observe earthworms burrowing behaviour more closely?

4. Life History Studies

Many species have been well documented and much is known of their life history, but for example, ask any researcher to tell you what age an earthworm can live to, or which life stage is responsible for dispersal and you may find that no simple answer is forthcoming (even for *L. terrestris*). Great scope exists for gathering fundamental information on aspects of the life histories of most earthworm species. In Britain, where earthworms are reasonably well documented and a synopsis of species has been available in a number

of revised forms for over 60 years [38, 39], information is still lacking in a number of quarters. For example, *Dendrobaena attemsi* is described from a single British record from Cumbria; yet we have collected this species easily from wooded areas on the Isle of Rum in Scotland. Equally for the same species, and more importantly with respect to life history, entries such as "presumably biparental" and "capsules unrecorded" [39] show that much is still to be learned—and perhaps this can be achieved relatively simply.

Wherever a researcher is based, there will be opportunities to collect local species of earthworm, as previously described. Providing that identification is not a problem, there are then chances to answer basic questions on the life history of the species. Using the soil from where the animals were collected, it should be possible to maintain them in containers of a chosen size, appropriate for the given species and its ecological group. The situation is to then ask relevant questions and seek to answer these through segregation of life stages and sampling at given time intervals. An initial question might relate to the mode of reproduction shown by the given species; is it amphimictic (requiring sexual reproduction) or parthenogenetic? To solve this, in the least amount of time, immature individuals need to be isolated and kept thus until they mature. This will naturally require consideration of their requirements in terms of, for example, soil, food, moisture, temperature, and space [40]. Inspection at appropriate time periods, monthly, weekly, or more frequently for rapidly maturing animals will determine when maturity (possession of a swollen clitellum) is reached. At this point the animals might usefully be subdivided into two groups 1 : 2. The smaller third should be left in isolation and the larger two-thirds put into groups of two to give an equal number of singletons and pairs. These labelled containers can then be monitored for cocoon production over the following weeks.

Sampling for cocoons can be straightforward and require a water supply and a mesh of appropriate size—depending on cocoon size—which is a function of clitellum diameter. Contents of containers in which adults have been kept can be sieved to obtain cocoons. These can then be incubated in Petri dishes, or equivalent, on moistened filter paper or similar at an appropriate temperature for the given species [40] (Figure 6). If animals have been kept, for example, in soil columns, then the depth at which cocoons are deposited might be considered by sieving away different levels from the column (more easily achieved if the cylinder in which they are housed is presplit (and taped together) along its length [41]). Incubation of cocoons can then occur and time to develop and hatch can be monitored. To obtain cocoons more rapidly for any species, mature animals which are field-collected can be employed directly in cocoon production studies and number produced per individual per time can be recorded from the given conditions under which they are maintained. Cocoons may be kept in groups or individually (depending on space available). The advantage of individual incubation is that the number of hatchlings can more easily be assessed, as many epigeic species produce more than one hatchling per cocoon. To complete life cycle records, growth of hatchlings to maturity can be assessed. This requires the

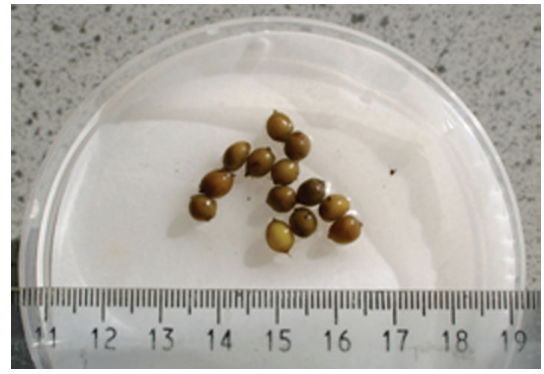


Figure 6: Freshly produced cocoons of *L. terrestris* housed on a moistened filter paper in a Petri dish. These were produced by 1 earthworm over 1 month.

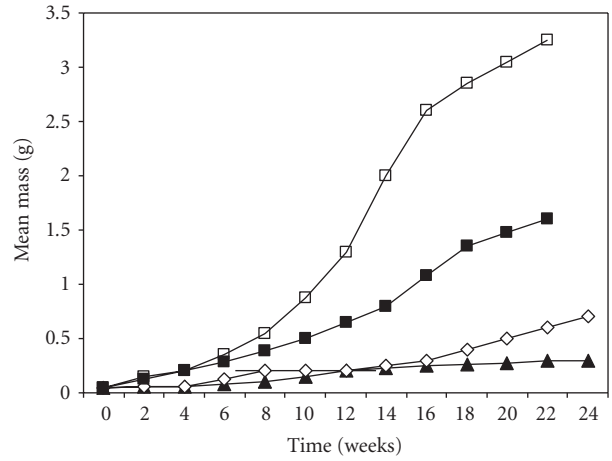


Figure 7: Typical earthworm growth curves obtained from periodic measurement of masses. Here *Lumbricus terrestris* was examined under constant temperature conditions (□ − 20; ■ − 15; ◊ − 10; ▲ − 5°C; adapted from [44]).

type of conditions previously described but with periodic monitoring (and mass determination) until maturity is reached (see Figure 7 for typical results). Manipulation of biotic and abiotic factors influencing the growth and reproduction of the earthworms, such as population density [42], food quality [40], interspecific interactions [43], temperature [44], and a host of others and combinations thereof, can be considered. Finally to ascertain the age to which earthworms can live, animals might need to be kept for some time.

One relatively simple technique that might assist life history/population studies is the ability to permanently mark (tag) individual earthworms. Recent work [45] has shown that it is possible, through injection of Visual Implant Elastomer (VIE), available from Northwest Marine Technology [46] to visually colour tag earthworms. In addition these tags have been shown to be retained in a number of earthworm species for more than 2 years and have no detrimental effects on growth to maturity, mating, and cocoon production in one closely studied species, *L. terrestris* [47]. This technique

may well prove to be valuable in earthworm age determination but may also reveal much from studies of population dynamics, in terms of capture, mark recapture exercises. Tagging captured animals and then recapture data could assist in learning much more of these organisms particularly in a variety of habitats. Cohorts of the same species could be tagged with different colours in different years to permit a better understanding, for example, of survival. Used in combination with density manipulation experiments, this type of exercise has already revealed aspects of *L. terrestris* dispersal and settlement under field conditions in managed woodland plots [24]. It should be noted that at current (2009) prices, this material is relatively inexpensive, with a trial pack of VIE costing $42. Such an amount will permit tagging of hundreds of earthworms (see Figures 8(a) and 8(b)).

By collecting earthworms and maintaining them under controlled environmental conditions, it ought to be possible to answer most of the following questions on life histories.

(i) What mode of reproduction is exhibited by a given species?

(ii) Where in the soil are cocoons produced?

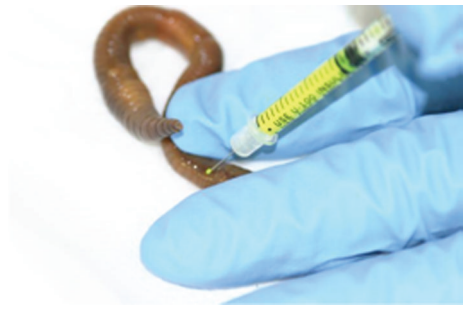
(iii) How long does cocoon incubation take before hatching occurs?

(iv) How many hatchlings are produced per cocoon?

(v) How long does it take for growth to maturity and at what mass is this reached?

(vi) Which factors (biotic or abiotic) may have a major influence on the above?

(vii) To what age does this species live?



(a)

(b)

Figure 8: (a) Injection of yellow Visual Implant Elastomer (VIE) into *Lumbricus terrestris*; (b) *Octolasion cyaneum* with a red VIE tag inserted.

5. Behavioural Studies

Many activities of earthworms, known for decades, still present uncertainties in terms of interpretation. Equally, where glimpses of the subterranean world are provided, much can be learned. Some behaviours relating to burrowing and casting have already been discussed; so this section will concentrate on just a small number of behaviours, such as mating and dispersal and focus on techniques which may be of use to further investigate them. Many authors have reported mass emergence and dispersal of earthworms [2]. The timing of this may be seasonal or associated with particular weather conditions. The species concerned may vary but perhaps this behaviour has a common underlying cause? Often occurring at night, in urban settings it might normally go unnoticed, but for the fact that "stranded" earthworms may be found the following morning on surfaces such as concrete or tarmac, into which they are unable to burrow.

En masse emergence of earthworms may often follow periods of rain. Opinions vary, but some suggest that this is a function of earthworms exiting their burrows as a response to inundation which might lead to potential death. We dispute this idea, as earthworms are able to survive lengthy periods of submergence in water, and support the hypothesis [28] that it is more closely related to dispersal. A question posed earlier related to the life stage at which earthworms disperse. Perhaps other related questions that need addressing are why would earthworms seek to disperse and what factors might encourage this? The "why" part may relate directly to evolutionary biology. Even though they are hermaphrodite, many earthworm species show sexual reproduction. Therefore mating may normally occur with near neighbours. To bring about greater possible exchange of genetic material and avoid inbreeding, movement away from place of birth (dispersal) is required at some point in the life cycle. This then moves on to the "how" part. Movement through the soil is slow and may only average a few metres per year [26, 48], but over surface movement by earthworms may be much more rapid [49]. To this end, some earthworm species may utilise periods following rain to disperse as the wet conditions prevailing will enhance movement across the soil surface and decrease the chances of desiccation and death from exposure. It could be argued that such behaviour would not therefore be found in parthenogenetic species if this were the only reason for emergence, and this is not the case as *Octolasion cyaneum* is such a species regularly located on the surface after some rains. Nevertheless, the latter may be utilising wet conditions to try and disperse to increase its distribution. All of this may seem very academic, but it does give reason for what is now described.

To assess surface movement of earthworms, fencing of the type already described could be employed, along with

traps if desired. In addition it is possible to "encourage" dispersal by simulating the stimuli that might be responsible. In the simplest terms, inundation experiments could be derived to sprinkle water on to enclosed plots, at known rates, and record qualitatively and quantitatively the (nocturnal) emergence of earthworm species (and life stages). Naturally other factors such as temperature, brightness of the moon, and more may have an influence and need to be considered. Nevertheless, such manipulations might reveal a great deal on the dispersal behaviour of some of the species present in known communities.

Another series of relatively simple experiment (with countless subtle developments) might be employed to measure direct actions of earthworms on organic matter incorporation into soils. So-called "litter bag" experiments require known quantities of (air dried) organic matter from a site, to be enclosed in mesh constructions which permit, by the size of the mesh, access to certain groups of detritivorous soil organisms [9, 50]. This requires some knowledge of the groups present and the type(s) of earthworm at the site but comparisons across different habitats, for example, or below different stands of trees can be revealing (see Figure 9). The litter bags need to be located at the soil surface (pinned down) or buried at chosen soil depths, to permit access to different ecological groups. In addition or instead, choice chamber experiments can be derived by offering laboratory-held earthworms different types of known food materials in specifically designed mesocosms [51]. Where an earthworm, such as *L. terrestris*, feeds directly from the soil surface, experiments can also be set up to determine which food is eaten/removed to the burrow and if choices are made [52]. This can be determined by observation of which material has been disturbed after the event or more directly through recording of the actual behaviours in progress. Results from such laboratory experiments can show, for example, the preference for different agricultural/industrial waste organic materials spread on fields where *L. terrestris* is present (see Figure 10). Details on the type of technology required for this are provided below.

Surface-related behaviours, as described with respect to dispersal, can be recorded indirectly through trapping. Nevertheless feeding and mating at the soil surface, where it occurs, may be better recorded through direct means. The full mating behaviour of *L. terrestris*, including premating burrow visits by partners and the 3.5-hour mating itself, was first described after use of video recording using a simple security-type camera setup, linked to a basic video recorder [53]. More recent work has examined details of the mating more thoroughly [54]. This same technology was also used to obtain results for food choice in this species [52]. However, such work may now be considered costly and has been overtaken by more recent developments in the IT world, whereby a "webcam" can now be obtained relatively inexpensively for similar use. This may seem to be a contradiction of the ethos of this article, but as will be seen, costs here may be negligible. Recent work [55] has investigated, for example, the effects of pesticides and water inundation on earthworm behaviour. For further experiments in progress, examining light effects on surface-related behaviours of earthworms, equipment was obtained including web cams ($n = 4$) and the appropriate software (for use on a standard PC) for less than $100 (at 2009 prices). Figure 11 was obtained during this particular set of experiments housing animals in plastic tubes (drainpipes).

Behavioural work with earthworms may still be regarded as in its infancy, although some major revelations have occurred, particularly with *L. terrestris* [53, 54]. From basic

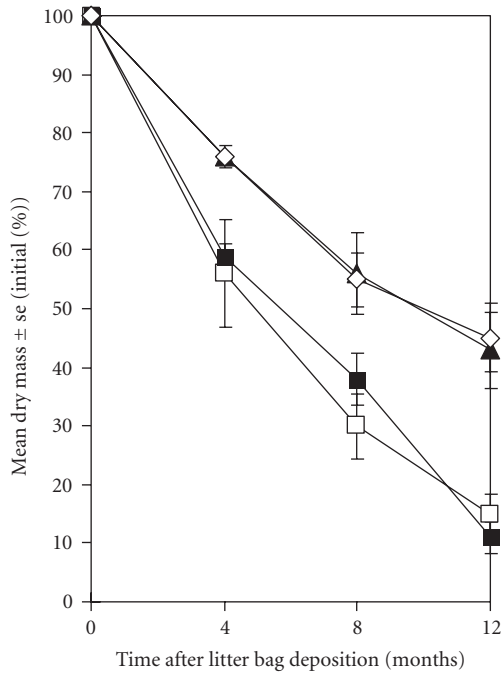


Figure 9: Decreasing mass of birch leaf litter from 3 mm mesh litter bags related to tree plots on the Isle of Rum; from known earthworm communities below pure stands of oak-□; birch-■; pine-▲; on moorland-◇ (adapted from [9]).

Figure 10: Results after one night of feeding by a single *L. terrestris* offered equal amounts of barley straw and waste paper pulp in a 0.25 m^2 arena with the earthworm housed in a central cylinder of attached soil (from above).

Figure 11: A pair of mating *L. terrestris* on the soil surface, revealed and photographed after sun rise.

Figure 12: Two litre Earthworm Inoculation Units (EIUs) ready for inoculation into an organically-enriched landfill cap in the south of England.

observations of mating behaviour, through mate selection, to close scrutiny of copulatory interaction, has all been examined. Great scope still exists in the area of earthworm behaviour and some of the following questions could be addressed.

(i) Which species exhibit mass dispersal and which life stages are involved?

(ii) How much leaf litter is removed or consumed by earthworms in given habitats?

(iii) Can removal of organic matter into the soil be harnessed for soil improvement?

(iv) Is *L. terrestris* the only species that mates on the soil surface?

(v) Do other earthworm species show mate choice?

6. Field Manipulation of Populations (Assisting the Plough)

Where soils require an input of earthworms, augmentation can be brought about using the above information—collection, selection for activities, and even selection for mass culture before field-release. Earthworms, because of their activities in the soil, are, where appropriate, considered as vital components of a healthy, fully functional system. Reviews of research have shown that, across the world and in numerous habitats, the provision of earthworms to sites where they were absent, assistance with recolonisation, or improvements to the type of conditions conducive to their survival can bring about marked positive changes in soil properties [56, 57].

Should areas exist that are devoid of earthworms, for known or unknown reasons, then one approach might be to (re)introduce them to site. Numerous methods are available to achieve this but most can be described simply as "collection and broadcast" using the type of collection techniques previously mentioned or "turf transfer", digging up and translocating soil with grass attached. Both have been used and have positive attributes but equally have less attractive features (see Table 3). To assist the reintroduction process, information gathered on earthworm life histories and requirements for culture have been coupled with further data relating to activities in the soil and interactions with other earthworm species [34, 40–43]. In this way a relatively simple technique, the Earthworm Inoculation Unit (EIU), was devised [26] seeking to overcome the problems associated with the existing techniques. Irreverently known as "worms in bags" this technique seeks to cultivate a starter culture of adults under optimal conditions over a period of a few months. After this time, population development within the plastic-bound units means that all life stages, adults, cocoons, and hatchlings ought to be present. The EIUs can then be transported to the desired inoculation site ready for introduction (see Figure 12). Inoculation requires the contents of the EIUs to be inserted into an appropriately sized hole in the soil, after the plastic envelope has been carefully removed.

The contents thereby retaining their original position in the soil profile and providing a protective microenvironment. Over the past two decades, results from both agricultural and post-industrial settings have been positive [26, 48]. Spread of earthworms over one site at Calvert site was completed within a decade and positive interactions were recorded with the presence of alder trees (*Alnus glutinosa*—which fix nitrogen) and earthworm density [27]. At one of the sites, further investigations developed the EIU technique with addition of organic matter. This was a response to use of manure as "earthworm attractant traps" to augment assessment of the numbers and species present on site [26].

Addition of earthworms to sites where they are absent (for some reason) may be valuable and permit a number of questions to be addressed.

(i) What factors brought about the removal of earthworms?

(ii) What can be done to remedy the situation?

(iii) How can the success of the operation be measured (in terms of earthworms and soils)?

(iv) Can more be learned of earthworm populations from this type of work?

Table 3: Relative Merits of existing Earthworm Inoculation Techniques (adapted from [26]).

Technique	Advantages	Disadvantages
Turf Cutting and relaying	Protective microenvironment	Densities usually low
	Cocoons transferred	Little control over species/numbers
		Mainly shallow working worms
		Cutting machines/labour required
		Damage to collection site
Chemical/physical extraction with broadcasting	High densities possible	Protective micro-environment absent
	Species selection possible	No cocoon transfer
		Mainly deep burrowing worms
		Worms may be injured during extraction
		Laborious and expensive
		Damage to collection site
Earthworm Inoculation Unit (EIU) method	Protective microenvironment	Laborious and potentially expensive
	Species selection possible	(compared with above methods)
	Worms of known origin	
	Cocoons transferred	
	High densities possible	

7. Conclusion

This article set out to demonstrate that low-technology methods are able to gain insights into fundamental questions relating to earthworms. Examples have been provided and direction given towards investigations asking relatively simple questions that can utilize these techniques. In addition to the sections described on collection, burrowing, behaviour, life history, and manipulation of earthworms, others which have only been hinted at or perhaps overlooked can also be developed, and many of those included have an amount of overlap within them. It is for the prospective researcher to identify the preferred niche area of investigation and progress it to potentially create a new angle within the existing fields of knowledge. Science tends to require funding in order to advance, but one critical aspect is the development of ideas and the creative use of available resources. Earthworm ecological research still has room for the use of basic tools.

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Review Article

The Role of Earthworms in Tropics with Emphasis on Indian Ecosystems

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The paper highlights the research carried out by different scientists in India on aspects of earthworm population dynamics and species diversity, associated with other soil fauna and microflora. It also deals with the importance of earthworm activity on physicochemical properties of soil with reference to India and other tropical countries. Stress is laid on the earthworm plant association and importance of the secretions of earthworms as plant growth stimulators. Moreover, the earthworm species reported and being utilized for vermicomposting in India are discussed, since vermicomposting is the ultimate technology which renders for the improvement of soil fertility status and plant growth. Earthworms serve as indicators of soil status such as the level of contamination of pollutants: agrochemicals, heavy metals, toxic substances, and industrial effluents; human-induced activities: land-management practices and forest degradation. In all these fields there is lacuna with respect to contributions from India when compared to the available information from other tropical countries. There is lot of scope in the field of research on earthworms to unravel the importance of these major soil macrofauna from holistic ecological studies to the molecular level.

1. Introduction

Earthworms belonging to Phylum Annelida, Class Chaetopoda, and Order Oligochaeta occupy a unique position in animal kingdom. They are the first group of multicellular, eucoelomate invertebrates who have succeeded to inhabit terrestrial environment. They form major soil macrofauna. Their species richness, abundance, and distribution pattern reflect on edaphic and climatic factors of the geographical zone. They serve as "bioindicators" to understand the physicochemical characteristics of their habitat. Their horizontal and vertical stratification and abundance contribute to pedogenesis and soil profile. Encouraging their establishment through no tillage or shallow ploughing and enriching soil with organic matter incorporation has resulted in improving soil fertility. This has been experimented for several decades at Rothamsted Research Station, U.K. The interaction of earthworms and other microflora and fauna has given much scope for understanding of soil community and its influence on above ground primary production.

Distinctive habitat, food niches, and adaptive mechanisms of earthworms have opened up new fields for investigations on their role in organic waste management. One of the advantageous factors in this field is the use of earthworms to minimize the degradable organic matter and to use the same as bioresource for organic manure production. The manure produced serves as good source of soil amendment. The ecologically distinguished epigeic earthworms are used for producing the organic manure, "vermicompost". This has gained attention of garden lovers, agriculturists, and agroindustries to convert organic matter generated at different levels into rich, odorless, free flowing compost to support sustainable agriculture.

2. Earthworms: Components of Soil Biota

Earthworms form one of the major macrofauna among soil biota to maintain dynamic equilibrium and regulate soil fertility. Their existence depends on adequate moisture, soil

texture, pH, electrolyte concentration, and food source in the given ecosystem. This clearly indicates the interdependency of the environmental factors to the survival of earthworms; when such conditions are created, they further contribute to soil fertility through their activity.

3. Food Niches of Earthworms

Degradation of leaf material commences from the time it detaches itself from the plant and drops to ground to add to litter. Earthworms are the major secondary decomposers in the soil faunal community. They feed on decomposed organic material at different levels of degradation. Lee [1] has suggested that earthworms survive on microorganisms, micro- and mesofauna associated with ingested dead tissue. According to him, earthworms that feed near the surface on decomposing litter and at the root zone on dead roots are the detritivores and those remain at subsurface and consume large quantities of soil are geophagous earthworms.

Lavelle [2] has categorized geophagous earthworms as polyhumic, oligohumic, and mesohumic based on the proportion of humus and soil in their feed. Through factorial analysis, he has given the explanation that temperature differences with latitude and litter characteristics like quantity and decomposability determine the variations observed with reference to their distribution. The detritivorous epigeic earthworms form the major component of earthworm fauna in temperate regions and mesohumic endogeic earthworms are predominant in tropical forests. There is minimum representation of mesohumic earthworms in temperate regions. Oligohumic earthworms that feed on soil having very low level of organic matter are abundant only in tropical regions.

Lavelle [2] considers polyhumic earthworms as more stable fraction of earthworm community occupying different soil strata as topsoil feeders to species of rhizosphere in tropical regions. Thus, tropical earthworms depend more on soil mixed with different levels of humic substances rather than surface litter. More stable environments like heavy rainfall areas (2000 to above 4000 mm rain/annum) in the state of Karnataka, India, have greater diversity of earthworms than the dry areas (<600 to 900 mm rain/annum). The geophagous earthworms of mesohumic and polyhumic types are widely distributed in places receiving heavy rainfall in this subtropical part of the country (Tables 1 and 2).

The acceptance level of various leaf litters shows positive correlation to nitrogen and carbohydrate contents and negative correlation to polyphenol content [3]. Ganihar [4] studied the litter feeding of *Pontoscolex corethrurus* in a multiple-choice test. He found variations in degree of acceptability of different litter that showed positive correlations to levels of organic carbon and nitrogen content. The least preference for *Eucalyptus camaldulensis* and *Acacia auriculiformis* was linked with high levels of polyphenols. It has been shown that *Lampito mauritii* exhibited similar preference either for partially decomposed large pieces of leaf material of different types or for powdered leaves mixed with agar base [5]. It could be inferred that apart from physical nature of leaf matter, chemical compounds in them serve as attractants or repellants (Tables 3 and 4). Ganihar [4] is of the view that in land reclamation sites, if earthworms have to be introduced, it is essential to develop above ground plant community. Litter from such plants when mixes with soil, at different levels of decomposition, serves as feed to developing earthworm population. The available carbon source encourages population growth of earthworms [6]. In India, *Lampito mauritii* is the most widely distributed earthworm in different agro-ecosystems [7–12]. This earthworm preferred decomposing grass of paddy (*Oryza sativa*) and finger millet (*Eleucine coracana*) to other leaf litter [5]. The grasses when developed in reclamation sites can form an ideal base for establishment of *Lampito mauritii* to bring about improvement in soil structure and finally chemical and biological activities. Food preference and sensitivity to other edaphic factors determine the possibility of introduction of earthworms for land reclamation.

4. Earthworm Activity on Physicochemical Properties of Soil

Earthworms are the major macrofauna in the soil community. They are distributed at different depths in soil strata. The litter feeders, which are not burrowers, constitute a very small number in tropical situations. The burrowing endogeic earthworms live in horizontal and vertical burrows constructed in soil strata. They make these burrows partly by ingesting soil particles through their way and partly by pushing the soil to the sides [13]. The ingested soil along with organic matter passes through the gut and undigested matter is released at the opening of the burrow on soil surface or at the subsurface as castings. The subsurface castings contribute to soil profile [1].

The burrows of earthworms, which run horizontally or vertically depending on burrow forming ability of species, will determine the possible physical effects on soil characteristics. In temperate regions where deep burrowing anecic earthworms are of common occurrence, it is opined that infiltrations can bring about leaching of nutrients from soils to ground water. The leachate volume may show an increase of four to twelve folds due to their activity [14]. Introduction of *Aporrectodea caliginosa* into coniferous forest soils resulted in fifty fold increase in concentration of nitrate and cations in soil solution. But the amount that entered ground water or plant system remained undetermined [15]. One of the major contributions of burrowing activity of earthworms is in affecting soil porosity [16, 17]. The major impact on hydrology has been worked out with respect to activity of anecic earthworm *Lumbricus terrestris* [18]. Information is lacking in India with respect to burrows of earthworms, their structure, and any variations observed depending on soil type. Influence of organic matter, agricultural practices on earthworm population, and similarly the role of earthworms in modifying the situations in cultivable lands are very meager in a country having diversity and abundance of the populations in different agro-ecosystems. Reddy et al. [19] reported the influence of various management practices

Table 1: Earthworm distribution in Southern Karnataka (India) in different agroclimatic zones including coastal plains, hilly regions, and interior plains.

Sl. No.	Species	Moisture level (%)	Soil type	Vertical distribution (cm)	Food niche	Population density (no./100 m^2)
1	*Curgeona narayani*	Wet land-in waterlogged soil	Red loamy soil	Up to 45	Mesohumic	640–11,250
2	*Dichogaster affinis*	20–40	Red loamy, alluvial and lateritic	5–10	Mesohumic to polyhumic	60–250
3	*D. bolaui*	20–40	〃	〃	〃	60–450
4	*D. curgensis*	20–40	Red loamy	〃	Polyhumic	25–200
5	*D. modigliani*	20–40	Red sandy	〃	Mesohumic	10–25
6	*D. saliens*	20–40	Red sandy	〃	〃	65–265
7	*Drawida ampullacea*	>40	Red loamy	10–20	Polyhumic	275–930
8	*D. barwelli*	>50	Red loamy to sandy soil	10–30	〃	275–576
9	*D. barwelli impertusa*	>50	Red loamy	〃	〃	120–430
10	*D. calebi*	>50	Red loamy to sandy soil	10–30	Polyhumic	80–1200
11	*D. ferina*	40–50	Red loamy	20–30	Mesohumic	40–340
12	*D. ghatensis*	40–50	〃	10–20	〃	450–1350
13	*D. kanarensis*	40–50	〃	〃	〃	85–400
14	*D. lennora*	40–50	Red sandy soil	〃	〃	15–30
15	*D. modesta*	40–50	〃	10–30	〃	4–500
16	*D. paradoxa*	>40	Red loamy to alluvial	10–20	Polyhumic	1700–2500
17	*D. pellucida pallida*	>40	Lateritic to Red loamy	〃	Mesohumic	4–500
18	*D. scandens*	>40	Red sandy loam	5–10	Polyhumic	10–350
19	*D. sulcata*	>40	Alluvial soil	10–30	Polyhumic	65–235
20	*Glyphidrillus annandalei*	>40	Sandy bed to Red loam	20–45	Oligohumic	130–1600
21	*Gordiodrilus elegans*	>40	Red sandy loam	10–40	Mesohumic	24–200
22	*Hoplochaetella kempi*	30–40	Lateritic to alluvial	10–30	Polyhumic	10–430
23	*H. suctoria*	30–40	Alluvial	10–20	〃	50–240
24	*Hoplochaetella sp.*	40–50	Red loam	20–40	〃	460–3330
25	*Howascolex sp.*	30–40	Red loam	10–30	〃	145–2500
26	*Lampito mauritii*	20–30	Red sandy to lateritic	10–30	Mesohumic	720–2190
27	*Mallehula indica*	30–40	Red loam	10–20	Mesohumic	180–880
28	*Megascolex filiciseta*	30–40	Lateritic	5–10	Polyhumic	15–330
29	*M. insignis*	30–40	Alluvial	5–20	Polyhumic	65–800
30	*M. lawsoni*	30–40	Red loam to sandy loam	10–30	Mesohumic	120–1000
31	*M. konkanensis*	30–40	Lateritic to alluvial	20–45	Mesohumic	20–3900
32	*Metaphire houlleti*	>40	Alluvial and Red loam	10–40	Polyhumic	18–2140
33	*Octochaetona albida*	30–40	Red loam	10–20	Polyhumic	150–650
34	*O. beatrix*	20–30	Sandy loam	〃	〃	40–335
35	*O. rosea*	30–40	Alluvial	10–20	Mesohumic	15–120
36	*P. excavatus*	>40	Organic layer	0–5	Detritivore	18–8000
37	*Plutellus timidus*	30–40	Alluvial	10–15	Mesohumic	60–460
38	*Polypheretima elongata*	>40	Sandy loam to Red loam	30–60	Mesohumic	194–4000
39	*Pontoscolex corethrurus*	30–50	Sandy, alluvial, loamy, lateritic	5–15	Mesohumic to polyhumic	250–7100

Table 2: Habitat preference of widely distributed earthworm species *Lampito mauritii* and *Pontoscolex corethrurus* at study sites.

District	Agroclimatic zone	Mean annual rainfall (mm)	Soil type	Earthworm species	Habitat preference
Bangalore	Eastern dry zone	700–900	Red loamy soil	*Lampito mauritii*	Arable lands
Kolar	″	600–800	Lateritic and red sandy soil	″	Grasslands
Tumkur	″	″	Red sandy soil	″	Grasslands and arable lands
Chickmagalur	South transition zone	900–1000	Red loamy soil	Species richness than species dominance	Varied habitats
Chickmagalur	Hilly zone	2000–3000	Red loamy soil	*Pontoscolex corethrurus*	Plantations
Coorg	Hilly zone	2000–>4000	″	″	Grasslands and plantations
South Kanara	Coastal zone	3000 > 4000	Coastal alluvial soil	*P. corethrurus* and *Megascolex konkanensis*	Grasslands, plantations, arable lands

Table 3: Disintegration of different leaf matters due to selective feeding by earthworm *Lampito mauritii* [5].

Leaf matter	1	2	3	4	5	6	7	8	9
Millet straw	70.00	50.00	55.00	—	—	—	—	—	—
Paddy straw	48.00	11.00	27.50	22.00	13.00	33.00	—	—	—
Cashew litter	—	—	—	38.00	24.00	39.00	22.50	2.60	67.00
Mango litter	—	—	—	48.00	30.00	50.00	30.70	28.60	6.30
Guava litter	—	—	—	44.00	14.00	83.00	25.00	23.00	—
Eucalyptus litter	—	—	—	32.00	10.00	61.00	31.00	24.40	11.60

Note: *Col: 1–3 data for first month*
(1) Percent loss of litter per month due to microbial degradation and feeding by earthworms.
(2) Percent microbial degradation per month.
(3) Rate of litter consumption (mg) for hundred earthworms per day.
Col: 4–6 data for second month
(4) Percent loss of litter per month due to microbial degradation and feeding by earthworms.
(5) Percent microbial degradation per month.
(6) Rate of litter consumption (mg) for hundred earthworms per day.
Col: 7–9 data for third month
(7) Percent loss of litter per month due to microbial degradation and feeding by earthworms.
(8) Percent microbial degradation per month.
(9) Rate of litter consumption (mg) for hundred earthworms per day.
The table also shows the acceleration of litter breakdown in presence of earthworms.

affecting density and surface cast production. The casts of the earthworm, *Pontoscolex corethrurus*, and the surrounding soil in an undisturbed forest floor in Sirumalai Hills, Tamil Nadu (South India) showed that the percentage of moisture content, organic carbon, and total nitrogen in the worm casts were higher and significantly differed from the values obtained in the surrounding soil [20].

According to the recent report by Julka et al. [21], in India, there are 590 species of earthworms with different ecological preferences, but the functional role of the majority of the species and their influence on the habitat are lacking. Recently Karmegam and Daniel [11] reported the correlation of soil and environmental parameters on the abundance of ten different earthworm species belonging to four families, namely, Megascolecidae (*Lampito mauritii, L. kumiliensis*, and *Megascolex insignis*), Octochaetidae (*Dichogaster bolaui, D. saliens*, and *Octochaetona thurstoni*), Moniligastridae (*Drawida chlorina, D. paradoxa*, and *D. pellucida pallida*), and Glossoscolecidae (*Pontoscolex corethrurus*) in the study that was carried out at different locations in Dindigul District (South India). The fluctuations in populations of earthworms were observed during the monthly collections in course of three years in all the selected sites. In the survey carried out from 1997 to 1999, the predominant species that were recorded as maximum number of earthworms/m^2 in sites 1–10 were *D. pellucida pallida* (Jan. 1998-70.44), *D. pellucida pallida* (Dec. 1999-32.30), *L. mauritii* (Feb. 1998-55.22), *D. pellucida pallida* (Dec. 1999-25.54), *L. mauritii* (Dec. 1997-66.78), *L. mauritii* (Nov. 1997-43.40), *L. mauritii* (Jan. 1999-44.60), *P. corethrurus* (Nov. 1997-58.34),

P. corethrurus (Dec. 1999-64.30), and *P. corethrurus* (Dec. 1998-107.60) [22].

The biomass dynamics also showed wide fluctuation among the species in relation to the months of collection from different collection sites. The highest worm biomass was recorded during December to February and certain species were totally absent during certain periods of the survey. The total biomass of different species recorded in the monthly observation over a period of three years (1997 to 1999) varied in various study sites. The highest biomass of the respective earthworm species as well as the month and year of its occurrence in the study sites 1 to 10 as recorded includes *D. pellucida pallida* (30.63 g/m^2 during Feb. 1998), *D. pellucida pallida* (22.88 g/m^2 during Jan. 1998), *D. pellucida pallida* (29.27 g/m^2 during Dec. 1999), *D. pellucida pallida* (20.20 g/m^2 during Dec. 1999), *D. pellucida pallida* (44.65 g/m^2 during Dec. 1999), *D. pellucida pallida* (22.38 g/m^2 during Dec. 1999), *D. pellucida pallida* (29.66 g/m^2 during Jan. 1998), *P. corethrurus* (15.20 g/m^2 during Dec. 1998), *D. bolaui* (19.79 g/m^2 during Jan. 1999), and *P. corethrurus* (26.34 g/m^2 during Dec. 1998), respectively [22]. Among the earthworm species studied, *L. kumiliensis* has been reported for the first time in Sirumalai Hills of Tamil Nadu, India [23]. This is the only study to highlight the cyclic fluctuations in the earthworm populations for a continuous period of three years and variations in the species structure at different time intervals. Still the information on the physicochemical changes in the soil with respect to species composition at given time is not clear. A composite study on microbial association with the predominant earthworm species at a given time may provide necessary information on its ecological role.

5. Factors Influencing the Abundance of Earthworm Populations

The percentage abundance of different species of earthworms in the 10 collection sites during the survey period (1997–1999) is shown in Figures 1 and 2. In most of the study sites, that is, 1–7, *L. mauritii* was the dominant species and it showed its presence during the premonsoon, monsoon, and postmonsoon months. *P. corethrurus* showed its abundance in the sites 8–10. Various parameters, that is, pH, electrical conductivity (EC), organic carbon (OC), nitrogen (N), atmospheric temperature (AT), soil temperature (ST), soil moisture (SM), humidity (HUM), and rainfall (RF) observed during the survey period (1997–1999) are given in Table 5 and in Figure 3. All the parameters showed fluctuations in all the ten study sites. Here, for the convenience of statistical analysis the parameters were categorized into two major groups: (a) physicochemical parameters which included pH, EC, OC, and N; and (b) climatic parameters which included ST, SM, HUM, and RF.

In Tamil Nadu, India, very limited information is available on the distribution pattern of earthworms. The data on earthworm distribution is available for the stations like Palni Hills [24], Madras [25], and Sirumalai Hills [11, 23, 26, 27]. Dindigul, a District in Tamil Nadu, was considered as study site for its variety of habitats to assess the earthworm species diversity, density, and biomass. The population and biomass dynamics of different earthworm species and their percentage abundance in relation to physicochemical characteristics of the soil and the climatic factors were recorded in selected sites. The correlation of earthworm population to physicochemical characteristics of the soil and the climatic parameters was carried out to find out the possibility of arriving at a suitable endemic earthworm species for vermicomposting operations in this part of the country. Since the populations of earthworms are extremely variable in size ranging from only a few individuals (sometimes totally absent) to more than 1000/m^2, the assessment of the size distribution and structure of earthworm population is difficult. The seasonal change, demography, and vertical distribution of the populations make it more complicated, and hence, it is absolutely essential to follow a uniform method of determining the number of earthworms in small sample areas as it has been done in this study. The regular monthly survey carried out for three years (1997 to 1999) showed the presence of ten species of earthworms, with four species restricted only to the hilly region and six species to the plain, including the foothills (Table 6). This observation indicates that species such as *L. kumiliensis, D. bolaui, D. saliens*, and *P. corethrurus* are specific only to the hilly region and they are not found in the foothills. Though *L. kumiliensis* and *L. mauritii* both belong to the same genus, *Lampito, L. kumiliensis* was found only in the hilly region and *L. mauritii* in the plains. This observation indicates that the distribution of different earthworm species is limited even though they are closely related. Such niche differences for closely related species have been reported by earlier workers in the field [28, 29].

The results of the percentage abundance of different species of earthworms showed that *L. mauritii* and *P. corethrurus* were the most abundant in the study sites 1 to 7 and 8 to 10, respectively. Formation of aggregation of species has been observed in sites 1 to 7; that is, wherever *L. mauritii* was found, it was in association with *D. chlorina* and *D. pellucida pallida*. This sort of association of earthworm species sharing the same habitat is not

Table 4: Artificial diet (1 : 8 by weight) of agar and different leaf litter powder on feeding of earthworm *Lampito mauritii* in relation to C/N of diets [5].

Litter powder in agar	Daily food intake mg/day/adult	C/N of the feed
Paddy straw	8.05 ± 0.28	37
Millet straw	7.07 ± 1.23	45
Mango litter	8.67 ± 1.27	19
Guava litter	3.25 ± 0.79	45
Cashew litter	4.44 ± 1.10	30
Eucalyptus litter	1.62 ± 0.59	42
Agar only (control)	2.53 ± 1.23	38

Number of observations = 3; Palatability depends on texture as well as chemical nature of the feed.

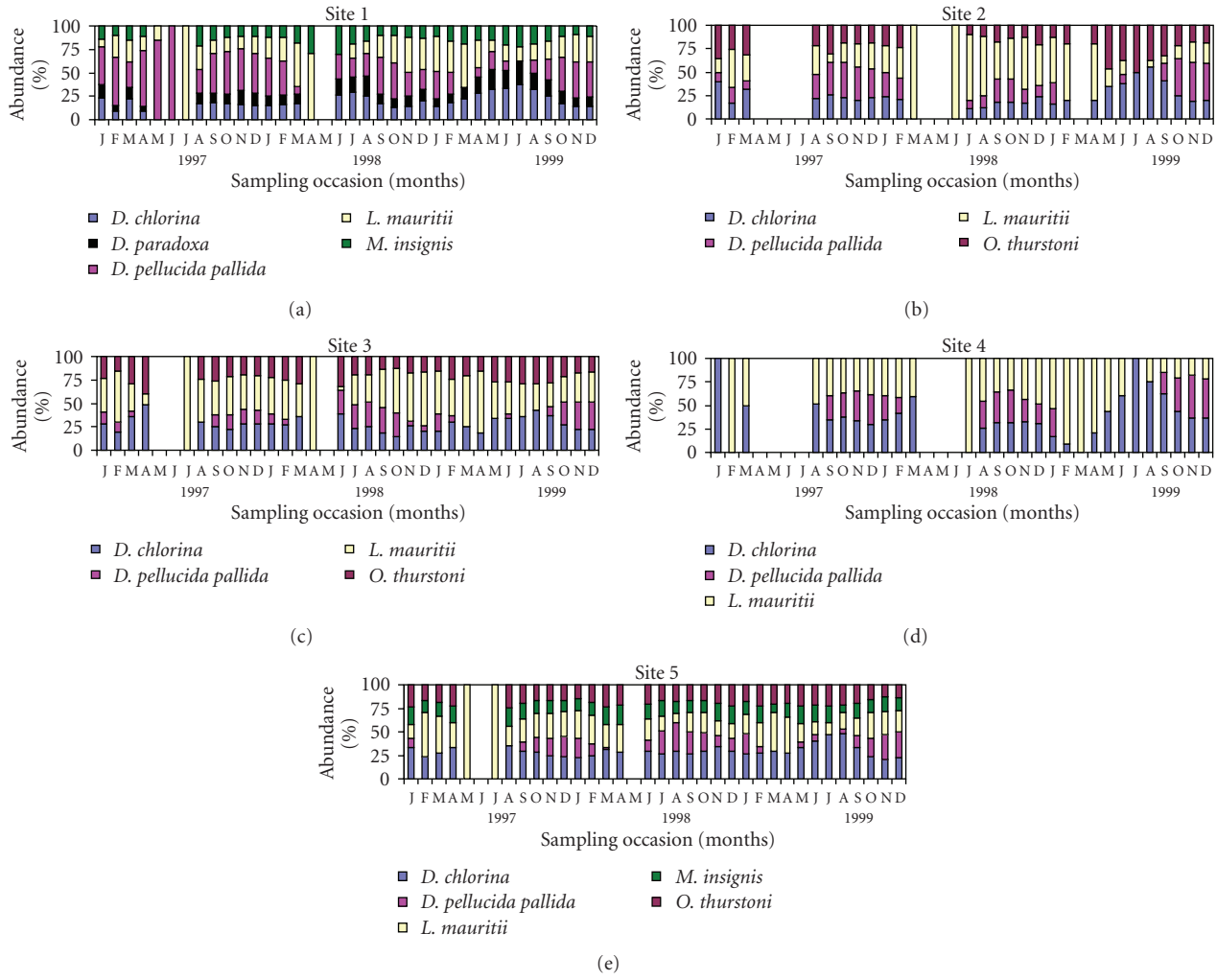

Figure 1: Percentage abundance of earthworm population in study sites 1 to 5 (1997–1999).

uncommon [1, 30]. *L. mauritii* is the dominant species found almost all over India along with other earthworm species such as *Drawida modesta, Octochaetona pattoni, O. thurstoni, Ramiella pachpaharensis, Polypheretima elongata*, and *Pontoscolex corethrurus* [8, 31], but Bano and Kale [32] reported that *L. mauritii* was not found in some forest areas and coastal Karnataka. The population densities of earthworms observed in the 10 collection sites ranged from 0 to 228 /m^2. Other authors observed population densities (earthworm no./m^2) of 53.5 in plain grass land, 73 in deciduous forest, 543 in the fallow phases of shifting agriculture, and 58.2 in the maize crop land [33–36]. In rubber plantations of Tripura (India) about 20 species of earthworms, namely, *Eutyphoeus gigas, E. gammiei, E. comillahnus, E. assamensis, E. festivas, Eutyphoeus sp., Dichogaster bolaui, D. affinis, Lennogaster chittagongensis, Octochaetona beatrix, Metaphire houlleti, Perionyx sp., Kanchuria sumerianus, Kanchuria sp.*1, *Kanchuria sp.*2, *Drawida nepalensis, Drawida sp.*1, *Drawida sp.*2, *Pontoscolex corethrurus*, and *Gordiodrilus elegans* were distributed and it was observed that the largely dominating species were endogeics [37].

Evans and Guild [38] have shown that nitrogen rich diets help in rapid growth of earthworms and facilitate more cocoon production than those with little nitrogen available. Due to the influence of nitrogen content of the soil, the percentage contribution of nitrogen to earthworm population might have shown a very high degree of dependence in the present study. Some of the reports from the country well support qualitative dependence of earthworm population on soil nitrogen content [26, 27, 39, 40].

Soil moisture plays a major role in the distribution and occurrence of various earthworm species. The same has been observed by other workers in their studies [25, 28, 29, 41, 42]. The abundance and species diversity are dependent on climatic conditions, especially the occurrence of dry and/or cold periods, and regional variation in vegetation, soil texture, and nutrient content. The climatic parameters, that is, soil temperature, soil moisture, humidity, and rainfall show seasonal fluctuations (Table 6 and Figure 3). The highest rainfall was recorded during October-November and the earthworm population was also the highest at this period. The soil moisture content corresponded with earthworm

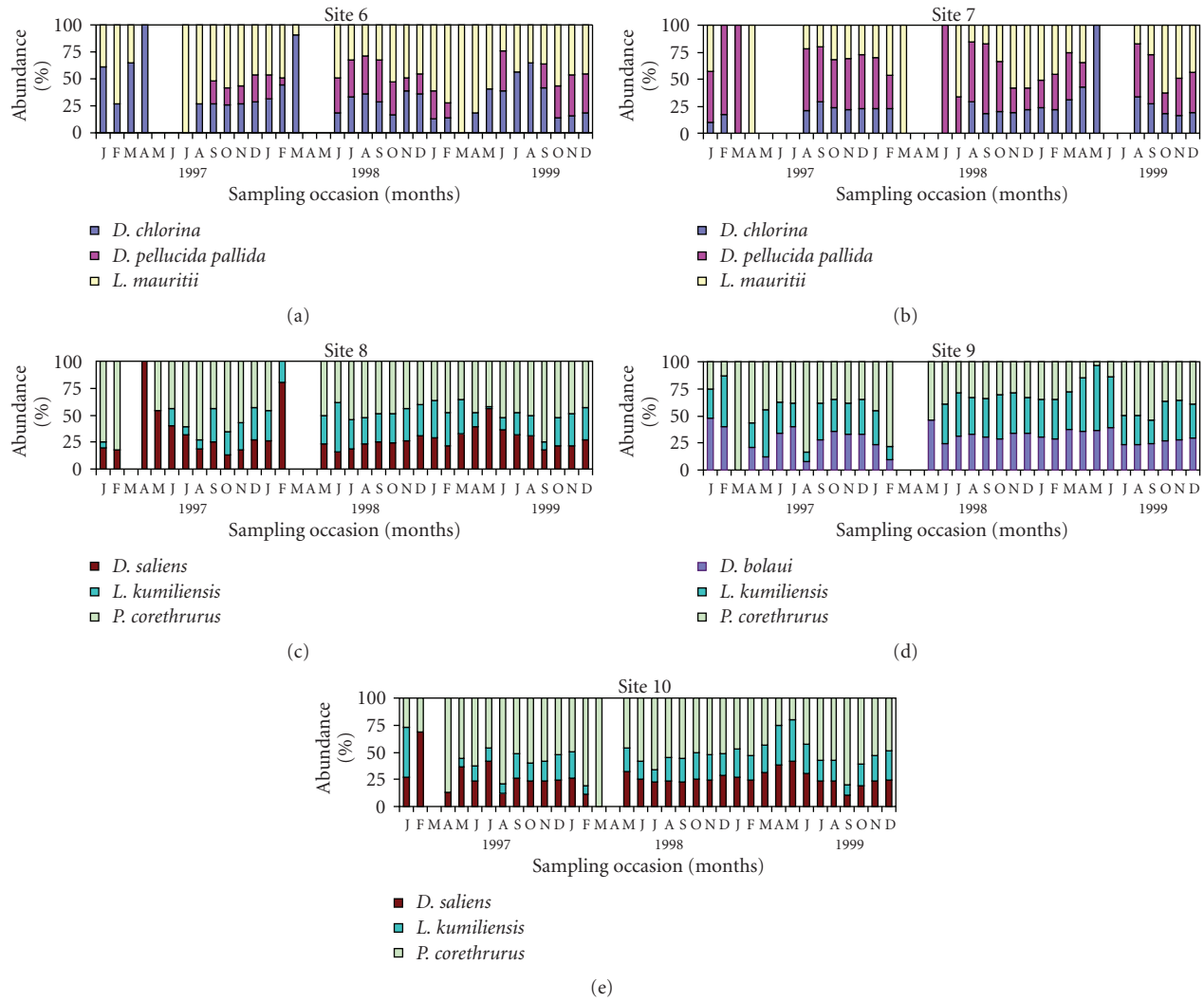

Figure 2: Percentage abundance of earthworm population in study sites 6 to 10 (1997–1999).

population. Total annual rainfall of 1130, 1284, and 959 mm was recorded during 1997, 1998, and 1999 in the plains and foothills of Sirumalai (study sites 1–7). The highest rainfall of 304 and 357 mm was received during October and November 1997 in the above study sites. The highest rainfall months in Sirumalai Hills (study sites 8–10) were October to December. The soil moisture content directly matched with the rainfall. The soil moisture content ranged from 2.0 to 30.4 percent in the study sites 7–10 during the three years of the study. The humidity also showed fluctuations in both the plains and hilly region of the study area. Soil moisture can explain the increase in earthworm population, since soils are moist under a mulch cover because of the restricted evaporation. There are many indications, to show that the population of endogeic earthworms is controlled mainly by soil moisture [42].

The influence of climatic factors on the populations of earthworm is not uncommon. The populations of *Millsonia anomala* are dependent on climatic conditions as well as vegetational patterns. Earthworm activity and populations are determined essentially by the moisture content of the soil [43]. The temperature and moisture are usually inversely related and higher surface temperature and dry soils are limiting factors to earthworms than low and water logged soils [44]. The soil temperature plays an important role in the maintenance of earthworm population in an ecosystem and available information also indicates the negative correlation of soil temperature to earthworm population [11, 25, 40, 45]. In rubber plantations of Tripura (India), the earthworms experienced 25.9°C, 24.8%, 4.85, and 1.8% mean soil temperature, moisture, pH, and organic matter, respectively [37]. Temperature largely affects activity of earthworms in temperate regions. Tropical species can withstand higher temperatures. *L. mauritii* is available throughout the year where the annual temperature is 30 ± 2°C. Population of *O. serrata* was active between 27 and 28°C. In tropical regions the temperature fluctuations are minimal when compared to temperate regions.

Moisture is another limiting factor for earthworm distribution as water constitutes a major portion of the body

Table 5: Physicochemical and climatic characteristics (average) of the study sites 1 to 10 (1997–1999) (refer to Table 6 for study site description) [22].

Parameter observed*	Study sites									
	1	2	3	4	5	6	7	8	9	10
1997										
pH	7.78	7.63	7.13	6.86	7.59	7.67	6.78	7.04	7.50	6.55
EC (dS/m)	0.34	0.20	0.38	0.11	0.21	0.18	0.32	0.14	0.27	0.39
OC (%)	1.42	2.29	4.44	2.75	1.47	2.94	3.42	3.05	4.20	7.99
TN (%)	0.41	0.35	0.23	0.23	0.25	0.40	0.42	0.31	0.22	0.40
ST (°C)	28.90	29.83	27.84	29.29	30.31	29.27	29.83	23.47	22.49	21.30
SM (%)	8.10	6.34	10.34	7.22	7.16	8.30	10.25	15.75	15.46	14.99
1998										
pH	7.95	7.51	7.25	6.66	7.51	7.62	6.45	7.15	7.34	6.44
EC (dS/m)	0.36	0.22	0.38	0.13	0.25	0.16	0.33	0.18	0.31	0.39
OC (%)	1.74	2.19	4.24	2.79	1.43	2.35	4.25	3.19	4.22	8.48
TN (%)	0.44	0.34	0.24	0.27	0.27	0.41	0.41	0.27	0.27	0.38
ST (°C)	29.23	30.18	28.27	30.63	29.92	29.30	30.18	24.14	22.68	21.30
SM (%)	12.14	9.93	15.18	9.73	12.83	12.03	14.35	16.00	17.09	16.29
1999										
pH	7.85	7.49	7.37	6.85	7.38	7.59	6.47	6.98	7.45	6.64
EC (dS/m)	0.35	0.23	0.34	0.14	0.24	0.17	0.35	0.16	0.26	0.39
OC (%)	1.40	2.45	4.34	2.90	1.36	3.02	4.05	3.45	4.37	9.99
TN (%)	0.46	0.37	0.27	0.26	0.26	0.43	0.41	0.31	0.28	0.39
ST (°C)	27.42	28.55	26.49	29.66	30.42	27.46	28.55	25.21	23.51	22.80
SM (%)	9.50	7.27	11.70	8.50	9.27	9.37	10.34	12.56	14.37	13.86

* EC: Electrical conductivity; OC: Organic carbon; TN: total nitrogen; ST: Soil temperature; SM: Soil moisture.

weight of an earthworm. Soil moisture and population estimates are positively correlated [35]. Water constitutes 75–90 percent of the body weight of earthworms. So the prevention of water loss is a major factor for their survival. They apparently lack a mechanism to maintain constant internal water content, so that their water content is influenced greatly by the water potential of the soil [46], which directly depends on the adequate availability of soil moisture.

The seasonal dynamics in an annual cycle shows that earthworm numbers and biomass were high in the rainy season with a gradual decline in number in the winter season. Earthworms were completely absent during the second half of January and February, when soil temperature was very low (4.9–6.2°C). Dash and Patra [7] and Kale and Krishnamoorthy [8, 47] have recorded maximum number of earthworms and biomass in the rainy and late rainy period. The relationship between earthworm activity and rainfall was observed by Fragoso and Lavelle [48] and Joshi and Aga [49]. The moisture requirements for different species of earthworms from different regions can be quite different [42]. The dependence of earthworm population on soil moisture is seen in the studies carried out for three years as of the highest degree when compared with other climatic parameters. This is because of certain physiological activities of earthworms such as cutaneous respiration and excretion of nitrogenous ammonia and urea, which need a moist environment, which, in turn, is essential for the maintenance of their life process.

Systematic correlation analysis results indicate that only about 80 percent of the population dependence can be explained by these physicochemical and climatic parameters and it is presumed that the remaining may depend on other environmental factors. The correlation analysis technique may be used to quantify and rationalize the effects of physicochemical parameters on the earthworm population. However, no single factor is likely to be solely responsible for the horizontal distribution of earthworms, but rather the interaction of several of the factors provides suitable soil conditions for the existence of earthworm populations [11].

6. Earthworm Casts: Abundance, Structure, and Properties

Earthworms' release "cast" at the opening of their burrows. Epigeic earthworms release the castings exclusively on soil surface. Their castings may be granular or spindle like masses that may be 2 to 3 cm high heaps as in *Eudrilus eugeniae* or *Perionyx excavatus*. There is no definite shape to the excreted matter to identify as castings of *Eisenia fetida*. *Eisenia fetida* releases fine, powdery, dark brown material as surface cast. Soil living endogeic earthworms that feed on different quantities of organic matter along with soil particles

Table 6: Population density of earthworms in different habitats in Dindigul District, Tamil Nadu studied during 1997–1999 [22].

Study site	Description	Earthworm species	Avg. population density (no./m^2)
(1)	Cultivated land	*Lampito mauritii* (Kinb.).	12.52
		Megascolex insignis Mich.	7.82
		Drawida chlorina (Bourne).	8.88
		Drawida paradoxa Rao.	5.10
		Drawida pellucida var. *pallida* Mich.	18.60
(2)	Unirrigated crop land	*Lampito mauritii* (Kinb.).	14.18
		Octochaetona thurstoni Mich.	5.46
		Drawida chlorina (Bourne).	5.04
		Drawida pellucida var. *pallida* Mich.	11.10
(3)	Uncultivated shaded fallow land	*Lampito mauritii* (Kinb.).	17.88
		Drawida pellucida var. *pallida* Mich.	13.27
		Octochaetona thurstoni Mich.	10.92
		Drawida chlorina (Bourne).	10.70
(4)	Uncultivated fallow land	*Lampito mauritii* (Kinb.).	10.30
		Drawida chlorina (Bourne).	4.73
		Drawida pellucida var. *pallida* Mich.	6.46
(5)	Garden	*Lampito mauritii* (Kinb.).	15.50
		Megascolex insignis Mich.	10.96
		Octochaetona thurstoni Mich.	13.04
		Drawida chlorina (Bourne).	17.26
		Drawida pellucida var. *pallida* Mich.	11.27
(6)	Orchard	*Lampito mauritii* (Kinb.).	8.92
		Drawida chlorina (Bourne).	6.32
		Drawida pellucida var. *pallida* Mich.	4.75
(7)	Foothills (Alt. < 450 m.)	*Lampito mauritii* (Kinb.).	5.63
		Drawida chlorina (Bourne).	6.22
		Drawida pellucida var. *pallida* Mich.	22.68
(8)	Grass land (Alt. 1,000 m.)	*Lampito kumiliensis* (Kinb.).	18.21
		Dichogaster saliens (Bedd.).	5.31
		Pontoscolex corethrurus (Muller).	10.30
(9)	Semi-evergreen forest (Alt. 1,100 m.)	*Lampito kumiliensis* (Kinb.).	29.52
		Pontoscolex corethrurus (Muller).	10.49
		Dichogaster bolaui (Mich.).	9.39
(10)	Sacred grove land (Alt. 1,300 m.)	*Lampito kumiliensis* (Kinb.).	19.42
		Dichogaster saliens (Bedd.).	9.37
		Pontoscolex corethrurus (Muller).	19.16

use part of their castings to strengthen their burrow walls and the rest is released as castings. Castings of these earthworms may be ovoid or irregularly shaped minute mounds. Though the nature of cast released is characteristic of a species, this cannot be criterion for their identification [50]. If pellet-like castings are released by *Pheretima posthuma*, *Perionyx millardi* releases thread-like castings. Thick and long winding columns of hollow mound of 5 cm long and 2.5 cm wide casts are characteristic of *Hoplochaetella khandalaensis.* The biggest cast of *Notoscolex birmanicus* weighing 1.6 kg after drying for four months is reported from Burma [50]. *Polypheretima elongata* and *Pontoscolex corethrurus* excrete the ingested soil as sticky, thick lumps on soil surface.

Amount of cast produced can serve as an index for assessing earthworm activity. Immediately after rains, release of surface casts will be at a maximum level. At this point of time, majority of earthworms are found at 0 to 10 cm depth and very few of them are found at 20 to 30 cm depth (Kale and Dinesh, 2005, unpublished). Surface cast production has been quantified in different agro-ecosystems to relate it to their abundance [51–53]. Influence of seasonal variation and land use pattern was observed with respect to cast production in shifting agriculture [34]. Norgrove and Hauser [54] have recorded around 30 to 35 t/ha of cast production in tropical silvicultural system. Reddy [55] has reported annual production of 23.4 to 140.9 tonnes by *Pheretima alexandri*. According to Lavelle [56], cast production is rhythmic and

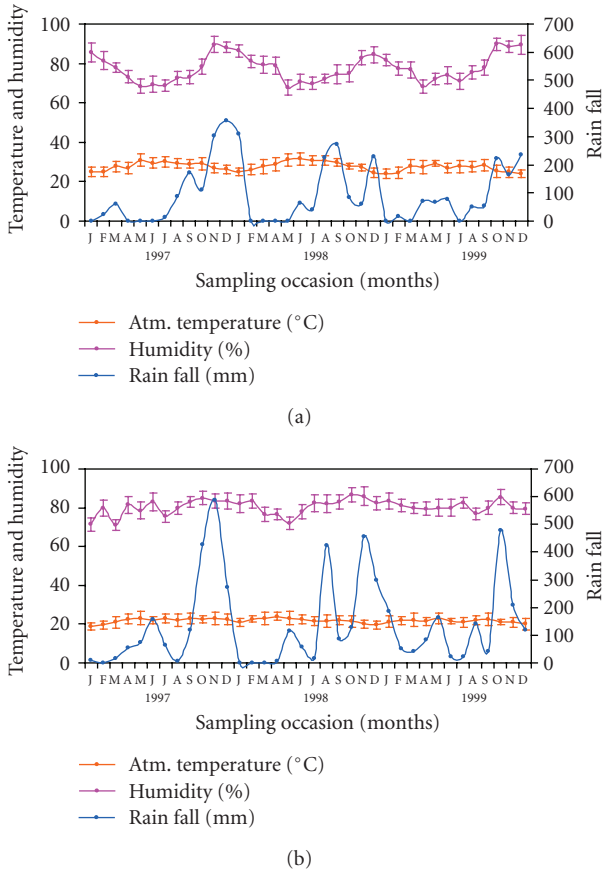

Figure 3: Atmospheric temperature (mean ± SD), Humidity (mean ± SD), and average rainfall of the study sites 1–7 (a) and 8–10 (b).

Table 7: Earthworm cast production during early postmonsoon period (Nov. 2004) at different agro-ecosystems in Kuti village of Somavarpatna Taluk of Karnataka State (Kale and Dinesh 2004, unpublished).

Land uses	Castings (Kg/Sq. M)
Natural forest	11. 20 ± 0.46
Coffee plantation	17. 2 ± 0.53
Cardamom plantation	16. 80 ± 1.00
Paddy fields (after harvest)	13. 60 ± 1.00
Acacia plantation	2.40*
Grassland	0.8*

* Due to dryness prevailing at the collection spots castings could be collected only from single spots out of 6 and 8 monolith points.

it will be at maximum at early morning hours. In general cast production in tropical countries is restricted to wet seasons. Table 7 provides the information on earthworm cast production in different agro-ecosystems during onset of postmonsoon season in the state of Karnataka, India.

The physicochemical properties of casts depend on the habitat soil and species of earthworm [57]. Their aggregate stability depends on the available organic matter [58]. The stability of casts and stability of fragmented casts on disintegration are the important factors to determine the soil structure [1]. Aggregate stability may result from addition of mucus secretion from earthworm gut and of associated microorganisms in the gut. It may also be due to macerated organic particles in the castings that encourage microbial activity after its release from the gut [59]. According to Parle [60], stability of casts is due to fungal succession that takes place in the cast. Habibulla and Ismail [61] are of the opinion that soil texture, particle size, and porosity play an important role in burrowing and surface cast production. As casting activity is restricted to wet seasons, not much of attention is paid to assess the quantum of cast produced and its influence on soil physical, chemical, and biological properties as is available from other parts of the world. It is essential to know the physicochemical and biological variations that may be seen in cast produced by the same species of earthworm inhabiting places that differ in physiographic and edaphic conditions. This will provide the information on interrelationship of earthworms, original soil characters, and nature of available organic material that influence the change in soil characters through deposition of earthworm cast. The fertile lands turning unproductive in Himachal Pradesh, India, due to sticky castings of earthworms that turned the soil into cement-like clods had been reported [62]. Puttarudraiah and Sastry [63] had observed stunting of growth in root crops like carrot, radish, and beetroots due to castings of *Pontoscolex corethrurus* in pot culture studies.

Castings of earthworms are the "store house" of nutrients for plants. The increased earthworm activity with increase in availability of carbon and in turn a raise in available nitrogen and phosphorus in their castings was also reported [6]. Earthworm activity has shown to improve the soil aggregates and soil minerals that are more available to plants than from soil [54, 64]. It is clear from various studies that earthworm casts may have more important role in plant nutrition and nutrient cycling than it was assumed previously [65, 66]. In India, very early reports are available on such observations on the chemical properties of earthworm castings that can play a positive role in plant growth [57, 67, 68]. The chemical composition of casts, which is widely studied, is of holonephric lumbricid earthworms. In subtropical country like India where majority of earthworms are meronephric, their castings may show higher level of available plant nutrients than surrounding soil. Dash and Patra [7, 53] had reported higher levels of nitrogen in casts of *Lampito mauritii* than in surrounding soil. Ganeshmurthy et al. [69] have found higher rate of mobilization of micronutrients in earthworm castings. It requires further studies on meronephric Megascolecid earthworms and their castings on available and exchangeable forms of nutrients to assess their contribution to soil fertility. Kale and Krishnamoorthy [70] had shown increased levels of soluble calcium and carbonates in castings of *Pontoscolex corethrurus.* Soluble carbonates contribute to exchangeable base contents of castings (Table 8). The physicochemical properties like pH, EC, organic C, total N, available P, K, Na, Ca, and Mg of casts did not differ in zero tillage land treated with mulch of residues of annuals or perennials [19]. The population dynamics of a peregrine earthworm,

Table 8: Calcium and carbonates in castings of *Pontoscolex corethrurus* compared with that of habitat soil [70].

Constituents	μg/g dry weight	
	Soil	Castings
Ionic Calcium	12.24 ± 0.41	145.50 ± 9.81
Exchangeable Calcium	12.83 ± 0.37	95.23 ± 7.28
Insoluble Calcium	179.62 ± 0.02	32.09 ± 0.93
Ionic/Insoluble Carbonate	0.15 ± 0.01	6.98 ± 2.22

Table 9: Microbial population in neem cake enriched vermicompost [80].

Microbial population no./g vermicompost	Vermicompost with 2% neem cake	Vermicompost without neem cake
Fungi no. $\times 10^4$	22.3	5.2
Bacteria no. $\times 10^6$	15.0	7.8
Nitrogen fixers no. $\times 10^5$	54.1	6.6

Pontoscolex corethrurus, in undisturbed soil of Sirumalai Hills clearly showed that the parameters like rainfall, humidity, soil moisture, and organic carbon influence the population positively [26, 27]. It has also been reported that in rubber plantations of Tripura, a part of north-east India, *Pontoscolex corethrurus* was the dominant species, representing 61.5% biomass and 72% density of the total earthworm population where it might be linked to individual tree species effect (*Hevea brasiliensis*) that favoured *P. corethrurus* over other species [37].

7. Earthworms and Microflora

Earthworm activity is closely associated with microbial activity. Lavelle [2] is of the opinion that there may exist competition between microorganisms and earthworms for easily digestible and energy rich substrates. Such competition may depend on availability of nutrients in the medium. Contrary to this, earthworms may derive benefit from microorganisms when they have to survive on materials rich in cellulose or hemi cellulose. So there exists mutualistic relation between earthworms and microorganisms. Tiunov and Scheu [6] have shown that earthworms deprive easily available carbon to microorganisms and availability of carbon increases effective mobilization of N and P by earthworms. The complex interrelationship of earthworms and microorganisms is at the level of their digestive tract, castings, and burrow walls [71]. This establishes the probable mutualism that exists between earthworms and microorganisms. Joshi and Kelkar [68] demonstrated higher microbial activity in earthworm castings and their role in mineralization of nitrogen. They incubated known weights of groundnut cake in a pot containing earthworm castings and other containing soil from the same place. The release of N from groundnut cake was at a higher level in pot containing castings than from one having soil as the medium.

Bhat et al. [72] were the pioneer contributors to report on role of microorganisms in the gut of earthworms. Khambata and Bhat [73] had made a detailed investigation on intestinal microflora of *Pheretima* sp. They had isolated *Pseudomonas*, Corenyform bacteria, *Nocardia*, *Streptomyces*, and *Bacillus* from the intestinal tract. There is no report of nitrosofying and nitrifying bacteria in their observations in the gut of earthworms. Dash et al. [74] have reported about isolation of 16 fungi from different parts of the gut out of 19 found in their habitat. In the fresh castings of the same earthworms there were only seven fungi with antibiotic properties or with thick spore coats. This suggests the selective fungal feeding by earthworms.

Drillosphere is the focus for understanding earthworm microbe interrelationship. This association is also associated with land use and metabolizable carbon present in the soil. Metabolizable carbon has positive effect on both microorganisms and earthworms [75]. Microbial activity will be at a higher level in the drillosphere than in surrounding soil and other edaphic factors determine the microbial diversity in drillosphere [76]. According to Kretzschmar [77], interaction of soil fauna and microflora determines soil dynamics. The contribution of their activity for formation of humus is an index for soil fertility. Bhatnagar [78] had expressed that at 20 to 40 cm depth in drillosphere zone there were 40% aerobic N-fixers, 13% anaerobic N-fixers, and 16% of denitrifiers. He attributed low C/N ratio in soils rich in earthworm population because of stimulation of N-fixers in drillosphere. Drillosphere provides necessary substrate for growth and establishment of microorganisms.

Recent developments in the country as well as at the global level are the application of detritivorous epigeic earthworms for organic manure/vermicompost production from biodegradable organic materials recovered from agricultural lands, agro-based industries, and municipal solid waste. This field of study is closely associated with earthworm microbe interaction. The quality of the manure or vermicompost depends on microorganisms associated with the process of decomposition. Bhat [79] had reported that the diet formulation or the composition of organic matter used as feed influences the microflora associated with earthworm activity. Similar studies were made on enhanced N-fixers activity on using 2% neem cake in the feed mix of earthworm *Eudrilus eugeniae* [80] (Table 9).

During winter months in Himalayan region, fungal population was higher in vermicomposting system than in the native soil [81]. Maintenance of temperature in vermicomposting system at a favourable level for earthworm activity might have been the reason for establishment of fungal population. Press mud, a by-product of sugar industry, is often used as one of the substrates in vermicomposting. Subjecting of this material to earthworm activity along with other organic matter has resulted in changes in microbial populations [82]. Rajani et al. [83] have related the microbial density and enzyme activity as a measure to assess the effectiveness of process of vermicomposting. It is essential to make an in-depth study to understand the mutualistic association between microflora and earthworms in mechanism of decomposition of organic matter.

An increase in actinomycetes population was observed in the gut region of earthworms. Some of the isolates from gut region of earthworms have expressed growth stimulatory effect when used in pot cultures of tomato and finger millet [84].

The colony forming units (CFUs) of bacteria and fungi in the casts of *P. corethrurus* significantly deviated from the CFU found in adjacent soil. The correlation between the physicochemical parameters and microbial populations of the casts of *P. corethrurus* showed that the establishment of microbial population requires optimum moisture, organic carbon, and nitrogen content [20]. The vermicasts of *P. ceylanensis* showed 14 different fungal species belonging to the genera, *Aspergillus, Chaetomium, Cladosporium, Cunninghamella, Fusarium, Mucor, Penicillium*, and *Rhizopus.* Total nitrogen, phosphorus, potassium, calcium, copper, iron, and zinc were higher in vermicasts than in control (substrate without earthworms) while organic carbon and C/N ratio were lower in vermicasts. The total organic carbon was 42.3% in the control whereas it was 35.2% in the vermicasts of *P. ceylanensis*. The incubation of vermicasts (45 days) showed significant correlation with that of the increase in fungal population ($r = 0.720$; $P < .05$) and decrease in moisture content ($r = -0.984$; $P < .001$), and the decrease in moisture content statistically had no effect on the total fungal population in the vermicasts of *P. ceylanensis* [85]. The total microbial population, namely, bacteria, fungi, and actinomycetes was found to be manyfold higher than in the initial vermibed substrate and in substrate without earthworms (control). The initial count of bacteria, fungi, and actinomycetes in the control was $123.42\,\text{CFU}\times10^{7}\,\text{g}^{-1}$, $159.64\,\text{CFU}\times10^{3}\,\text{g}^{-1}$, and $86.90\,\text{CFU}\times10^{4}\,\text{g}^{-1}$ whereas in castings (vermicompost) of *P. ceylanensis* the reported microbial populations were 268.62, 223.39, and 141.09 [86]. These observations clearly indicate the importance of microorganisms associated with earthworms in creating suitable environment for the standing crops as well as for vermicomposting of different organic wastes. It is still at the infancy to draw any inference regarding earthworm, microbe, and plant association.

Studies are also in progress to assess the inhibitory effects of the principles present in the body wall, gut extract, and of coelomic fluid on some selected plant and animal pathogens. The studies are at preliminary stages and it will require some more time to draw any conclusions based on the available data. Such interdisciplinary applications of earthworm research help to understand the functional complexity of these organisms other than their contribution to management of organic biodegradable residues as the major secondary detritivorous group.

8. Earthworms as Bioindicators

Earthworms can also serve as indicators of several changes/factors associated with soil. Many studies clearly showed that the earthworms are best indicators of heavy metals, toxic pollutants, and direct and indirect anthropogenic changes in soil [87–89]. A study conducted in northern semiarid region of India showed the presence of earthworms to the maximum level wherever the farmers followed integrated farming (100%) practice and this was followed by organically managed (70%) and conventional (18.9%) agro-ecosystems. The earthworm abundance was directly related to the management practices and the values of ecological indices like Shannon diversity (H′), species dominance (C), the species richness (S), and evenness (E). This clearly illustrates the anthropogenic pressure on earthworm communities in arable lands [90]. Similar report from Ivory Coast is available on the impact of land-use changes and land-use intensification on earthworm populations and diversity in intermediate-disturbed systems [91]. Even though these studies suggest the use of earthworms as bioindicators of man-made changes, it necessitates more field and laboratory investigations to find out earthworm community structure, species interrelations, and the most efficient species to be used in biomonitoring of ecosystem degradation due to anthropogenic activities in the forest areas.

Certain toxic substances in soil affect the behaviour and physiology of earthworms that can serve as biomonitoring tool for their systematic effect on soil organisms and other higher organisms. For example, the presence of tetra ethyl lead (TEL) in leaded gasoline and lead oxide has a significant effect on behaviour, morphology, and histopathology of earthworms. Absorption of TEL into the tissues of earthworms produced severe effects, rupture of the cuticle, extrusion of coelomic fluid, and inflexible metameric segmentation. This led to desensitization of the posterior region and its fragmentation [92].

The efficient potential of earthworms in bioaccumulation of heavy metals in their tissues serves as ecological indicator of soil contaminants. As per the recent report from India, the level of DTPA extractable metals in casts of earthworms, *Metaphire posthuma* (endogeic) and *Lampito mauritii* (anecic) collected from cultivated land, urban garden and sewage soils were higher than those of surrounding soil. The concentration of Zn, Fe, Pb, and Mn in earthworm casts was higher in sewage soil followed by cultivated land and urban garden, respectively. There exists a close relationship between metal concentration in earthworm tissues and surrounding soils. The study also revealed the presence of species-specificity in metal accumulation in earthworms. Higher level of metal concentrating in the tissues was found in endogeic *M. posthuma* than in tissues of anecic *L. mauritii.* The difference in burrowing patterns may influence the patterns of bioaccumulations of metals apart from other contributory factors. Further, more detailed study is still required to elaborate the proposed hypothesis [93]. Analogous study conducted in Egypt also suggests that the variation in heavy metal concentration in soil and earthworms in different sites may be significant depending on soil properties and pollution status [88]. Sizmur and Hodson [94] evidently suggested that earthworms increase metal mobility and availability but more studies are required to determine the precise mechanism for this. So, this field of research with earthworm requires in depth research to understand the functional role of earthworms as bioindicators and bioconcentrators.

9. Earthworms and Vermicomposting: Indian Scenario

The familiar earthworm species, *Eudrilus eugeniae, Eisenia fetida, Lumbricus rubellus*, and *Perionyx excavatus*, are well known for their efficiency in vermicomposting. It is desirable to know about other species of earthworms that may be as efficient or better in their performance over the above mentioned species in a country having rich diversity of fauna for *in situ* and *ex situ* vermiculture. There are more than a dozen of earthworm species that have been reported to be efficient in vermicomposting. Most of the species that are included under genus *Perionyx* show great potential to work on organic matter. Apart from the well-known *P. excavatus*, other *Perionyx* species such as *P. ceylanensis, P. bainii, P. nainianus*, and *P. sansibaricus* are recently considered to be the potential vermicomposting earthworms [20, 95–97]. Future investigations provide scope for identifying more species with vermicomposting potential.

In natural systems, if earthworms are ecosystem engineers, in man made seminatural systems of organic residues, the detritivorous earthworms are saviors of biosphere from organic pollutants. From the review, it is very clear that the earthworm ecology needs much attention with reference to their functional role in different ecosystems. By the way of exploration, it might be possible to understand the significant role of earthworms in plant-microbe interactions. With regard to vermiculture, it is necessary to work on the idea of developing the consortia of earthworm species for vermiculture practices in India. It is always better to develop and encourage polycultures rather than maintaining monoculture. Moreover, with diversity in agricultural residues and by-products from agroindustries, it is essential to identify earthworms that will accept these materials with minimum effort and investment.

There are more than 500 species of earthworms distributed in different geographical regions in India, in different ecosystems. Being partly subtropical and partly temperate, majority of earthworms are endogeic or geophagous. Even among the epigeic earthworms (*ca*. 8%), those that are voracious feeders, are efficient biomass producers, and have short life cycle, high rate of fecundity, and high rate of adaptability to changing physicochemical properties of feed material can only serve as successful species for vermiculture. One has to look for these characters before recommending any species for vermiculture. The species that is promising under protected laboratory conditions in a small scale may fail to perform under field conditions when it is expected to work on large amount of organic matter. The present scenario in India shows that there is good response from the farmers to adopt the technology for producing vermicompost to use as soil amendment. They are reaping the benefits of using the recommended species for producing required quantity of vermicompost to fulfill the needs of their land and also to market the production to other neighbourhood farmers. Still many avenues remain open for the scientists to carry out research in this field to unravel various problems associated with the technology.

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Review Article

Role of Earthworms in Soil Fertility Maintenance through the Production of Biogenic Structures

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The soil biota benefits soil productivity and contributes to the sustainable function of all ecosystems. The cycling of nutrients is a critical function that is essential to life on earth. Earthworms (EWs) are a major component of soil fauna communities in most ecosystems and comprise a large proportion of macrofauna biomass. Their activity is beneficial because it can enhance soil nutrient cycling through the rapid incorporation of detritus into mineral soils. In addition to this mixing effect, mucus production associated with water excretion in earthworm guts also enhances the activity of other beneficial soil microorganisms. This is followed by the production of organic matter. So, in the short term, a more significant effect is the concentration of large quantities of nutrients (N, P, K, and Ca) that are easily assimilable by plants in fresh cast depositions. In addition, earthworms seem to accelerate the mineralization as well as the turnover of soil organic matter. Earthworms are known also to increase nitrogen mineralization, through direct and indirect effects on the microbial community. The increased transfer of organic C and N into soil aggregates indicates the potential for earthworms to facilitate soil organic matter stabilization and accumulation in agricultural systems, and that their influence depends greatly on differences in land management practices. This paper summarises information on published data on the described subjects.

1. Introduction

Protection of the soil habitat is the first step towards sustainable management of its biological properties that determine long-term quality and productivity. It is generally accepted that soil biota benefits soil productivity but very little is known about the organisms that live in the soil and the functioning of the soil ecosystem. The role of earthworms (EWs) in soil fertility is known since 1881, when Darwin (1809–1882) published his last scientific book entitled "The formation of vegetable mould through the action of worms with observations on their habits." Since then, several studies have been undertaken to highlight the soil organisms contribution to the sustainable function of all ecosystems [1]. Soil macrofauna, such as EWs, modify the soil and litter environment indirectly by the accumulation of their biogenic structures (casts, pellets, galleries, etc.) (Table 1). The cycling of nutrients is a critical ecosystem function that is essential to life on earth. Studies in the recent years have shown increasing interest in the development of productive farming systems with a high efficiency of internal resource use and thus lower input requirement and cost [2, 3]. At present, there is increasing evidence that soil macroinvertebrates play a key role in SOM transformations and nutrient dynamics at different spatial and temporal scales through perturbation and the production of biogenic structures for the improvement of soil fertility and land productivity [4, 5]. EWs are a major component of soil fauna communities in most natural ecosystems of the humid tropics and comprise a large proportion of macrofauna biomass [6]. In cultivated tropical soils, where organic matter is frequently related to fertility and productivity, the communities of invertebrates—especially EWs—could play an important role in (SOM) dynamics by the regulation of the mineralization and humification processes [7–9].

Table 1: Some properties of casts of *Pheretima alaxandri* and their underlying soils with and without litter cover [10].

	Soil without litter		Soil with litter	
	Surface soil	Worm cast	Surface soil	Worm cast
pH	5.65	7.70	6.25	6.30
Organic Carbon (%)	1.52	1.70	2.66	3.36
Available P_2O_5 (mg $100\,g^{-1}$)	0.15	0.24	0.19	0.22
Available K_2O (mg $100\,g^{-1}$)	3.31	4.78	5.98	7.36

Table 2: Effect of land conversion and management practices on changes in functional catagories of earthworms in the Indo-Gangetic plains, (±SE, $n = 10$).

Sites	Density (*Anecic*) (Individuals m^{-2} $year^{-1}$)	Biomass (*Anecic*) (gm^{-2} $year^{-1}$)	Density (*Endogeics*) (Individuals m^{-2} $year^{-1}$)	Biomass (*Endogeics*) (gm^{-2} $year^{-1}$)
Primary forest	$_1$41 (±3.2)^a	$_1$23 (±11.6)^a	$_2$127 (±13.8)^a	$_2$255.8 (±20.6)^a
Productive agroecosystem	$_1$141 (±11.6)^b	$_1$323 (±23.5)^b	$_2$75 (±6.3)^b	$_2$157.5 (±13.3)^b
Low productive agroecosystem	$_1$106 (±7.9)^c	$_1$318 (±27.8)^b	$_2$45 (±3.2)^c	$_2$94.5 (±6.8)^c
Agriculture fallow	$_1$64 (±3.8)^d	$_1$42 (±2.9)^c	$_2$274 (±14.6)^d	$_2$518.7 (±42.6)^d
Sodic ecosystems	0	0	0	0
5-year-old reclaimed agroecosystem	0	0	143 (±12.7)^e	114.4 (±5.8)^c
10-year-old reclaimed agroecosystem	0	0	282 (±24.7)^d	160.6 (±15.3)^b
Acacia plantation in reclaimed soils	$_1$44(±5.3)^a	$_1$132 (±5.9)^a	$_2$133 (±9.6)^a	$_2$279.3(±21.5)^e

Values followed by the different superscript letters are significantly different in different sampling sites. Values followed by different subscript numbers are significantly different in same sampling sites [11].

1.1. Functional Significance of Earthworms. The effects of EWs on soil biological processes and fertility level differ in ecological categories [12]. Anecic species build permanent burrows into the deep mineral layers of the soil; they drag organic matter from the soil surface into their burrows for food. Endogeic species live exclusively and build extensive nonpermanent burrows in the upper mineral layer of soil, mainly ingested mineral soil matter, and are known as "ecological engineers," or "ecosystem engineers." They produce physical structures through which they can modify the availability or accessibility of a resource for other organisms [13]. Epigeic species live on the soil surface, form no permanent burrows, and mainly ingest litter and humus, as well as on decaying organic matter, and do not mix organic and inorganic matter [14]. In the majority of habitats and ecosystems (Table 2), it is usually a combination of these ecological categories which together or individually are responsible for maintaining the fertility of soils [15–17].

1.2. Role of Earthworms in Nutrient Availability to Soil. EWs influence the supply of nutrients through their tissues but largely through their burrowing activities; they produce aggregates and pores (i.e., biostructures) in the soil and/or on the soil surface, thus affecting its physical properties, nutrient cycling, and plant growth [19, 20]. The biogenic structures constitute assemblages of organo-mineral aggregates. Their stability and the concentration of organic matter impact soil physical properties and SOM dynamics. Besides they affect some important soil ecological processes within their "functional domain" [21, 22] where they concentrate nutrients and resources that are further exploited by soil microorganism communities [23, 24]. The effect of EWs on the dynamics of organic matter varies depending on the time and space scales considered [25]. The activity of endogeic EWs in the humid tropical environment accelerates initial SOM turnover through indirect effects on soil C as determinants of microbial activity. Due to selective foraging of organic particles, gut contents are often enriched in organic matter, nutrients, and water compared with bulk soil and can foster high levels of microbial activity [26, 27]. They have been reported to enhance mineralization by first fragmenting SOM and then mixing it together with mineral particles and microorganisms, and thereby creating new surfaces of contact between SOM and microorganisms [28]. In the short term, a more significant effect is the concentration of large quantities of nutrients (N, P, K, and Ca) that are easily assimilable by plants in fresh cast depositions [18]. Most of these nutrients are derived from earthworm urine and mucus [29]. In highly leached soils of humid tropics, earthworm activity is beneficial because of rapid incorporation of the detritus into the soils [30]. In addition to this mixing effect, mucus production associated with water excretion in the earthworm gut is known to enhance the activity of microorganisms [31]. This is followed by the production of organic matter. So fresh casts show high nutrient contents

Table 3: Variation in nutrient concentration of earthworm casts and noningested soils during cropping under shifting agriculture in North East India (±SE, $n = 5$) [18].

	5-year-cycle		15-year-cycle	
	Soil	Worm cast	Soil	Worm cast
Organic Carbon (%)	2 (±0.1)	*2.5 (±.13)	3.2 (±.17)	**4.5 (±.23)
Total Nitrogen (%)	0.22 (±0.01)	*0.29 (±.17)	0.4 (±.03)	*0.6 (±.04)
Available Phosphorus (mg/100 g)	0.9 (±0.03)	*1.4 (±.09)	2.0 (±.06)	**2.8 (±.15)
Potassium (meq/100 g)	0.5 (±0.02)	0.54 (±.04)	1.2 (±.05)	*2.0 (±.09)
Calcium (meq/100 g)	0.9 (±0.01)	*1.2 (±.08)	1.5 (±.04)	**2.5 (±.13)
Magnesium (meq/100 g)	1.2 (±0.05)	*1.8 (±.09)	3.1 (±.17)	*4.0 (±.34)

* $P < .05$, ** $P < .01$.

Table 4: Variation in nutrient concentration of earthworm casts and non ingested soils in abandoned agricultural fallows in North East India (±SE, $n = 5$) [18].

	5-years-old fallow		10-years-old fallow		15-years-old fallow	
	Soil	Worm cast	Soil	Worm cast	Soil	Worm cast
Organic Carbon (%)	1.2 (±.07)	*3.5 (±.09)	1.9 (±.09)	**4 (±.03)	2.2 (±.13)	**5.2 (±.04)
Total Nitrogen (%)	0.22 (±.01)	*0.55 (±.02)	0.25 (±.03)	**0.59 (±.02)	0.21 (±.04)	*0.62 (±.05)
Available Phosphorus (mg/100 g)	0.38 (±.02)	*1.1 (±.05)	0.5 (±.01)	**1.8 (±.07)	0.54 (±.01)	*1.7 (±.05)
Potassium (meq/100g)	0.24 (±.01)	*0.61 (±.32)	0.4 (±.03)	*1.0 (±.05)	0.42 (±.01)	*0.90 (±.02)
Calcium (meq/100 g)	0.19 (±.03)	*0.60 (±.03)	0.22 (±.02)	**0.75 (±.01)	0.22 (±.01)	*0.85 (±.02)
Magnesium (meq/100 g)	0.22 (±.01)	*0.50 (±.01)	0.2 5 (±.04)	*0.60 (±.01)	0.32 (±.01)	*0.70 (±.01)

* $P < .05$, ** $P < .01$.

(Table 3). The chemical characteristics of casts differ from those of noningested soil [32] and are rich in plant available nutrients. Upon cast deposition, microbial products, in addition to earthworm mucilages, bind soil particles and contribute to the formation of highly stable aggregates [33, 34]. Although EWs may speed up the initial breakdown of organic residues [35, 36], several studies have indicated that they may also stabilize SOM through its incorporation and protection in their casts [37–40]. Over longer periods of time, this enhanced microbial activity decreases when the casts dry, and aggregation is then reported to physically protect SOM against mineralization. Thus C mineralization rate decreases and mineralization of SOM from casts may be blocked for several months [37, 41]. It might become accessible again for the microflora once these are degraded into small fragments [42–44]. In addition EWs seem to accelerate the mineralization as well as the turnover of SOM [45]. Furthermore, studies have also indicated that organic matter in the casts, once stabilized, can maintain this stabilization for many years [46, 47]. Nevertheless, chemical mechanisms may also contribute to the stabilization since evidence shows that the casts are held together by strong interactions between mineral soil particles and SOM that is enriched in bacterial polysaccharides and fungal hyphae [48, 49]. Earthworm casts are enriched in organic C and N, exceeding the C and N contents of the non ingested soil by a factor of 1.5, and 1.3, respectively (Table 4). This enrichment appears in all particle-size fractions, not restricted to certain organic compound dynamics of a cultivated soil [50]. These results clearly indicate the direct involvement of EWs in providing protection of soil C in microaggregates within large macroaggregates leading to a possible long-term stabilization of soil C [51] (Table 5). It has also been reported that EWs increase the incorporation of cover crop-derived C into macroaggregates, and more important, into microaggregates formed within macroaggregates. The increased transfer of organic C and N into soil aggregates indicates the potential for EWs to facilitate SOM stabilization and accumulation in agricultural systems [52].

EWs are known also to increase nitrogen mineralization, through direct and indirect effects on the microbial community (Table 6). Our studies on the role of EWs in the nitrogen cycling during the cropping phase of shifting agriculture in North East India showed (Table 7) that the total soil nitrogen made available for plants through the activity of EWs was higher than the total input of nitrogen to the soil through the addition of slashed vegetation, inorganic and organic manure, recycled crop residues, and weeds [54]. An important role of EWs is the dramatic increase in soil pH as observed through our studies in shifting agroecosystem in North East India, in a sedentary terrace agroecosystem in central Himalayas, and in intensive agroecosystem in Indo-Gangetic plains. This increases microbial activity and N fixation in the soil, so that nitrogen in the worm cast may be due at least in part to this rather than to concentration by gain worms. Nitrogen mineralization by microflora is also quite intense in the earthworm gut and continues for several hours in fresh casts [55, 56], respectively, by incorporating organic matter into the soil and or by grazing the bacterial community. EWs have been found to either enhance or decrease bacterial biomass [57–59], and to stimulate bacterial activity [60, 61]. The influence of EWs on N cycling,

Table 5: C and N contents and C : N ratio in particle-size organic fractions in control soil and cast of *Pontoscolex corethrurus* (±SE) [53].

Particle size (μm)	Laguna Verde		La Mancha	
C(mg g^{-1} soil)	Soil	Casts	Soil	Casts
2000–250	32.8 ± 5.1	51.2 ± 2.8	13.8 ± 8.4	7.1 ± 2.4
100–50	48.8 ± 4.7	54.1 ± 1.3	1.6 ± 0.6	1.5 ± 0.9
50–20	48.5 ± 7.6	63.4 ± 4.8	21.9 ± 9.6	17.1 ± 2.3
20–2	50 ± 4.2	22.4± 13.7	15.2 ± 6.7	29.5 ± 5.1
N(mg g^{-1} soil)				
2000–250	4.72 ± 1.2	4.35 ± 0.10		
100–50	4.35 ± 0.2	5.24 ± 0.60	0.21 ± 0.01	2.2 ± 0.22
50–20	4.06 ± 0.4	5.04 ± 0.04	1.91 ± 0.20	2.4 ± 0.20
20–2	4.20	4.76 ± 0.40	2.46 ± 1.02	2.8 ± 0.9
C : N ratio				
2000–250	8.8	11.8		
100–50	10.8	10.3	7.6	6.8
50–20	12.0	12.6	11.5	7.1
20–2	11.9	4.7	6.2	10.5

Table 6: Total and mineral nitrogen content in soil and fresh casts from earthworms incubated in different soil types (Barois et al., 1992 [53]).

Soil type	Layer (cm)	Earthworm species	Soil		Worm cast	
			N total (%)	Mineral N (μg g^{-1})	N total (%)	Mineral N (μg g^{-1})
Andisol, Martinique	0–10	Pontoscolex corethrurus	15.5	516.8	15.7	1095.1
Andisol, Mexico	0–10	Pontoscolex corethrurus	4.8	55.4	4.9	625.1
Luvic, Cuba	0–10	Onychochaeta elegans	2.6	55.4	2.4	212.5
Ultisol, Yurimaguas	0–10	Pontoscolex corethrurus	1.37	30	1.47	150.5
Vertisol, Lamto	0–10	Protozapotecia australis	3	52.1	4	560.9

Table 7: Nitrogen input/output budget during the cropping phase under 5- and 15-year Jhum cycle, (±SE, $n = 5$) [54].

	Nitrogen balance (kg ha^{-1} yr^{-1}) in different shifting agriculture cycles	
	5-years	15-years
INPUT		
Slash	27.60 (±1.30)	51.4 (±3.6)
Organic manure	14.0 (±1.1)	—
Inorganic fertilizer	0.80 (±.04)	—
Crop biomass	0.42 (±.05)	0.9 (±.01)
Weed biomass	2.85 (±1.1)	0.7 (±.03)
Precipitation	4.20 (±.28)	4.2 (±.26)
Input subtotal	**49.90**	**57.2**
Worm casts	27.0 (±1.3)	65.6 (±4.8)
Worm tissues	9.5 (±.13)	12.1 (±1.4)
Mucus production	75.9 (±3.2)	95.3 (±4.5)
Input total	**112.4	**173.0
OUTPUT		
Fire	277.6 (±23.2)	657.9 (±23.9)
Sediment	158.0 (±10.2)	116.0 (±4.5)
Percolation	1.0 (±.04)	1.2 (±.08)
Runoff	7.3 (±0.3)	14.0 (±1.3)
Weed removal	14.25 (±3.86)	3.33 (±.26)
Crop removal	15.24 (±1.28)	43.52 (±3.20)
Output total	**474.39**	**835.96**
Input-Output difference	**312.12**	**605.75**

however, appears also to be largely determined by cropping system type and the fertilizer applied (mineral versus organic). Various experimental studies suggest that EWs have potentially negative consequences on fertilizer-N retention studies [62]. The earthworm species and species interactions present in the system also effect nitrogen mineralization and crop production [63]. This may result in enhanced nitrogen immobilization or mineralization depending on species characteristics and substrate quality. The review thus highlights the important effects that EWs have on C and N cycling processes in agroecosystems and that their influence depends greatly on differences in management practices [64]. Further the EWs can also increase nutrient availability in systems with reduced human influence and low nutrient status, that is, no tillage, reduced mineral fertilizer use, and low organic matter content [65–67]. The role of EWs in improving soil fertility is ancient knowledge which is now better explained by scientific results emerging from different studies. This is an important field of study where the research is directly linked to the social welfare [68]. Every involved step requires appropriate protocols and reproducible results. This is a feedback mechanism where the technology adopted in the fields is further improved in the laboratories based on the feedback received from the technology adopters so as to provide more convincing information to technology adopters.

2. Future Research Needs

Most of the studies conducted to assess the role of earthworm casting in nutrient cycling and soil structure are related to surface casting species, and only a few have dealt with casts deposited under field conditions [5, 18, 54]. To reach a better understanding of the ecological impact of in-soil casts, the assessment of nutrient dynamics in earthworm burrows and on the effect of in-soil casts on plant growth would be of immense help. For below-ground casting earthworm species, the ecological impact of their below-ground casts is likely to be as important as their surface casts in relation with nutrient availability, especially for biological management of degraded and disturbed ecosystems. Therefore more research is needed to be done in this area to complete our knowledge of the role of EWs in nutrient dynamics so as to evolve strategies for better soil management techniques.

3. Conclusions

Considering the potential contribution of EWs to soil fertility management, there is the need to consider them in agroecosystem management decisions. The EWs can specifically affect soil fertility that may be of great importance to increase sustainable land use in naturally degraded ecosystems as well as agroecosystems. Proper earthworm management may sustain crop yields whilst fertilizer inputs could be reduced. Since farming can involve many soil disturbing activities, the understanding of the biology and ecology of EWs will help devise management strategies that may impact soil biota and crop performance.

Abbreviations

EW: earthworm
SOM: soil organic matter.

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Research Article

Casting Activity of *Scherotheca gigas* in No-Till Mediterranean Soils: Role in Organic Matter Incorporation and Influence of Aridity

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The behaviour of earthworms, their role in organic matter incorporation into the soil, and the influence of aridity in such processes in arid and semiarid regions have scarcely been studied. In this study, physico-chemical analyses of the casts and the surrounding no-till agricultural soils of three experimental sites representing an aridity gradient in Navarre (NW Spain) were done. The casts were formed by the activity of the only anecic species, *Scherotheca gigas* (Dugès, 1828), ubiquitous in no-till soils in this region. We observed a significant depletion of clay and higher concentration of total organic C and labile C in the form of particulate organic matter (POM) in the casts as compared to the surrounding soil, suggesting selective ingestion of soil by *S. gigas*. This, together with the observation of increased concentration in POM with increasing aridity, suggests a major role of this species in the observed progressive gains of organic C stocks in no-till soils in the region.

1. Introduction

It is well known that the abundance of earthworms in the soil depends on soil properties and on climate. Most of the studies related to the earthworms behaviour are from the tropical and subhumid temperate soils. Their behaviour in more limiting conditions, such as agricultural soils in semiarid or arid regions, has been less studied [1]. Some particularities of earthworms such as large body size, slow growth rate, ability to feed on soils poor in organic matter, occupying deeper soil profile, have been described in Mediterranean soils [2]. However, our knowledge is still limited. For instance, in the Iberian Peninsula, much less information on earthworm occurrence and activity exists for the Mediterranean domain than for the more humid NW area [3]. The reality is that in the majority of agricultural soils with Mediterranean type of climate, like in the Ebro Valley of Spain, located in the Mediterranean-Iberian-Levantine biogeographical superprovince [2], earthworms are scarce and often absent when tillage is intense [4].

It is also well known that, in general, when tillage is reduced earthworm activity increases. No-till and other methods of conservation tillage such as chisel plowing can thus increase earthworm populations [5]. Decreased tillage disturbances particularly benefit anecic species, which can move in the same burrow between deeper soil layers and the soil surface in search of food. This is so because when tillage destroys these burrows, some earthworms may not have the energy reserve needed to form a new burrow to their food source [6]. Previous studies in rainfed agricultural soils, cropped to cereals in the Ebro Valley (Navarra, Spain), provided evidence of increased earthworm density and biomass with reduced tillage [7, 8]. The three species commonly found in no-till soils in the area are *Allolobophora rosea, Scherotheca gigas, and Prosellodrilus praticola*. *S. gigas (Dugés 1828)* is the only anecic species to deposit cast on the soil surface. Though this species is widespread in the area, it

has a narrow geographical distribution worldwide [9]. In a study in the role of earthworms in soil aggregate formation, *S. gigas* was cited by Bouché and Al-Addan [10] as one of the most abundant species in a dry grassland on calcareous soil.

As for most earthworm species, soil moisture and temperature affect the activity of *S. gigas*. Field observations led us to distinguish two periods of surface casting activity over the year [8], each interrupted by a period of inactivity in January/February and June/September. As observed in other semiarid areas [11], during the dry summer months the species undergo diapause and cannot be found at the soil surface.

The contribution of earthworms to organic matter incorporation into the soil can be studied by comparing surface casts and the surrounding soil. Properties of surface casts usually differ from those of the bulk soil, but contradictory findings have been reported. Differences are related to the type of soil [12] and/or to earthworm species [13]. Brown et al. [14] have described in detail the role of earthworms in organic matter stabilization. These authors consider that the inclusion of the active fractions of the soil organic matter (SOM) into earthworm casts can contribute to its protection and stabilization when casts dry up, a process that can happen within hours or days after its deposition. It has also been demonstrated that soil management can greatly influence this process [15–17].

The protection of SOM from biological degradation in surface casts [18, 19] has a special significance for soils with high mineralization rates and low contents in SOM, as is the case for the soils of the Ebro Valley. In addition, soils rich in calcium carbonate also contribute to the persistence of casts over longer time periods and thus to long-time SOM protection.

The purpose of this study was to gain knowledge on the behavior of *S. gigas* in no-till carbonated Mediterranean soils by studying its role in the incorporation and preservation of organic matter into the soil and the influence of aridity in this process.

2. Methodology

2.1. Study Sites. The study was conducted at three long-term experimental sites that include different soil management treatments in the Ebro Valley in Navarre (NE, Spain). These sites were located in Pamplona (42°47′32″N; 1°37′54″W), Olite (42°27′33″N; 1°41′07″W), and Santacara (42°23′44″N; 1°32′32″W). Only the plots under no-till (NT) of each experimental site were chosen for earthworms and soil sampling. This was done because greater earthworm activity has been observed in the area in NT plots [8]. The three sites contain carbonated soils which are cropped to rainfed barley (*Hordeum vulgare* L.), but differ in some soil properties and, especially, in their agroclimatic characteristics (Table 1). They represent an aridity gradient because average rainfall is greater in Pamplona (Mediterranean sub-humid type of climate) and decreases southward to Olite (semiarid Mediterranean) and then Santacara (arid Mediterranean), while average annual temperatures and reference evapotranspiration (ET_0) increase in the same order.

A direct seeder that opens a seed-furrow 30 to 50 mm deep is used for barley seeding in fall in the studied NT plots at the three sites, using similar seeding doses (~160 kg/ha) and fertilization treatments. Average barley yield decreases with increasing aridity (Table 1).

2.2. Sampling Methods. As already mentioned, earthworm activity in the region is clearly seasonal, and coincides with the two rainfall peaks and mild temperatures observed in spring and fall. Three field NT replicates (plots) were sampled in November 2007 at each study site, in order to identify the earthworm species present in the soil. Two soil blocks (0.20 by 0.20 by 0.20 m) were taken in each plot. Earthworms were sampled by hand-sorting and counted in the field. Individuals were fixed with ethanol-formalin, and preserved in 10% formalin [20]. They were classified at the Department of Ecology and Animal Biology of the University of Vigo (Spain). Thirty to 50 individuals of *S. gigas* were found per square meter at the three study sites. Considering the size and the ability of this species to dig deep into the soil, these numbers are likely an underestimation of the actual abundance of *S. gigas* in the studied soils. It was observed that *S. gigas* was the only anecic species found, which indicates that surface casts present in the three soils were created by individuals of this species. This also illustrates the ubiquity of this species in NT soils in the region, regardless of soil characteristics and agroclimatic conditions.

Simultaneously, four replicates of surface earthworm casts were collected at three random points in the NT plots at each study site. Casts were air-dried, ground to pass through a 2-mm sieve and stored for further analyses.

Soil (0–30 cm) was sampled using an Edelman-type auger. Disturbed subsamples were collected at four random points per plot, and combined to obtain a composite sample per plot, so that three field replicates were analyzed at each site. Samples were air-dried and ground to pass through a 2-mm sieve.

2.3. Laboratory Analyses. Clay, total organic C content, and C in the form of particulate organic matter (C-POM [21]) were determined both in the casts and the bulk soil. Clay content was determined by the pipette method [22], using a modified Robinson pipette. Samples were chemically dispersed with a solution of 5% $(NaPO_3)_6$ before analysis. Due to the elevated carbonate content of the three soils (Table 1), wet oxidation was used to analyze total organic C [23].

Particulate organic matter is acknowledged to represent the most labile fraction of organic matter in the soil, and it is related to the formation of stable aggregates [24]. For this study, POM was isolated by dispersion and sieving of 10 g of air-dried soil, as described by Marriott and Wander [25]. Briefly, samples were dispersed with 150 mL of 5% $(NaPO_3)_6$ and the fraction >53 μm (which includes the POM and sand-size mineral components of the soil) was collected on a 53-μm-opening polycarbonate mesh (Gilson Co. Inc.,

Table 1: Agroclimatic and soil characteristics of the three studied sites.

Study site	Pamplona	Olite	Santacara
Rainfall (P, mm)	830	525	423
Evapotranspiration* (ET_0, mm)	972	1163	1145
P/ET_0	0.85	0.45	0.37
Temperature (°C)	12.4	13.5	14.2
Average barley yield (t grain ha^{-1})	3.93	3.70	2.75
Soil type (FAO, 2003)	*Calcic Cambisol*	*Calcic Cambisol*	*Haplic Calcisol*
pH (0–30 cm) (1 : 2.5)	8.63	8.25	8.48
$CaCO_3$ (0–30 cm) (g $kg\ soil^{-1}$)	252	359	363

*Reference evapotranspiration, (FAO).

Columbus, OH) after 3 rinses with distilled water, and then oven-drying at 50°C. Samples were then ground to a powdery consistency before measuring their C content to determine POM-C by wet oxidation [23].

2.4. Statistics. Data are presented as ± standard error of the mean for casts and soil characteristics. Data were analysed using ANOVA (univariate linear model), and means were compared among the three soils and between soil and casts at each study site using significant differences. Post hoc analysis was performed by Duncan's multiple range test. Significant results are based on a probability level of $P < .05$. All statistical analyses were performed using the SPSS 16.0 software [26].

3. Results and Discussion

3.1. Role of S. gigas in the Incorporation of Organic Matter into the Soil. Lower clay contents were observed in the *S. gigas* casts than the bulk soil in the three studied fields (Table 2). Our findings support thus the suggestion of Schrader and Zhang [27] that selective particle ingestion by earthworms leading to increased clay contents in casts than in the surrounding soil [28] appears to be confined to sandy soils, and/or might be more common in other smaller species of earthworms than the anecic *S. gigas*. An alternative explanation could be the existence of ultra-stable silt-size or sand-size aggregates in the casts, which would resist chemical dispersion.

Results from the analysis of the organic components of casts and bulk soils revealed that *S. gigas* casts contained more total organic C and POM than the soil (Table 2), as has also been reported through other studies [14]. More interestingly, the percentage of C in the form of POM was also greater within the casts than in the soil at the three studied sites, suggesting a selective ingestion of the more edible fresh plant residues and labile organic materials by *S. gigas* [14]. This finding is of special importance in the studied soils, which are naturally poor in organic matter, because POM entrapped within casts is likely to be more protected against microbial activity than elsewhere in the soil [12]. Guggenberger et al. [29] reported that the incorporation of organic matter in the casts of anecic earthworms on an Oxisol occurred preferentially in the form of POM. They related this to the higher aggregate stability observed in the casts in comparison to the surrounding soil. They further suggested that the mechanisms that protect organic matter from decomposition during aggregate formation and stabilization, as also described by Tisdall and Oades [30] and Six et al. [24], can be enhanced or facilitated by earthworm casting activity. Direct incorporation of POM into stable microaggregates through earthworm transit in the soil has also been described by Pulleman et al. [15] as an "alternative pathway" to the microbial-mediated one for the stabilization of POM by occlusion within microaggregates in undisturbed soils.

Long-term stabilization of organic matter occluded within aggregates inside earthworm casts is in fact possible [31] and depends on the type of soil [32], earthworm species [33], the age of casts, the way they dry [34–36], and the level of disturbance to which casts are subjected [12]. A number of studies have shown that the quantity of organic matter incorporated into the soil by earthworms also significantly affects the stability of casts. In the present study elevated clay and carbonates contents of the soils allow for overall high aggregate stability [8]. In Olite and Santacara, scarce rainfall and high average temperatures cause a rapid dehydration of casts and, as a result, intact earthworm casts are observed several months after their formation at these two study sites. In addition, NT techniques ensure low disruption of the soil surface and protection against raindrops by the mulch. Considering the observed contribution of *S. gigas* to POM preservation within the casts, the contribution of this species to the stabilization of organic materials and the overall increase of organic C observed in NT soils in the region [37, 38] is likely to be of importance.

3.2. Influence of Aridity in the Behavior of S. gigas. Differences related to the agroclimatic context of the three studied soils were found in the enrichment factor and composition of casts in relation to bulk soil (Table 3).

While in all the three sites casts contained less clays than the bulk soil, the relative amount of clay found in the casts of the more humid soil in Pamplona was significantly greater than in Olite and Santacara (Table 3). If we consider that the soil clay content was similar in Pamplona and Santacara and

Table 2: Content in clay, total organic C, and C in the form of POM (C-POM) in bulk soil samples and in *Scherotheca gigas* casts at the three study sites. Values marked with $*$ are significantly different ($P < .05$) between soil and casts for each parameter and site. Values followed by the same letter in the same row belong to the same Duncan's homogeneous groups ($P < .05$) for each parameter among sites. Means ± standard error ($n = 3$).

Study site		Pamplona	Olite	Santacara
Clay (g/kg)	Soil	$306.2 \pm 6.7^{*a}$	$426.2 \pm 2.04^{*b}$	$284.8 \pm 2.6^{*a}$
	Casts	$233.4 \pm 2.5^{*b}$	$234.8 \pm 22.2^{*b}$	$165.2 \pm 6.8^{*a}$
Total OC (g/kg)	Soil	$14.03 \pm 0.42^{*b}$	$14.32 \pm 0.66^{*b}$	$12.55 \pm 0.22^{*a}$
	Casts	$21.93 \pm 0.78^{*a}$	$26.23 \pm 1.81^{*b}$	$19.13 \pm 0.36^{*a}$
C-POM (g/kg)	Soil	$2.77 \pm 0.12^{*ab}$	$3.66 \pm 0.52^{*b}$	$2.16 \pm 0.07^{*a}$
	Casts	$6.47 \pm 0.37^{*a}$	$9.52 \pm 0.27^{*b}$	$8.47 \pm 0.89^{*ab}$
C-POM/Total OC	Soil	$0.20 \pm 0.01^{*a}$	$0.25 \pm 0.03^{*b}$	$0.17 \pm 0.00^{*a}$
	Casts	$0.29 \pm 0.01^{*a}$	$0.36 \pm 0.02^{*ab}$	$0.44 \pm 0.04^{*b}$

Table 3: Cast-to-soil ratios of clay, total organic C, and C in the form of POM (C-OPM) at the three study sites. Values followed by the same letter in the same row belong to the same Duncan's homogeneous groups ($P < .05$) for each parameter among sites. Means ± standard error ($n = 3$).

Study site		Pamplona	Olite	Santacara
Cast-to-soil ratios	Clay	0.76 ± 0.02^{b}	0.53 ± 0.07^{a}	0.59 ± 0.02^{a}
	Total OC	1.57 ± 0.11	1.82 ± 0.12	1.50 ± 0.04
	C-POM	2.44 ± 0.19^{a}	3.00 ± 0.37^{ab}	4.01 ± 0.45^{b}

greater in Olite (Table 2), this finding suggests that climate is a more important factor for this relative diminution in the clay content of the casts than the soil texture itself. Not much information is available on the influence of climate on the burrowing and casting activity of any particular earthworm species. In temperate areas, annual variations in the casting activity of anecic species (*Lumbricus terrestris*) have been described as related to rainfall and soil moisture [39].

In tropical semiarid areas, no earthworms are found during the dry seasons in soils that show earthworm activity during the rainy season [17].

Higher moisture in the soil in Pamplona than in the more aridic Olite and Santacara could be one reason for the enhanced casting activity and thus for more intense remixing of the casts with bulk soil. Lower concentration of carbonates in this soil (Table 1) probably also favored the formation of less cemented casts with less nondispersible sand- and silt-size aggregates within casts. Further research is needed in this sense.

Regarding the incorporation of organic materials into the casts, it was observed that the enrichment factor in total organic C in the casts compared to bulk soil was similar among the three studied soils, but a clear and significant trend towards higher enrichment ratios in POM was observed with increasing aridity (Table 3). The role of *S. gigas* in incorporation of POM was most significant in the arid soils of Santacara, which had the lowest total SOC and POM values as well as the lowest crop yield (Table 1), as compared to the other two sites. This more evident preferential ingestion of POM in this soil probably is due to fresh stubble being the most significant available source of C in this field.

4. Conclusions

By studying the composition of surface casts of *S. gigas* and the surrounding soil under different agroclimatic conditions in NT fields in the Ebro Valley, we found that this species has an important role in the incorporation of organic matter, and in particular of the most labile fractions, to the soil. The casting activity of *S. gigas* seems thus related to the observed progressive increments of organic C stocks in NT soils in the region. We also found a proportionally greater enrichment in labile organic particles in casts with increasing aridity, which suggests that the importance of *S. gigas* in organic matter stabilization under NT increases with aridity in the region. The relationship between climate, *S. gigas* casts texture, and organic C content remains however incompletely understood and merits further research.

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Review Article

Effect of Soil Physical State on the Earthworms in Hungary

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Hungarian authors have long been discussing the role of earthworms in improving soil productivity. Earthworm counts in our higher quality soils are similar to those found in soils where more attention is paid to earthworm activity. Negative impacts that are independent of farming—such as sustained dry spells in the summer—also affect earthworm counts. Negative impacts that definitely depend on farming include land use causing soil moisture loss, deep stubble treatment leaving the soil without cover, and ploughing in the summer without subsequent pressing. The climate change is having both positive and negative impacts. Weather patterns are causing losses but adopting climate mitigating tillage are generating benefits. In the trials results so far show that tillage focusing on preserving soil moisture, structure, and organic materials, covering the surface in the critical months as well as adequate soil loosening are fundamental pre-requisites for making the soil a favourable habitat for earthworms.

1. Introduction

> *"These entirely deaf and blind little creatures do immense good to the soil".*
>
> Róbert BALLENEGGER, 1938

We have a relatively rich technical literature in the field of agriculture in Hungary dealing with the importance of the activities of earthworms, including text from before the birth of Christ, from China, Egypt, and Rome. Some [1–3] refer to Darwin's work dated 1881 [4] as providing more precise data on these soil habitats than those originating from practical observance. Hungarian books written for farmers to improve their skills drew, initially, on works of Roman authors—Cato, Columella, Varro, Vergilius—and later on, since the 1800s, as referred to by Jolánkai [5], on books written by West European authors, Liebig, Lawes, Schultz-Lupitz, Thaer, Wolny, and so forth as well. Works of Hungarian classical authors [6–8] refer to the soil biological life only in general terms but they do not mention earthworms' activity. The science of soil physics developed impressively from the 1920s on. Consequently, authors began to pay increasing attention to soil biological activity in books on tillage and soil science [9–13]. Although the role of earthworms in enhancing the fertility of soils has long been recognised, concrete reports in books on tillage and soil science did not appear until as late as 1938. Ballenegger [1] refers to earthworms as useful beings in the soil. According to Fekete [14] the deep layer of chernozem soils with high humus content results from earthworms' mixing activity. Grábner [2] noted that earthworm casting contains a variety of nutrients for plants (soluble phosphorous, potassium, calcium, magnesium, trace elements) originated from the plant and animal remains they had consumed together with the soil. Kreybig [15] estimated the quantity of earthworm casting to be as much as 7.5–18.2 tonnes per hectare, depending on the suitability of the habitat. Others [16] consider that the quantity of soil passing through earthworm intestines is as much as 25 tonnes per hectare. This is fairly important since the earthworms concentrate the lime what is available in their structures taking it from the bulk soil. Unfortunately, acidic soils are not favourable for earthworms. Sipos [3] noted, in particular, the soil loosening and fertility improving effects of the activities of earthworms. Others [17–20] consider earthworms to be indispensable elements of the chain of what is known as "biocoenosis." The first proponent of biologically based tillage in Hungary, Kemenesy, found higher earthworm activity in undisturbed soils not damaged by traffic and in soils under perennial papilionaceous plants and grasslands than soils subject to frequent disturbance [17, 18]. He [18] also observed higher

earthworm numbers under lathyrus grown for being used as green manure. He found soil under conserving tillage to be a good habitat for them, in contrast to ploughing in the summer, creating definitely adverse conditions for earthworms. He noted that earthworms are hard hit both by summer drought and heat alike, finding a 15°C soil temperature as being optimal for these organisms. Today they have been observed to have a broader range of tolerance in terms of temperature, but weeks of extremely high temperatures will definitely reduce their activities. Other authors also found, relatively early, the poor habitat value of cloddy and dry soil after ploughing, for instance, Tischler [21]. Kemenesy [17, 18] noted that soils in their pristine condition, that have never been disturbed, offer the best habitat for earthworms. Some authors have reported [22–25] that the application of excessive amounts of chemicals reduce the value of sites as habitat. In land under crops sown by direct seeding where crop protection is limited practically to the application of chemicals, authors still found higher earthworm numbers [26–28]. Accordingly, factors affecting a site's value as a habitat can be assessed only by adopting a complex approach. Wide-ranging tests were carried out by Zicsi [29, 30] in Hungary to produce qualitative and quantitative assessments of the earthworm populations in various soil types. In the new publications the authors discuss earthworm counting for the most part as supplementary aspects instead of primary goals of experiments. Between 1996 and 1998 Gyuricza [31, 32] assessed earthworm activity in tillage experiments set up in Chromic Luvisol and in Typic Argiudoll between 1996 and 2006, along with László [33] between 1998 and 2006 also in long-term tillage experiments in brown gleyic forest soil. Birkás et al. [34, 35] used earthworms as indicators of the soil state in their soil quality experiment set up in chernozem soils (Calcic Chernozem) and at different sites under field conditions from 1994 on. This study provides an overview of the findings of experiments carried out during recent years.

2. Earthworm Activities in Soils Cultivated in Different Ways

There are 40 different earthworm species [18, 25, 29] in Hungary, the most commonly found species easily found in arable fields and in gardens. The most frequently encountered species is called common earthworm (*Lumbricus terrestris*), whose specimens dig deep vertical burrows in the soil. Satchell [22] and Lee [23] note that the presence or the absence of earthworms is a rather good indicator of the quality of the soil, so they can be used as biological soil indicators. This particular form of soil quality assessment is also applied by Hungarian scientists in tillage experiments. In comparison to other soil-borne organisms there is a definite relationship between the number of earthworms and the state of the soil, and the—ISO 23611-1:2006—testing methods are relatively simple [33]. Earthworms are collected from underneath a known soil surface area—for example, a quadrant (0.25 m^2)—after excavating and screening a certain volume of soil. Under Hungary's climatic conditions in natural habitats the earthworms that are longer than 5 mm can be picked out of the soil. In the experiments, counts were taken every 10 days during the period concerned, in six repetitions, from the soil down to depths of 20–30 cm as appropriate, as a consequence a negligible number of earthworms were not included in the count. The findings of László [33] apply to settling brown gleyic forest soil—Pyhra, Austria—in the case of three different types of tillage or soil disturbance. Measurements were taken by László—using the ISO 23611-1:2006 testing method—in mid-June when the soil was well-shaded by maize (Table 1). Since the soil had a good supply of potassium, no potash was applied, and the P fertiliser dose was between 42 and 112 kg ha^{-1} according to the residual supply from the previous year, while the quantity of N fertiliser was—also according to the soil analysis—between 92 and 154 kg ha^{-1} [32], as was fit for the soil concerned. Findings were processed with the aid of *nonparametric* variance analysis.

Table 1: Number of earthworms (pcs m^{-2}) in the top 20 cm layer of a brown gleyic forest soil, under maize (Pyhra, 1998–2006, from [33]).

Tillage variants	1998	2000	2002	2004	2006	Mean
Direct drilling	54	72	66	88	288	113.6
Ridge till	27	32	42	28	72	40.2
Ploughing	10	12	31	16	56	25.0
Years	rainy	dry	rainy	average	average	

Table 2: Earthworms' live weight (g m^{-2}) in the top 20 cm layer of a brown gleyic forest soil, under maize (Pyhra, 1998–2006, from [33]).

Tillage variants	1998	2000	2002	2004	2006	Mean
Direct drilling	52.7	24.2	55.1	64.3	217.8	82.8
Ridge till	9.8	11.3	41.9	17.8	67.1	29.6
Ploughing	14.7	3.5	23.8	11.7	42.3	19.2
Years	rainy	dry	rainy	average	average	

In 1998—in a rainy year—two times more earthworms (54) were found in soil under crop sown by direct drilling than in soil after ridge till (27) and 5.4 times more than under conventional tillage (10). In a dry year (2000) the differences were greater, while in another rainy season (2002) they were smaller. In average years (2004 and 2006) the density of earthworms was significantly higher after direct seeding (88 and 288) than that counted after the other two types of tillage. The differences also appeared in the five year average figures as well. Significant differences (7.9992 $P < .05$) were found between the types of tillage. The soil state modified by tillage also affected the live weight of earthworms (Table 2).

In a year of slightly more precipitation than the average—as is favourable for earthworms—László [33] found five times greater total of earthworm live weight (52.7) in soil under direct drilling than in soil under ridge till (9.8) and three and a half times more than in soil under conventional tillage (14.7). Earthworm populations were reduced by less precipitation in dryer years but differences between their populations in soils of different states remained significant.

Table 3: Earthworm burrow density (>2 mm burrows m^{-2}) in the top 20 cm layer of a brown gleyic forest soil, under maize (Pyhra, 1998–2006, from [33]).

Tillage variants	1998	2000	2002	2004	2006	Mean
Direct drilling	374	425	386	407	1372	592.8
Ridge till	211	293	305	319	584	342.4
Ploughing	97	198	287	113	460	231.0
Years	rainy	dry	rainy	average	average	

In the rainy year 2002 ploughing had a notably positive impact, yet it was still as not as favourable as those treatments involving less soil disturbance. The difference between the earthworm counts in 2006 between different tillage variants was also reflected in the weight of the earthworms. The difference between the live weights of earthworms in soils under different tillage variants was smaller than the difference between the numbers of earthworms counted. László [33] found no significant difference between tillage variants (5.6600 $P > .05$). It was also him who examined burrow numbers (Table 3), finding that there were 1.73 times more earthworm burrows than under ridge till and 2.56 times more than in soil under conventional tillage. He found both horizontal and vertical burrows. He found no significant differences between tillage variants (5.4600 $P > .05$), but he found a close relationship between earthworm density and burrow density ($R^2 = 0.79$).

László [33] underlined that in the given gleyic forest soil earthworms favoured soil under direct drilling, that is, less disturbed but adequately loosened soil in terms of the total porosity space. Their other requisites for life, that is, adequate moisture and—even in the dry year of 2000—food were continuously available for them in the soil.

3. Importance of the Looseness of the Soil and of the Depth of the Loosened Layer

Earthworm burrows play an important role—as "biopores"—in soils' water, material, and gas transport heat exchange processes [24, 31]. Horizontal burrows in the top soil layer enable primarily the soil aeration, while deep vertical earthworm burrows enabling the seeping of water into the soil function as important gravitational pores, making it possible, for instance, for quick transport of sudden downpours to deeper layers of the soil [33, 36, 37]. According to László [33] vertical earthworm burrows mitigate erosion in sloping sites as run-off is reduced by improved water absorption. Birkás [34] considered that a certain looseness of the soil is a prerequisite for the particular soil loosening activity of earthworms. This was concluded from findings of field experiments set up in the 1990s on various soil types—brown forest, chernozem, and meadow alluvial—by three different clay content levels (Table 4).

The earthworm count was an important factor in addition to monitoring soil state changes in the experiments whose results are summed up in Table 4. The three different clay content levels applied to three different soil types (forest, chernozem, and meadow alluvial) with humus contents of 1.8%, 3.1% and 3.4%, respectively. Ten to fifteen, years ago farmers did not consider earthworms to be of particular importance, therefore according to Birkás [34] the maximum of 36 earthworms (per m^{-2}) in the chernozem soil is to be considered to be very good. Incidentally, this was the soil in which the largest numbers of earthworms were found regardless of the soil states. According to the ranking based on earthworm counts undisturbed and deeply loosened soil state is the most favourable under a high (55%) cover. More deeply loosened soil with medium (35%) coverage was the 2nd in the rank, followed by shallower loosened layer and medium coverage. Ploughed soil came as the 5th in the rank, despite the fact that ploughing put the largest amount of plant residues in the soil ($5\,t\,ha^{-1}$), but inverting in the same depth was not found to be advantageous. Ploughed soil turned into a particularly disadvantageous habitat when not even field residues were mixed into the soil. Birkás [34] underlined that a compact tillage pan is not suitable for earthworms at all (more deeply loosened state was favourable for maize, surface cover was not, but a compact state was disadvantageous). Tests showed the importance of covering the soil—from the aspect of the earthworm habitat—therefore this factor was also taken into account in other experiments.

4. The Importance of Surface Cover

In an experiment conducted by Birkás et al. [38–41] the soil surface was covered as follows: ploughing 0%, loosening, disking 25%, tillage with cultivator 35%, and direct seeding 65%. These coverage ratios were kept up regardless of crops (2002: mustard, 2003: wheat, rye, 2004: rye, pea, 2005: wheat, mustard, 2006: wheat, phacelia, 2007: maize, and 2008: sunflower). The authors found earthworm counts increasing in parallel with increasing coverage ratios. On the other hand, some increase was found in all types of tillage during the first 5 years, thereafter earthworm counts dropped. The authors concluded that densely sown crops—eared cereals as main crops, followed by catch crops—created more favourable conditions, while more wide row crops created slightly less favourable conditions as a consequence of the modest shading such plants provide. In order to enable a more accurate evaluation the authors sought for a relationship between the depth of soil disturbance, soil coverage and typical earthworm counts (Table 5).

The authors found that increasing depth—that is favourable habitat at lower depths—, is favourable in the case of every mulch variant. Smaller numbers of earthworms were found under bare surface, almost in all cases, than under various percentages of coverage. The earthworm count under direct drilling was higher than in ploughed soils, as found by many other authors [28, 32, 33]. If, however, soils disturbed to greater depths were covered, even after ploughing, they were found to be better than settled soils. In the case of coverage between 0% and 25%, between 0% and 35%, and between 0% and 65%, an average of 28%, 43%, and 67%, respectively, were found in favour of the higher

Table 4: Relationship between the soil clay content, soil state, and earthworm count, under maize (1994–1999, June [34]).

Clay %, v/v			Rank of soil states based on earthworm counts
30	50	60	
Earthworm count per m^2 (0–20 cm)			
26	36	28	(1) Undisturbed soil, loosened to a depth of 45 cm, covered to an extent of 55%
25	34	26	(2) Soil loosened down to 40 cm, covered up to 35% (plant residue mixed in the soil: 3 t ha^{-1})
24	30	26	(3) Soil tilled with cultivator to a depth of 18–22 cm, under 35% coverage (plant residue mixed in the soil: 3 t ha^{-1})
21	26	23	(4) Soil tilled with cultivator to a depth of 16–18 cm, under 20% coverage (plant residue mixed in the soil: 3 t ha^{-1})
16	22	16	(5) Soil ploughed to a depth of 22–25 cm (plant residue inverted to the soil: 5 t ha^{-1})
16	20	9	(6) Undisturbed, uncovered, settled soil (field residues removed)
6	10	7	(7) Soil ploughed to a depth of 22–25 cm (plant residues removed)
0	0	0	(8) Plough pan and disk pan
1.786	1.908	1.216	$LSD_{5\%}$

Table 5: Effects of depth of soil disturbance and soil coverage on earthworm count (in loam soil of 19%–22%, w/w soil moisture contents as an average of 6 repetitions).

Tillage depth (cm)	Mulch %				Mean
	0	25	35	65	
0–5	14	18	20	21	18
16–20	24	24	27	31	26
20–25	22	31	32	39	31
30–35	25	34	41	47	37
Mean	21	27	30	35	

$LSD_{5\%}$.
Between different tillage depths under identical mulch treatment: 3.44
Between different tillage depths as an average of the mulch variants: 2.06
Between mulch variants under the identical depth treatments: 4.977
Between mulch variants as an average of depths: 4.146
Between two depth variants in the case of different mulch percentages: 4.670
Tillage depth: $P > .1\%$; Mulch %: $P > .1\%$; Depth × Mulch: $P > 1\%$.

coverage rates. Deeper disturbance increased earthworm counts by 44%, 72%, and 105%—in the order presented in the left-hand-side column in Table 5—, respectively, creating better habitats accordingly. It should also be noted that along with the increasing depth the amount of food for earthworms (that is, the mass of field residue) also increased, except in soils under direct drilling. The authors noted that the amount of field residue mixed in the soil equalled, on an average and per year, 0.2 (only in the case of direct drilling), 3.0, 3.7, and 4.3 t ha^{-1} [41]. Eventually, the authors established a close relationship between the depth of disturbance, the ratio of coverage, and the earthworm counts. The tillage-mulch interaction was reliable at a $P = 1\%$ level. They assumed that increased soil cover makes it possible to decrease tillage interventions by a reasonable measure. This was also confirmed by the study carried out in their stubble trial [42].

5. The Importance of Soil Moisture

As has been described above, earthworms favour soils loosened to increased depths. Soil coverage is also an important habitat as covered soil is less exposed to the risk of damage by heat or water stress and it also helps the soil keep its moisture content.

In their experiment Birkás et al. [40, 41] were seeking to identify the relationship between soil moisture and earthworm count. The data have been presented merely as an illustration, without supplementing them by a mathematical evaluation. The optimum moisture range for tillage of chernozem soil that is moderately prone to compacting—near the town of Hatvan—is between 19%, w/w and 25%,

w/w, with the optimum being 22%, w/w which is when tillage takes the smallest energy input, but this is also the soil moisture content preferred by earthworms. The authors found that during the period between 2003 and 2008 the shortage of water varied between 5% and 25%, but it was characteristically affected by tillage variants (Figure 1). The shortage of water had the smallest impact on ploughed soil, followed by direct drilling, while the greatest shortage of water was found in soil after disking. In the average of six successive years the earthworm number was the smallest in disked soil (35), hardly more (36) in ploughed soil. The number of earthworms in soil under direct drilling was 40 on an average, slightly more in soil loosened to a depth of 40 cm (43), while the highest (48) count was found in soil loosened with cultivator to a depth of 20 cm. The authors found [38, 40] that in the case of water shortage not heavily restricting cropping, earthworm activity, and so forth, other factors—for example, soil cover and the required looseness—will have increased impacts.

Accordingly, supplementary studies were carried out in the experiment site, involving soils of 8 different depths of the loosened layer and three different levels of soil moisture content. In any given soil an 11%, w/w soil moisture content qualifies as dry, while 22%, w/w and 28%, w/w qualify as humid and wet, respectively. The findings are presented in Figure 2.

Clearly, a 30–40 cm loosened layer and humid soil are the most favourable for earthworms in any given type of soil. If the soil is dry, a deeper loosened layer makes it more likely for earthworms to be able to survive the dry period (earthworms were found in a state of the rest). A shallow loosened layer combined with high moisture content was found to be the least favourable for earthworms.

6. The Soil Structural State, with a View in Particular to the Crumb Structure

The agronomical structure—the relative proportions of clods (>10 mm), aggregates (0.25–10 mm), and dust (<0.25 mm)—is suitable for assessing the effects of tillage and the trends in the condition of the soil (improving, unchanged, and deteriorating) [43]. The aggregate fraction can be divided into fractions of small (0.25–2.5 mm) and large crumbs (2.5–10 mm). In an experiment carried out by Birkás et al. in the Hatvan region on chernozem soils [40] the growing of densely sown crops entailed steadily increasing earthworm activity, which is likely to be related to the increasingly crumby structure of the soil (Figure 3). According to the authors, the earthworm activity and the ratio of crumbs dropped during years in which wide-row crops were grown.

This is the very reason why the authors were looking to establish a relationship between tillage (loosened layer) depth, the trend of crumb forming, and the trend of the changes in earthworm counts (Figure 4). They found that the depth of the loosened layer has an impact on crumb forming ($R = 0.76$), but other factors also play an important role. Earthworm counts varied between 26 and 49. As the loosened

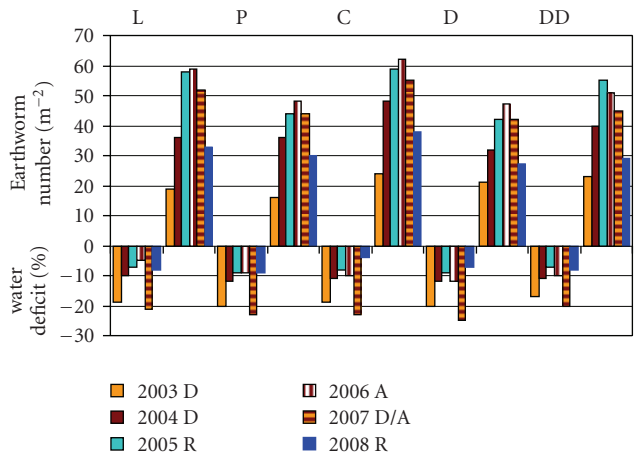

Figure 1: Coherence between water deficit of soil and earthworm number (Hatvan, 2002–2008). Legend: A: average season, D: dry, R: rainy, D/A: dry and average in one season, L: loosening 40 cm, P: ploughing 30 cm, C: cultivator use 20 cm, D: disk tillage 16 cm, and DD: direct drilling.

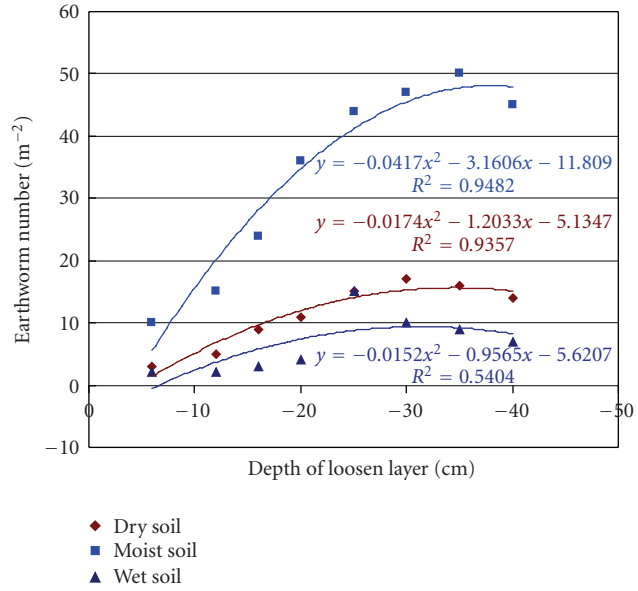

Figure 2: The depth of the loosened layer and the earthworm counts at three different soil moisture levels, in a loam soil.

layer increased earthworm counts increased too to a certain depth (30 cm), but after a certain depth the earthworm declined slightly. The R value (0.82) shows a considerably close relationship, but it also indicates that the evaluation should be extended to another factor (soil coverage) as well.

7. More Earthworms, Better Soil Condition

This study was carried out as a survey, to provide lead information. The authors hope that earthworm activity studies will increase in importance in tillage research in Hungary. The authors performed no ecological separation, on the

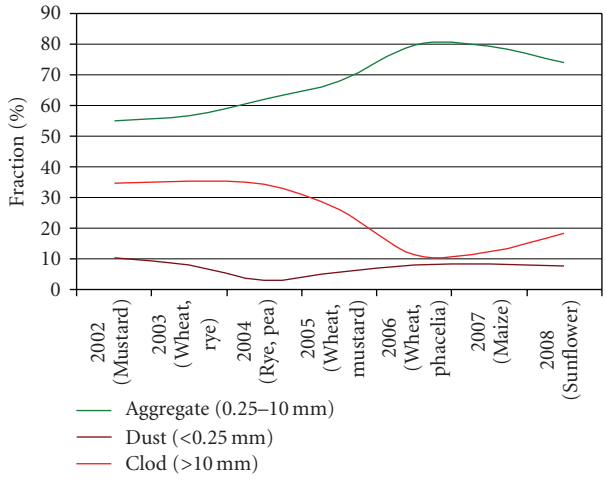

Figure 3: The trend of crumb forming in the average of 6 tillage variants, under different crops (Hatvan, 2002–2008, From Birkás et al. [41]).

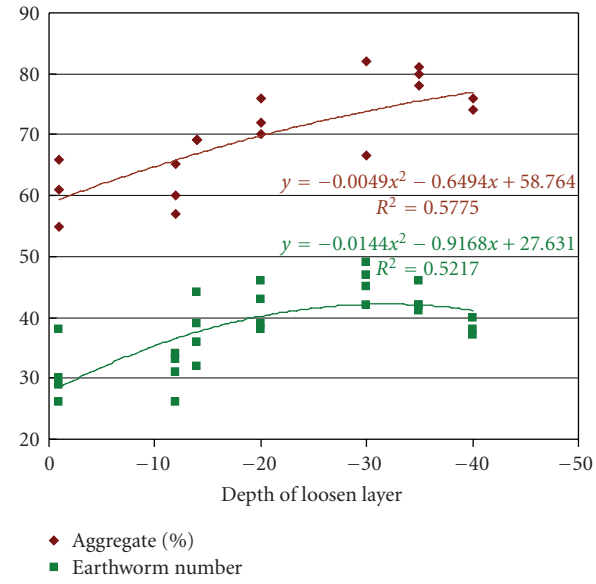

Figure 4: Relationships between the depth of the loosened layer ($n = 7$), crumb forming ($n = 21$), and earthworm counts ($n = 28$) in soils of favourable soil moisture contents (19–22%, w/w). (Hatvan, 2002–2008).

basis of the photos and site descriptions the earthworms counted probably fall in the anecic and the endogenic groups. Earthworm counts in well-tended soils in Hungary are similar to those measured in West European countries and in the USA.

The amount of the application of fertilisers and chemicals never reached the levels prevailing in countries with advanced agriculture sectors. By examining soils under sugar beets Birkás [44] found that in the case of reasonable application of chemicals the quality of tillage and mulch cover affected earthworm counts primarily. Tillage—soil disturbance—however, had a larger than expected impact. The earlier applied deep stubble tillage practices without surface mulching and ploughing in the summer without surface press [45] were likely to have reduced earthworm activity for quite some time. Moreover, the same mistakes were repeatedly made in the same fields [45, 46]. The climate change has had both negative and positive impacts in Hungary. Extreme weather patterns are causing losses but the recognition of the necessity and the application of climate damage mitigating tillage have been found to have favourable impacts on soils as well [41]. During recent years in soils under conservation tillage, under 15%–25% mulch coverage even after sowing, 6–8 earthworms were found under every plant grown in wide rows, after harvest. It should also be mentioned that no earthworms at all were found in soils of damaged structure. Accordingly, the solution—including increased earthworm activity—is offered by preserving the quality of the soil and mitigating the damage to be caused by climate conditions [46]. Results achieved so far show that soil moisture, structure, and organic material conserving tillage, covering the soil surface during the critical summer months and maintaining adequate looseness are essential prerequisites for making the soil a suitable habitat for earthworms as well.

8. Conclusions

Earthworms have been duly appreciated in technical literature in Hungary but precious little research has been focused on them. In tillage experiments supplementary types of evaluations have been produced concerning the relationships between earthworm count, depth of loosened layer, soil moisture, and soil surface cover. The authors found that earthworm-friendly tillage results in loosening and crumb forming, it creates minimised soil surface and is characterised by mulch cover on the soil. The depth of the layer loosened by tillage should be around 30–40 cm and it may even be shallower (even as shallow as 20 cm) if there is no compact tillage pan underneath the tilled layer.

Soil moisture content that makes the soil workable is also favourable for earthworms. Soil surface coverage may increase in importance in the future, during the warm and hot summer months. An at least 35% soil coverage is necessary, but the optimum is as high as 45%. Field residues mixed into the soil serve as food for earthworms therefore shortage of that is just as disadvantageous as a large, inadequately distributed mass. Climate damage mitigating tillage produces habitats favourable for earthworms as well.

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Research Article

The Effect of Earthworm (*Lumbricus terrestris* L.) Population Density and Soil Water Content Interactions on Nitrous Oxide Emissions from Agricultural Soils

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Earthworms may have an influence on the production of N_2O, a greenhouse gas, as a result of the ideal environment contained in their gut and casts for denitrifier bacteria. The objective of this study was to determine the relationship between earthworm (*Lumbricus terrestris* L.) population density, soil water content and N_2O emissions in a controlled greenhouse experiment based on population densities (90 to 270 individuals m^{-2}) found at the Guelph Agroforestry Research Station (GARS) from 1997 to 1998. An experiment conducted at considerably higher than normal densities of earthworms revealed a significant relationship between earthworm density, soil water content and N_2O emissions, with mean emissions increasing to 43.5 g $ha^{-1}day^{-1}$ at 30 earthworms 0.0333 m^{-2} at 35% soil water content. However, a second experiment, based on the density of earthworms at GARS, found no significant difference in N_2O emissions (5.49 to 6.99 g $ha^{-1}day^{-1}$) aa a result of density and 31% soil water content.

1. Introduction

The presence of earthworms can be seen as an added benefit to many agricultural systems since earthworms contribute greatly to the overall physical properties of agricultural soils [1]. Previous studies in sole cropping systems have focused on the ability of earthworms to facilitate soil mixing and the decomposition of organic matter, which is especially important in agricultural systems [2–4]. Earthworms also affect soil properties, by increasing soil porosity and decreasing bulk density and through bioturbation and cast deposition on the soil surface [1]. Earthworm activity stimulates mineralization of N in residues, which promotes the availability for plants and microorganisms of inorganic forms of N from plant material [1, 5].

However, increased earthworm population might increase the production of nitrous oxide (N_2O) emissions from agricultural soils. Over 50% of *in situ* N_2O emissions, in some soils, could be a result of earthworm activity [6]. Recent research suggests that, globally, earthworms could be producing up to 3×10^8 kg of N_2O annually [6]. Conventional agricultural practices, which aim to encourage earthworm populations due to their positive influence on soil properties are the highest anthropogenic sources of N_2O emissions. On a global scale, annual emissions of N_2O were 16.2 Tg in 2004 [7], and as a result, earthworms could be responsible for nearly 2% of global emissions.

One reason for this is that the earthworm gut is an ideal environment for denitrification [8–10]. Using microsensors, Horn et al. [9] determined that the earthworm gut is anoxic and contains copious carbon substrates for microorganisms and is therefore ideal for N_2O production. Denitrification is enhanced when the earthworm ingests denitrifier bacteria with organic matter [1, 8–10]. When gaseous N_2O is produced, it is able to escape the permeable epidermis of the earthworm and diffuses from the soil surface [9].

At the Guelph Agroforestry Research Station (GARS) in Guelph, Ontario, Canada, Price and Gordon [11] found that earthworm density was greater in a Tree-Based Intercropping (TBI) system than in a conventional agricultural

monoculture. A TBI system is defined as "an approach to land use that incorporates trees into farming systems and allows for the production of trees and crops or livestock from the same piece of land in order to obtain economic, ecological, environmental and cultural benefits" [12]. These systems incorporate leaf litter and increase soil water content, which could encourage higher earthworm populations compared to sole cropping systems. In turn, this could increase the overall volume of the earthworm gut, thereby facilitating denitrification and higher N_2O emissions from a TBI system. Price and Gordon [11] also speculated that the reason earthworm densities were higher in the intercropped system compared to the conventional monoculture was because earthworms move to an area with a lower soil temperature, which in turn are areas that also have higher soil water content.

Currently, very little information exists on the influence that earthworm density has on N_2O emissions from agricultural soils, and specifically those potentially associated with a TBI system. The objective of this study was to investigate the relationship, if any, between N_2O flux, earthworm density, and gravimetric soil water content, taking into account the earthworm densities calculated by Price [13] in the TBI and monoculture systems located at GARS and using the most common earthworm species found in GARS, the common nightcrawler (*Lumbricus terrestris* L). It was hypothesized that N_2O flux would be higher as earthworm density and soil water content increased.

2. Materials and Methods

2.1. Study Design. The first experiment was conducted in the Science Complex Phytotron at the University of Guelph, Guelph, Ontario, Canada. The purpose of the first experiment was to determine the optimal soil water content for earthworm activity resulting in the highest N_2O emissions. The experiment was a two factorial, completely random design with four replications for a total of 64 experimental units. The first factor was earthworm density (see below) and the second factor was gravimetric soil water content (15%, 25%, 35%, and 45%).

Soil was collected from GARS and homogenized using a 2 mm sieve. The soil is sandy loam in texture with an average pH of 7.2 [14]. A leaf litter mixture composed of silver maple (*Acer saccharinum* L.) and poplar (*Populus spp.*) leaves was also collected from GARS, dried at 60°C for one week, and mixed into the homogenized soil to achieve a soil organic matter content of approximately 3%. Four kilograms of the soil and leaf litter mixture was then put into each of the 5 L polypropylene mesocosms, equipped with an airtight lid and rubber septum for sampling. The lids were only placed on the mesocosms at the time of N_2O sampling. The surface area of each mesocosm was 0.033 m^2.

Earthworm density was calculated based on data collected in the spring of 1997 from GARS by Price [13]. The three earthworm densities included high, medium, and low earthworm densities, representing populations found 0 m, 3 m, and 6 m from the tree row in a TBI system, respectively. However, these values were tripled in order to ensure the detection of N_2O for the purpose of finding optimal soil water content and also to represent an earthworm invasion where populations could initially be very high and decline over time [15]. These values were 30, 20, and 10 earthworms per 4 kg of soil or 0.033 m, for the high, medium, and low treatments, respectively, and a control with no earthworms. *L. terrestris* were purchased from Kingsway Sports (Guelph, Ontario, Canada). Earthworms were counted and weighed prior to being added to the mesocosms.

Prior to adding the earthworms, each mesocosm was fertilized with urea (46-0-0, N-P-K), which represented the N fertilizer requirement for corn planted at GARS (215 kg ha^{-1}). Deionized water was applied to each mesocosm for one week prior to adding the earthworms in order to achieve the desired gravimetric soil water content for each treatment. A small hole in the bottom of each mesocosm allowed for proper drainage. During the course of the experiment, soil water content was maintained by weight. The mesocosms were weighed every day for the entire course of the experiment and deionized water was added to bring each mesocosm to the desired water content.

The mesocosms were placed in a greenhouse with a constant air temperature of 20°C and monitored light conditions of 16/8 hr cycles. Soil temperature was monitored using Priva soil temperature sensors (Priva North America Inc., Vineland Station, Ontario, Canada) to ensure a constant soil temperature of approximately 20°C. N_2O sampling technique and calculations will be explained in the following section.

A second experiment was conducted from February 2009 to March 2009 in the Science Complex Phytotron at the University of Guelph. Experiment 2 was a completely random design with four replications for a total of 16 experimental units. A control with no earthworms and earthworm densities of 9 (high), 6 (medium), and 3 (low) earthworms per mesocosm were used for a total of four treatments. The high, medium, and low density treatments were calculated based on actual densities found by Price [13] at GARS representing an earthworm density adjacent to the tree row, 3 m from the tree row, and 6 m from the tree row in a TBI system, respectively; a control with no earthworms was also included.

Optimal gravimetric soil water content was determined in Experiment 1 and was found to be 31%. This soil water content treatment was held constant for all four earthworm density treatments over the duration of the experiment. Methods for soil preparation, maintaining gravimetric soil water content, and monitoring temperature were the same as in Experiment 1.

2.2. Sampling Procedure. At the time of N_2O sampling, the airtight lid was placed onto each mesocosm and a 30 mL air sample using a 26-gauge needle and syringe was taken at $t = 0$, 30, and 60 min to calculate N_2O flux over an hour. Air samples were deposited into 12 mL Exetainers (Labco Limited, United Kingdom) and analyzed using a SRI Model 8610C Gas Chromatograph (Torrance, California, USA) at

Environment Canada (Burlington, Ontario, Canada). N_2O samples were taken once a week for four weeks beginning at 10:00 AM.

A soil sample was taken from each mesocosm, both before the addition of earthworms and after the last week of sampling. This was done to measure the initial and the final nitrate (NO_3^-), ammonium (NH_4^+), and total inorganic N (TIN) concentrations to determine if there was a change over the course of the experiment. Soil samples were stored in the freezer until analysis. N content was measured following a 2N KCl extraction [16], and samples were run through an Astoria 2-311 Analyzer (Astoria-Pacific Inc., Oregon, USA). Measurements of soil inorganic and organic carbon (C) were also done for initial and final C content using a Leco C determinator (Leco Corporation, St Joseph, MI, U.S.A.). However, results for soil N and C are not reported here and are part of a larger study.

2.3. N_2O Flux Calculation. N_2O flux was calculated using the ideal gas law; the molar volume of N_2O at 0°C and 1 atm is 44.0128 L/mol. The N_2O flux was adjusted for air temperature and pressure using the following formula:

$$\text{Flux adjustment} = 44.0128\,\text{L mol}^{-1} * \frac{[(273.16^\circ\text{K}+T^\circ\text{C})]}{273.16^\circ\text{K}} * \frac{(1013.2\,\text{hPa})}{P\,\text{hPa}}, \quad (1)$$

where T is the air temperature and P is the air pressure on the day of sampling. These values were taken into consideration because a temperature greater than 0°C increases molar volume and, air pressure that is greater than atmospheric decreases molar volume.

The volume of the mesocosm was then converted to mol of air and multiplied by the slope of the flux determined by hourly measurement. This value was then used to calculate the flux in μmol m^{-2} s^{-1}:

$$\text{Flux } (\mu\text{mol m}^{-2}\,\text{s}^{-1}) = \frac{\left(S\,\text{nmol mol}^{-1}\,\text{s}^{-1}\right)(M\,\text{mol})}{X\,\text{m}^2}, \quad (2)$$

where S represents the slope of the line (N_2O concentration at each measurement interval over one hour), M is the molar volume of the air in the mesocosm, and X represents the area of the mesocosm. This value was then converted into kg of N_2O ha^{-1} day^{-1}:

$$\text{Flux}\left(\text{g ha}^{-1}\text{day}^{(-1)}\right) = \left(F\mu\text{mol m}^{-2}\,\text{s}^{-1}\right)(1.0 * 10^{-9}\,\text{mol}) * \left(44.0128\,\text{L mol}^{-1}\right)(10000\,\text{m}^2) * (86400\,\text{s})(1000\,\text{g}), \quad (3)$$

where F is the flux calculated from (2).

Some of the flux values were negative as a result of a sink of N_2O being created rather than the N_2O being emitted through the soil surface during the extraction period from the mesocosms, which created negative flux values [17]. Therefore, a value of 100 was added to all flux values in order to complete statistical analyses and maintain positive values since the statistical program could only read positive values. The final flux values following analysis were then subtracted by 100 to present actual flux values in the following sections.

2.4. Statistical Analysis. All statistical tests were conducted using SAS v.9.1 (SAS Institute, Cary, NC, USA) at an error rate of $\alpha = 0.05$. An analysis of variance (ANOVA) using repeated measures in the PROC MIXED function was used to compare the effects of earthworm density and N_2O flux according to soil water content treatment to determine the variance in initial and final earthworm biomass between moisture treatments, as well as mortality rates between moisture treatments in Experiments 1 and 2. A response surface design using the PROC RSREG function [18] was applied to data from Experiment 1 to determine the optimal range levels of earthworm density and soil water content for the production of N_2O over ranges for these parameters that were not part of the original experimental design. The optimal soil water content found through the RSREG was then applied to Experiment 2.

3. Results

3.1. N_2O Emissions. The earthworm density and soil water content interaction on N_2O emissions was significant ($P = .0457$). Mean N_2O emissions ranged from 0.54 g ha^{-1} day^{-1} from the 15% moisture and no earthworm density treatment to 43.5 g ha^{-1} day^{-1} from the 35% moisture and high earthworm density treatment as illustrated in Figure 1. Patterns did exist in emissions, where N_2O emissions were highest at the high density across all moisture treatments and lowest in the mesocosms with no earthworms across all moisture treatments. The extent of emissions across all of the moisture treatments was high > medium > low > control. Emissions due to moisture were 35% > 25% > 45% > 15% across all earthworm densities except when earthworm density = 0, where emissions were 35% > 25% > 45% = 15%. Emissions were only significant at the high density and 25% and 35% soil water content treatments, as well as the medium density and 35% soil water content treatment compared to the rest of the treatments.

Over the course of the experiment, N_2O emissions only increased at 45% soil water content, where emissions were highest in the last week of sampling compared to the first week at all density treatments (Figure 2). At 15% and 25% soil water content, emissions peaked at week three and week two, respectively, and declined by week four. In the 25% moisture treatment, emissions had a significant peak at 56.6 g ha^{-1} day^{-1} at high density in week two compared to 1.5 g ha^{-1} day^{-1}, 3.2 g ha^{-1} day^{-1}, and 3.6 g ha^{-1} day^{-1} in the control, low, and medium densities, respectively. An outlier did exist in the 25% moisture and high density treatment during week two, but when left in, it did not significantly change the result. However, it may explain the peak in emissions during week two at the high density treatment.

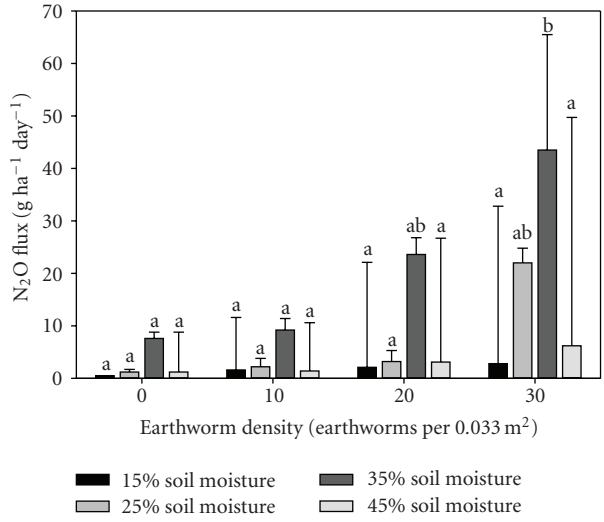

Figure 1: The relationship between N_2O emissions, earthworm density per 0.033 m^2, and gravimetric soil water content (**P** = **.0457**, SE = 4.07, 5.29, 6.10, and 4.52 for 15%, 25%, 35%, and 45%, resp.). Bars with same letter indicate no significant difference between treatments at **P** = **.05** according to Tukey-Kramer means adjustment.

N_2O emissions declined over the course of the experiment in all densities at 35% moisture except in the high density treatment where emissions were the highest in week two at 69.6 g ha^{-1} day^{-1}.

A response surface regression indicated that the lowest N_2O emissions would occur at soil water content of 15% and an earthworm density of 13 earthworms per 0.033 m^2, whereas the highest emissions would occur at a soil water content of approximately 31% and an earthworm density of 30 individuals per 0.033 m^2 as seen in Figure 3. The lowest and highest emissions correspond to −1.7 and 22.3 g ha^{-1} day^{-1}, respectively. These numbers represent emissions within the treatment range of the experiment. Emissions at soil water content or earthworm density outside of the treatment range can be determined using the equation found in the caption for Figure 3.

3.2. Earthworm Mortality and Biomass. Mortality rates were not significantly different between moisture treatments within the density treatments (Table 1). There was very little mortality in the low-density treatment across all soil moisture treatments. Mean mortality rates in the medium density treatment ranged from 3% to 11%, the highest mortality rate occurring in the 15% moisture treatment and the lowest in the 25% moisture treatment. Mean mortality in the high-density treatment ranged from 5% to 18%, the highest mortality rate occurring in the 35% soil moisture treatment and the lowest occurring in the 25% moisture treatment.

The difference in the initial and final earthworm biomass was significant according to soil water content across all earthworm density treatments as seen in Table 2. The largest increase in biomass in the low density treatment also occurred at 35% soil water content. The largest increase in biomass in the medium density treatment occurred at 35% soil water content where the final earthworm biomass was significantly higher than the initial biomass. Earthworm biomass declined in the 15% soil water content treatment due to a mortality rate of 11%; however, this decline was not significant. The highest increase in earthworm biomass over the course of the experiment occurred at 25% soil water content in the high density treatment; however, this increase was not significant. There was also a decline in earthworm biomass over the course of the experiment in the 15% and 35% soil water content treatment due to high mortality rates in the high density treatment; however this decline was not significant.

Table 1: Mean earthworm mortality in the low, medium, and high earthworm densities according to θ_g (%) treatment.

	Mortality Rate (%)		
θ_g (%)	Low[§]	Medium	High
15	2.0 a[†]	11.0 a	15.6 a
25	2.0 a	2.5 a	5.0 a
35	0.0 a	8.5 a	18.3 a
45	0.0 a	6.0 a	10.0 a
SE	0.6	0.6	1.0
P value	.1994	.2139	.0571

[†]Within columns, means followed by the same letter are not significantly different according to Tukey-Kramer means adjustment (0.05).
[§]Low, medium, and high refer to densities of earthworms per 0.033 m^2: 10, 20, and 30, respectively.

3.3. N_2O Emission at 31% Gravimetric Soil Water Content. Based on the gravimetric soil water content of 31% found in the response surface in Experiment 1, there was no significant difference in N_2O flux across all earthworm densities (P = .8085). Mean N_2O flux over the duration of the experiment was 6.99, 5.49, 6.36, and 5.63 g ha^{-1} day^{-1} for the control, low, medium, and high earthworm densities, respectively. There was also no significant difference in mean N_2O flux according to the week by density interaction (P = 0.7611, SE = 2.37 for the control, SE = 2.05 for low, medium, and high earthworm density). However, at all earthworm densities, N_2O flux peaked at week two and then declined below week one levels at week three.

3.4. Earthworm Mortality and Biomass at 31% Soil Water Content. Earthworm survival was 100% in the low and medium density treatments and 95% in the high density treatment. Initial and final earthworm biomass was significantly different across all earthworm densities. Earthworm biomass in the low density treatment increased from 12.6 g at the start of the experiment to 27.1 g at the end (P = .0007) as seen in Table 3. In the medium density treatment the initial earthworm biomass was 28.6 g and increased to 44.4 g by the end of the experiment (P = .0003). In the high density treatment, earthworm biomass increased from 36.9 g to 74.2 g by the completion of the experiment (P = .0001).

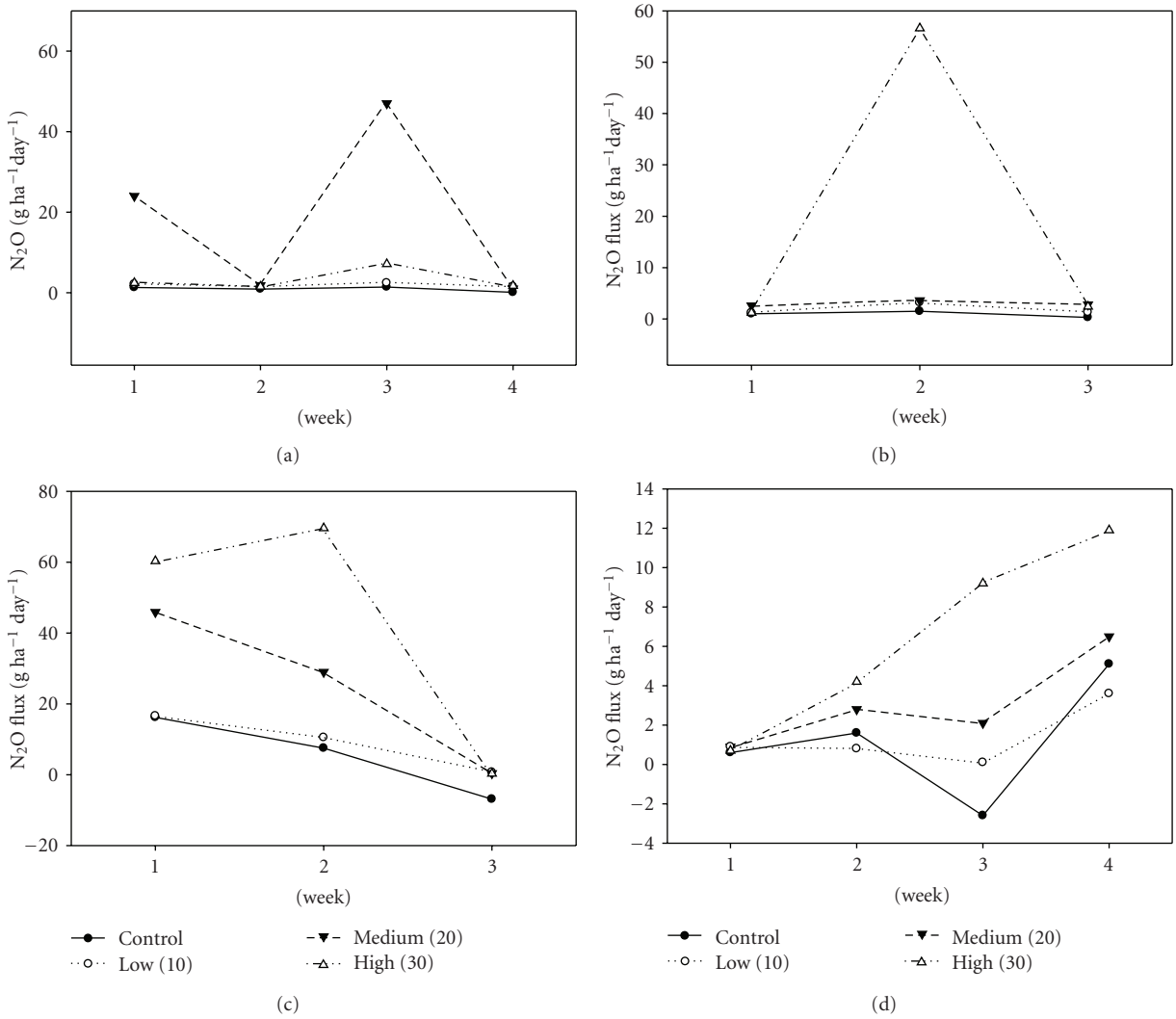

FIGURE 2: N_2O flux over the entire course of the experiment according to the control, low, medium, and high earthworm density at (a) 15% ($P = .1398$), (b) 25% ($P = .3912$), (c) 35% ($P = .2451$), and (d) 45% ($P = .0685$) gravimetric soil water content.

4. Discussion

Overall, emissions were highest at the 25% and 35% soil water content treatments and the lowest emissions were seen at 15% and 45% soil water content. Bertora et al. [19] found similar results with the presence of earthworms, where emissions increased significantly over the course of their experiment at 25% soil water content up to 62 days, when emissions began to decrease. N_2O emissions were significantly higher at 25% than at the lower moisture treatments (19%, 12.5%) where emissions were not significant.

Conversely, at 35% moisture, there was a downward trend in emissions over the course of the experiment, except at the high density where N_2O flux peaked at $69.6\,g\,ha^{-1}\,day^{-1}$ in week two with a significant decline in emissions in week three. This could mean that earthworms may only be able to tolerate high soil water content for a limited time. Therefore, the high earthworm mortality in this treatment could have occurred toward the end of the experiment, which could explain the decline in N_2O flux following week two. However, the 45% soil water content treatment also contradicts optimal soil water content for earthworm activity. N_2O emissions gradually increased across all earthworm densities at 45% soil water content showing that *L. terrestris* may have been adapting to the soil conditions. Increases in emissions were gradual and did not reach levels found at 25% and 35% soil water content, but mortality rates were lower, but not significant, compared to mortality rates at 35% soil water content showing some tolerance. El-Duweini and Ghabbour [20] also reported soil water content tolerance levels, but for two Australian species, *Allolobophora caliginosa* and *Metaphire californica*, to be 20%–45% and 35%–55%, respectively in a clay soil.

Earthworm mortality was the highest in the 35% moisture treatment, at the highest earthworm density even though emissions were significantly higher than at any other treatment combination. Dymond et al. [15] reported an initial earthworm invasion of 2,621 individuals m^{-2} of

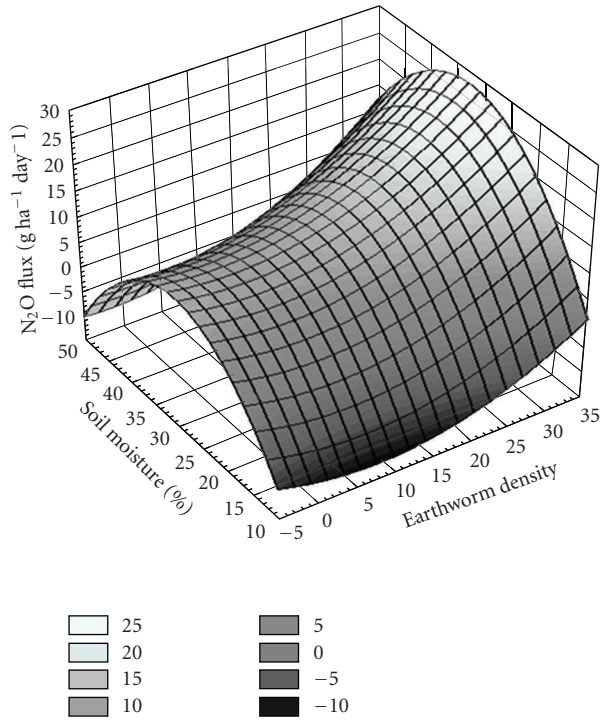

Figure 3: A response surface regression showing the relationship between N_2O flux (kg ha^{-1} day^{-1}), gravimetric soil water content (%), and earthworm density (number of earthworms 0.033 m^{-2}). Equation of the line is $36.7186 - (0.36143 * D) + (3.1095 * M) + (0.0174 * D * D) + (0.00810 * M * D) - (0.0518 * M * M)$ ($R^2 = 0.17$, $P \leq .0001$), where D is earthworm density and M is gravimetric soil water content.

Table 2: Mean initial and final earthworm biomass in the low, medium and high earthworm densities according to θ_g (%) treatment.

	Density Treatment Biomass (g)		
θ_g (%)	Low§	Medium	High
15 Initial	40.88 a†	86.97 a	130.55 abc
15 Final	50.92 a	83.85 a	121.48 abc
25 Initial	43.08 a	86.97 a	125.85 abc
25 Final	52.19 a	101.95 b	150.85 c
35 Initial	47.20 a	103.60 b	145.63 ac
35 Final	89.94 b	123.30 c	144.50 ac
45 Initial	33.28 c	70.29 a	103.90 b
45 Final	55.59 a	75.60 a	117.10 ab
SE	2.69	4.02	5.54
P value	<.0001	.0420	.0323

†Within columns, means followed by the same letter are not significantly different according to Tukey-Kramer means adjustment (0.05).
§Low, medium, and high refer to densities of earthworms per 0.033 m^2: 10, 20, and 30, respectively.

Dendrobaena octaedra into a northern Alberta pine (*Populus sp.*) and aspen (*pinus sp.*) forest. This population dropped to 76 individuals m^{-2} within just a few years as a result

Table 3: Mean initial and final biomass in the low, medium, and high earthworm densities at 31% gravimetric soil moisture content.

	Density Treatment Biomass (g)		
	Low§	Medium	High
Initial	12.6 a†	28.6 a	36.9 a
Final	27.1 b	44.4 b	74.2 b
SE	1.94	1.94	1.94
P value	.0007	.0003	.0001

†Within columns, means followed by the same letter are not significantly different according to Tukey-Kramer means adjustment (0.05).
§Low, medium, and high refer to densities of earthworms per 0.033 m^2: 3, 6, and 9, respectively.

of competition for resources. High competition could have been the reason for the drastic decline in emissions in the high density treatment at 35% soil water content and lower mortality in the medium (9%) and low (0%). Another reason for the decline in emissions after week two could be due to the ability of high earthworm populations to speed up residue decomposition [19]. Organic matter is more palatable to earthworms at higher soil water content; therefore, ingestion of organic matter is enhanced. Organic matter turnover could have been enhanced at the 35% moisture and high density combination by week two resulting in a decrease in preingested organic matter and a decline in earthworm activity.

The gravimetric soil water content treatments of 15%, 25%, 35%, and 45% are approximately equivalent to a water-filled pore space (WFPS) of 30%, 55%, 75%, and 100%. It is generally accepted that denitrification rates are optimal between a WFPS between 60% and 100%, where N_2O is the primary product between 60% and 90% [21]. Above 90% N is the dominant product [21], which could be the reason for lower N_2O flux measurements in the 45% soil water content, where the WFPS was 100%. The highest N_2O flux occurred at 35% soil water content or 75% WFPS, which is within the range of optimal denitirification rates. Furthermore, nitrification rates are highest between 45% and 75% [21]. The product of nitrification is NO_3^-, a primary input for denitrification. This means that in the 35% soil water content treatment, both nitrification and denitrification were optimal, which may have contributed to the significantly higher N_2O flux compared to the 15% and 45% soil water content treatments.

N_2O emissions could be lower at dryer soil water contents as a result of earthworm diapause or aestivation. In this state, earthworms will decrease their activity to prevent water loss from the body [2]. Ingestion of soil and organic matter content would decrease, thereby limiting microbial activity in the earthworm gut and reducing emissions. The same occurs at high moisture contents and could explain the lower N_2O emissions at the 45% moisture treatment in this study.

Perrault and Whalen [22] found that earthworm burrowing length decreased in wetter soils, which would indicate a decrease in earthworm activity. However, wetter soils caused

an increase in the ingestion of organic matter compared to dryer soils. Leaf litter is more palatable to earthworms when wetted, and as a result ingestion is increased. This could explain higher emissions in the 25% and 35% moisture treatments compared to 15% soil water content, as well as the decrease in earthworm biomass in the medium and high densities at 15% soil water content. Earthworms would ingest higher carbon substrates at these moisture contents, which would in turn provide energy for denitrifying bacteria found in the earthworm gut and increase N_2O production.

Earthworm surface casting also increases in wetter soils, which provides another ideal environment for denitrification. Earthworm casts contain higher populations of denitrifying bacteria compared to mineral soils due to higher amounts of carbon substrates, and as a result, higher N_2O emissions are produced [23]. Elliot et al. [24] found that denitrification was higher in earthworm casts than surrounding mineral soil. Denitrification rates from earthworm casts ranged between 0.2–0.9 $\mu g\,N\,g^{-1}$ during the fall compared to 0.05–0.3 $\mu g\,N\,g^{-1}$ from the soil within the same time period. This indicates that a portion of the emissions from this experiment could be due to increased surface casting in the 25% and 35% moisture treatments at the high density treatments.

Trends in N_2O emissions according to earthworm density did occur. The high, medium, and low density treatments represent 9.1×10^5, 6.1×10^5,and 3.0×10^5 earthworms ha^{-1}, respectively. Emissions consistently increased as earthworm density increased in all moisture treatments. However, emissions were only significantly higher at the medium and high densities in the 25% and 35% soil water content treatments (Figure 1). Frederickson and Howell [25] found no relationship between earthworm density and N_2O emissions in large-scale vermicomposting beds. However, in a subsequent laboratory experiment, emissions were correlated with earthworm density at five earthworm density treatments ($R^2 = 0.76$).

The reason for this may be a result of an increase in the ingestion of organic matter and, with that, denitrifier bacteria at higher earthworm densities; therefore, denitrification may occur at faster rates than in soils with lower earthworm densities. Denitrification occurs at higher rates in the earthworm gut due to the anoxic environment and sufficient supply of carbon for denitrifier bacteria compared to soil homogenates [6, 26, 27]. An increase in earthworm density results in an increase in this ideal environment of earthworms for denitrifier bacteria and therefore, could increase emissions. The number of denitrifier bacteria is also higher in the earthworm gut and surface castings than outside soil homogenates [8]. These authors calculated that there were 256-fold more denitrifier bacteria in the earthworm gut of *L. rubellus* than in the surrounding soil where the earthworms were found. This indicates that an increase in earthworm density also increases the number of denitrifier bacteria in the gut of the earthworms facilitating higher N_2O emissions as could be the case in this study.

Another reason why N_2O emissions were highest at the high density earthworm treatments could have been a result of an increase in the microbial biomass pool and subsequent increase in respiration causing lower O_2 levels in the soil. Groffman et al. [28] found that in areas with the presence of earthworms, microbial biomass was significantly higher in the mineral soil compared to areas without the presence of earthworms. In turn, Fisk et al. [29] discovered that this increase in microbial biomass due to the presence of earthworms increased respiration rates by 20% compared to areas without earthworms. Therefore, O_2 levels will decline providing a more ideal environment for denitrification to occur and subsequent gaseous N losses. However, even though microbial biomass may increase with earthworm presence, a subsequent increase in mineralization and nitrification rates may not occur. Bohlen et al. [30] and Groffman et al. [28] found that mineralization and nitrification rates in the soil did not differ significantly in plots with and without earthworms. They speculated that earthworms facilitated a C-sink in the soil and subsequently created an N-sink, preventing the increase in N mineralization and nitrification rates in the soil. This could mean that the majority of the N_2O released from the mesocosms was attributed to the presence of earthworms and earthworm gut, rather than denitrification occurring in the surrounding soil, since NO_3^- concentrations may have been low due to low nitrification rates.

In Experiment 2, N_2O emissions were not significantly different across earthworm population densities; however, the results were consistent to what was found in Experiment 1. N_2O flux in Experiment 2 across all earthworm densities (0, 3, 6, and 9) was in the same range as emissions in Experiment 1 between the control and low density earthworm treatments (0 and 10). This was expected since the earthworm densities used in Experiment 2 were within the range of the control and low density treatments in Experiment 1, and there were no significant differences in emissions between the control and low density treatments in Experiment 1. No significant differences in N_2O flux occurred even with significant differences in initial and final biomass between density treatments. This shows that even with an increase of approximately 3.0×10^5 earthworms ha^{-1} from zero earthworms, there would be no significant corresponding change in emissions between a TBI and sole cropping system, like the systems found at GARS. This could be a result of other compounding factors such as soil water content, soil temperature, residual soil N and C, and land management practices, which could all mask the earthworm effect on denitrification.

The same general trend of N_2O emissions occurred over time as in the 35% soil water content treatment in Experiment 1, where emissions hit a peak at week 2 and declined at week 3 to levels the same or lower than at week 1. This cannot be explained by earthworm mortality since earthworm mortality was insignificant or did not occur in Experiment 2 compared to Experiment 1. However, since soil water content of 31% was found to be optimal for earthworm activity, this may have sped up organic matter decomposition [18] between weeks 1 and 2 leaving the less palatable lignin compounds, thereby slowing earthworm activity between weeks 2 and 3.

5. Conclusion

A relationship was found between earthworm density, gravimetric soil water content, and N_2O flux in Experiment 1. As earthworm density increased, N_2O flux also increased; however, flux was only significantly higher in the high density treatment at 25% soil water content and at both the medium and high earthworm densities at 35% moisture. This could be attributed to optimal gravimetric soil water content for earthworm activity between 25% and 30%, which closely corresponds to the 31% moisture value reported by the response surface analysis in which emissions were also the highest.

Experiment 2 showed no relationship between earthworm density and N_2O emissions, which was expected because the earthworm densities used in Experiment 2 are within the range of the control and low density treatments used in Experiment 1 in which there was no significant difference in N_2O emissions. As a result, the results found here would only have implications in a TBI system where earthworm populations were triple to what is found at GARS. However, earthworms prefer environments with higher organic matter content and soil water content, both of which are present in a TBI system. This could result in higher emissions indirectly related to earthworm population as TBI systems have more favourable environments to earthworms. However, N_2O emissions as a result of the presence of earthworms could be dependent on the proximity of earthworm to the tree row, as well as the species of trees present in the TBI system.

The results of this study are important to consider when deciding on the implementation of agricultural practices to reduce N_2O emissions and also the invasion of earthworms into areas previously void of earthworms. The benefits that are normally seen from earthworms in agricultural systems may be masked by their influence on facilitating the production of N_2O and in turn, climate change.

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Review Article

Can We Predict How Earthworm Effects on Plant Growth Vary with Soil Properties?

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Earthworms are usually assumed to enhance plant growth through different mechanisms which are now clearly identified. It is however difficult to determine their relative importance, and to predict a priori the strength and direction of the effects of a given earthworm species on a given plant. Soil properties are likely to be very influential in determining plant responses to earthworm activities. They are likely to change the relative strength of the various mechanisms involved in plant-earthworm interactions. In this paper, we review the different rationales used to explain changes in earthworm effect due to soil type. Then, we systematically discuss the effect of main soil characteristics (soil texture, OM, and nutrient contents) on the different mechanisms allowing earthworm to influence plant growth. Finally, we identify the main shortcomings in our knowledge and point out the new experimental and meta-analytical approaches that need to be developed. An example of such a meta-analysis is given and means to go further are suggested. The result highlights a strong positive effect size in sandy soil and a weakly negative effect in clayey soil.

1. Introduction

Earthworms are among the most important detritivores in terrestrial ecosystems in terms of biomass and activity [1]. They are known to affect plant growth through five main mechanisms [2, 3]: (1) the enhancement of soil organic matter mineralization, (2) the production of plant growth regulators via the stimulation of microbial activity, (3) the control of pests and parasites, (4) the stimulation of symbionts, and (5) the modifications of soil porosity and aggregation, which induce changes in water and oxygen availability to plant roots. Although these mechanisms are well identified, it is difficult to determine their relative influence [4] and to predict the impact of a given earthworm species on a given plant species.

In a recent review, Brown et al. [2] proposed that the response of plants to earthworms should depend on soil properties such as texture, mineral nutrient levels, and organic matter content. However, most studies tackling earthworm effects on plant growth used soils containing more sand than clay (Brown et al. [2] and see Table 1). Comparatively, few studies [5–7] have tested in the same experiment earthworm effects on plant growth using different soils. Doube et al. [5] showed that the endogeic *Aporrectodea trapezoides* may increase wheat growth in sandy soils but may have no significant effect with a clayey substrate. They also found that the growth and grain yield of barley were both increased by *A. trapezoides* and *Aporrectodea rosea* in the sandy soil but reduced in the clayey one. On the contrary, Laossi et al. [7] showed that *Lumbricus terrestris* increased the shoot and total biomasses of *Trifolium dubium* in a clayey and nutrient-rich soil but not in a sandy and nutrient-poor one. The hypothesis that earthworm effects on plant growth should vary with soil type is based on two main reasons. (1) Soil properties may inhibit or stimulate some of the mechanisms through which earthworms tend to increase

plant growth. (2) If earthworms are able to alleviate limiting factor for plant growth, their impacts are expected to be weak in soils where the factor is not limiting. According to this rationale, the main mechanism through which earthworms affect plants should depend on soil type and in some soils earthworms might have no detectable or negative effect on plant growth.

2. How Soil Properties Should Modulate Earthworm Effects on Plant Growth?

Below, we go through the different mechanisms listed above and try to determine how soil properties should modulate their effect on plant growth.

(1) Earthworm activities usually have a positive impact on the mineralization of soil organic matter [8]. This effect is assumed to be a consequence of plant litter fragmentation and incorporation into the soil, as well as of the selective stimulation of microbial activity [9, 10]. Hence earthworms may enhance the release of nutrients that become available to plants and thus increase plant growth when they allow higher nutrient uptake than nutrient leaching [11, 12]. Anecic and endogeic earthworms have different feeding habits and affect differently soil organic matter composition and distribution [13]. Anecic earthworms feed on plant litter at the soil surface and tend to live in semipermanent vertical burrows while endogeic earthworms are active within the soil profile where they feed on soil organic matter [14]. This can lead to different effect on plant growth [15–17] which could also vary with soil properties such as organic matter and nutrient contents [18]. However, this rationale only holds if nutrients are limiting plant growth, that is, in soils where nutrients are poorly available. In contrast, in nutrient-rich soils, plants are less limited by the availability of mineral nutrients and earthworm-mediated mineralization should have less or no influence on plant growth [2]. Water is between the factors that limit plant growth and earthworms have been found to increase drought stress in plants [19]. This effect should be stronger in sandy soil which retains less water than in a clayey one.

(2) Earthworms affect plant growth through modifications of soil structure. They tend to increase soil porosity and the stability of organomineral aggregates by creating burrows and organomineral casts at different places within the soil profile [20, 21]. This effect is assumed to enhance plant growth in most situations [2] although opposite effects have also been reported [22]. It is difficult to predict how soil texture will modulate these effects. In clayey soils, earthworm might lead to very stable structures which could in turn strongly influence plant growth. This influence could be positive if the casts produced by earthworms do not lead to soil compaction [22], or negative with a physical protection of organic matter that impedes the release of mineral nutrients. In sandy soils, structures created by earthworms are more fragile [23] but more mineral nutrients can be released since the soil organic matter is less protected.

(3) Earthworm effects on plant growth via the release of plant growth regulators may be modulated by soil properties through several mechanisms, but here again the outcome is difficult to be predicted. First, plant growth regulators are though to be released by bacteria [24] and may be differently available depending on the levels of microbial activity in the soil. Sandy soils and soil with low organic matter contents usually have lower microbial biomasses and low potential for plant growth regulator production [25]. Thus, in such soils, earthworm effects via production of plant growth regulator could lead to weak effects on plant growth. Second, soil texture and soil organic matter could also affect the short-term availability of the produced phytohormones. For instance, clays and organic matter are known to adsorb organic molecules [26] and could reduce plant growth regulator availability to plants and weaken earthworm effect on plant growth.

(4) Earthworms are known to alleviate the negative effect of some parasites on plant growth by reducing strongly their density [27], ingesting and killing some pathogens in their intestine, or producing unfavourable conditions in cast material or tunnel lining [28]. This kind of mechanism may be influential for plant growth, especially in soil properties (such as moisture and temperature) that allow the development of abundant parasite populations. We can thus expect more parasites and greater negative effect of earthworms on them in a clayey soil.

(5) Similarly, earthworms can increase plant growth through the stimulation of symbionts or the increase in the contact between plants and symbionts [29]. Besides, if symbionts such as mycorrhizae provide nutrients to plants, symbiont-mediated earthworm effect (as their effect through mineralization) on plants should be more marked in poor soils than in rich soils where mineral resources are already available.

Taken together, these elements show that earthworm effects on plants vary with soil type but that it is difficult to predict the direction and the intensity of these variations. To make relevant predictions, we need to develop studies comparing in the same experiment earthworm effects on plants under different soil conditions. It is also necessary to set up meta-analyses using data of previous earthworms—plants studies. We provide below an example of what could be done through computing the effect size of earthworms on plant growth using meta-analysis with the data of the studies listed in Table 1.

3. How Can We Go Further?

To determine how earthworms effects on plant growth change with soil properties a first approach would be to compare earthworm-induced effects in different soils but in the same experimental conditions (same plant and earthworm species, same watering protocol, same greenhouse, etc.). Such experiments have been so far very scarce (but see [5–7]). To help predicting earthworm effects on plant growth in different soil types one could also use the "all-minus-one" tests proposed by Brown et al. [2]. In such experiments, only one factor such as mineral nutrition [4] or a root parasite [27] is limiting plant growth so

Table 1: References included in the survey of C and N contents in soil and the soil texture used in earthworm effects on plant growth.

References	Soil classification	Soil texture	N total	Clay	Sand	C Total
Aira and Plearce 2009	John Innes potting compost no. 2	Loamy compost	?	?	20%	?
Blouin et al. 2005	Ultisol	Sandy soil	0.05%	6%	78%	0.91%
Blouin et al. 2006	Ultisol	Sandy soil	0.05%	6%	78%	0.91%
Blouin et al. 2007	Ultisol	Sandy soil	0.05%	6%	78%	0.91%
Bonkowski et al. 2001	?	Loam soil + sand	0.1%	?	>50%	1.52%
Clapperton et al. 2001	Chernozem	Loamy soil	?	?	?	?
Derouard et al. 1997	?	Sandy soil	0.11%	5%	87%	0.91%
Devliegher and Verstraete 1997	Ardoyne	Sandy soil	?	?	?	0.9%
Daube et al. 1997	Xerosol, Palexeralf, wiesenboden	Sandy soil, loamy soil and clayey soil	?	?	?	?
Eisenhauer et al. 2009	Eutric Fluvisol	Loam soil	0.3%	?	?	4.6%
Eisenhauer et al. 2008	Gleyic cambisol	Silty soil	?	22%	9%	1.1%
Eisenhauer and Scheu 2008a	Eutric Fluvisol	Loam soil	0.3%	?	?	4.6%
Eisenhauer and Scheu 2008b	Eutric Fluvisol	Loam soil	0.3%	?	?	4.6%
Eriksen-Hamel and Whalen 2007	Typic endoquent	Sandy loam soil	?	12%	58%	2.45%
Eriksen-Hamel and Whalen 2008	Typic endoquent	Loam soil	?	?	?	5.6%–13.3%
Fraser et al. 2003	Udic dystrochrept	Silt loam soil	0.2%	?	?	2.6%
Gilot 1997	Ferralsol	Sandy soil	0.44%–0.59%	6%–10%	>75%	>1%
Gilot-Villenave et al. 1996	Ferralsol	Sandy soil	0.4%–1.2%	2.4%–4.5%	>80%	0.28%–1.18%
Hale et al. 2008	Eutroboralf	Silty clay loam soil	?	?	?	?
Hale et al. 2006	Eutroboralf	?	?	?	?	?
Hopp and Slatter 1948	?	Clayey soil	?	70%	16%	?
Kreuzer et al. 2004	?	?	<0.1%	?	?	1.8%
Taossi et al. 2009a	Cambisol	Sandy soil	0.12%	6.9%	74%	1.47%
Taossi et al. 2009b	Cambisol and leptosol	Sandy soil and clayey soil	0.12%; 0.46%	6.9%; 34.4%	74%; 27%	1.47%; 5.67%
Milcu et al. 2006	Eutric Fluvisol	Loam soil	0.3%	?	?	4.6%
Milleret et al. 2009	Anthrosol	Sandy soil + compost	?	26.7%	45.3%	?
Newington et al. 2004	?	Sandy loam + aquatic compost + leaf mulch	<0.01%	?	?	?
Ortiz-Ceballos et al. 2007a	Fluvisol	Silty clay loam soil	0.25%	26.8%	41.5%	?
Ortiz-Ceballos et al. 2007b	Fluvisol	Silty clay loam soil	0.25%	26.8%	41.5%	?
Partsch et al. 2006	Eutric Fluvisol	Loam soil	0.3%	?	15%	4.6%
Pashanasi et al. 1996	Paleudult	Sandy loam soil	?	23%	55%	?
Patron et al. 1999	Inceptisol	Sandy soil	0.08%	11%	82%	0.85%
Poveda et al. 2005a	?	?	?	?	?	?
Poveda et al. 2005b	?	?	?	?	?	?
Schmidt and Curry 1999	Podzol	Loam to clay loam soil	0.18%	19%	47%	1.88%
Stephens and Davoren 1995	Calcic Natrixeralf	Calcic soil	?	?	?	1.5%

Table 1: Continued.

References	Soil classification	Soil texture	N total	Clay	Sand	C Total
Stephens and Davoren 1997	Calcic Natrixeralf	Calcic soil	?	?	?	?
Thompson et al. 1993	?	Loam soil	?	?	?	?
Welke and Parkinson 2003	Dystric brunisols, grey-brown luvisols	Sandy loam soil	0.1%–1.07%	?	?	21.63%–43.73%
Wurst et al. 2008	?	Loamy sandy mineral soil	0.13%	?	?	2.1%
Wurst et al. 2005	Cambisol	Loam soil	0.087%	?	?	1.58%
Wurst et al. 2003	?	Loam soil	0.087%	?	?	1.58%
Zaller and Arnone 1999	Rendzina	Calcareous soil	?	?	?	?

that the capacity of earthworms to alleviate this limiting factor can be tested. This allows testing main mechanisms through which (see introduction) earthworms affect plant growth in particular conditions. Such experiments could be repeated in soils differing by only one property to determine how this property modulates the strength of each of these mechanisms. For example, previous experimental studies conducted in greenhouse conditions [27, 30] and using sandy soil have showed that earthworms enhanced the tolerance of plants to nematodes. This kind of study should be carried out using sandy and clayey soil in the same experiment to test whether soils properties change the strength and direction of this earthworm effect.

While comparing earthworm effect in different soils differing by only one parameter is easy, this is not likely to allow disentangling all factors because soil properties are often correlated. Clay soils are generally rich in organic matter. A solution would be to directly manipulate soil properties. Hence, it would be possible to add, for example, clay, sand, organic matter, or mineral nutrients to a soil. We would then study the effect of a gradient in clay, sand, organic matter, or mineral nutrient on earthworm-induced effect. In the same vein, earthworm effect on the nutrient input-output balance of ecosystems should determine the long-term effect of earthworm on plant primary production [31]. Thus, comparing earthworm effects in different soils could also allow measuring their effects on nutrient leaching in these different soils and to identify the type of soil in which nutrients made available are leached and in which other it remains in the superficial soil layers. This is important to determine the long-term effect of earthworm-soil type interaction on plant growth. Another possibility would be to conduct meta-analyses to take advantage of the numerous studies already published on the issue. We give below a first example of such meta-analyses.

4. Example of Meta-Analysis

We used the results of 25 experiments (Table 1) to perform a meta-analysis and calculate the effect size of earthworms influence on plant growth in different soil types with contrasting texture properties (sandy, clayey, or loamy soil). The effect size was computed as $(M1-M2)/\sigma$, with $M1$: mean plant biomass in the presence of earthworms, $M2$: mean plant biomass without earthworm, and σ: standard deviation without earthworm [32]. An ANOVA was then used to test for the effect of soil texture on the effect size, that is, on the magnitude of earthworm impact on plant biomass. This shows that soil texture influences significantly earthworm impact on plant growth ($r^2 = 0.11$ and $P = .02$). LS mean comparisons show that the effect size was in sandy and loamy soils, respectively, 60- and 17.5- fold higher than in clayey soil. The result highlights a strong positive effect size in sandy soil and a weakly negative effect in clayey soil (Figure 1).

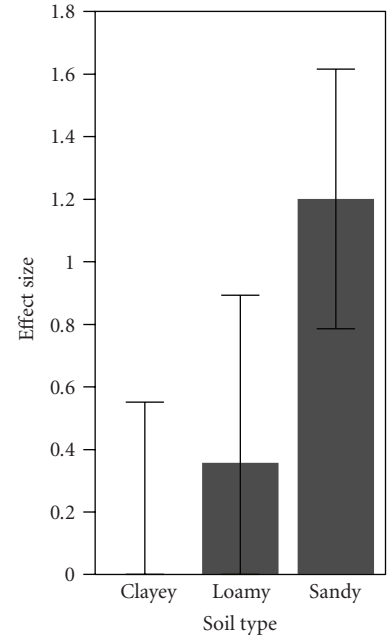

Figure 1: Effect size of earthworm effects on plant growth based on results of 25 experiments presented in Table 1. Mean effect size calculated as $[M1 - M2]/\sigma$ with $M1$: mean plant biomass in the presence of earthworms, $M2$: mean plant biomass without earthworm, and σ: standard deviation without earthworm. $P = .02$

This result supports the assertion of Brown et al. [2] that positive effects of earthworms on plant growth are more pronounced in sandy soils (generally nutrient-poor soils) that in clayey soils (generally nutrient-rich soil). However, as showed in Table 1 most studies used sandy soils while only few studies have used clayey ones. We thus need to release this bias by developing more studies for clayey soil. Nevertheless, our meta-analysis is the first formal test of the influence of soil properties on earthworm effect on plant growth.

5. Conclusion

Although the majority of authors provided detailed data on soil characteristics, this basic information was not available in all studies in earthworm impacts on plant production (Table 1). Further studies should pay attention to providing a standardized description of soil characteristics, which would thus be available for meta-analyses on earthworms—plants studies. For example, data on soil texture (sand and clay percentage), total C, total N content, NH_4^+, and NO_3^- should be systematically published. Because such information, is not always given (see Table 1), we have only compared the effect of wide texture classes on earthworm effect. Finally, we have shown that these texture classes only explain 11% of the variations in effect sizes. This is probably due to a variety of other factors that we have not taken into account: soil properties mentioned above but also earthworm species (or its functional group) and plant species (or its functional group), and so forth [2, 3]. Gathering more studies on earthworm effects on plant growth and documenting for each of these studies all these factors would allow disentangling, through a unique meta-analysis statistical model, the respective effect of all these factors on earthworm-induced effect on plant growth, as well as interactions between these factors. This kind of general and systematic approach is required to derive general results on soil ecology and to develop the theoretical background needed to base soil ecology on solid bases [33].

Taken together, while a given earthworm species could have positive effects in a soil, it could have negative effects in another soil. To restore soil fertility or to enhance the sustainability of crop production [14], the right earthworm species has indeed to be chosen according to soil properties and crop type. Developing applications based on the use of earthworms would thus also require implementing the general meta-analysis (as suggested above) and the subsequent development of a general and comprehensive framework on earthworm-induced effect on plant growth that is so far missing.

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Research Article

Earthworms and Plant Residues Modify Nematodes in Tropical Cropping Soils (Madagascar): A Mesocosm Experiment

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Free-living nematodes present several characteristics that have led to their use as bioindicators of soil quality. Analyzing the structure of nematofauna is a pertinent way to understand soil biological processes. Earthworms play an important role in soil biological functioning and organic matter dynamics. Their effects on soil nematofauna have seldom been studied. We studied the effect of the tropical endogeic earthworm, *Pontoscolex corethrurus*, on nematode community structure in a 5-month field mesocosm experiment conducted in Madagascar. Ten different treatments with or without earthworms and with or without organic residues (rice, soybean) were compared. Organic residues were applied on the soil surface or mixed with the soil. The abundance of nematodes (bacterial and fungal feeders) was higher in presence of *P. corethrurus* than in their absence. The type of plant residues as well as their localisation had significant effects on the abundance and composition of soil nematodes. The analysis of nematode community structure showed that earthworm activity led to an overall activation of the microbial compartment without specific stimulation of the bacterial or fungal compartment.

1. Introduction

Soil organisms play a leading role in decomposition and mineralization of organic matter (OM) [1]. They are involved in processes that affect carbon (C) sequestration as well as in the modification of soil physical structure and chemical properties. They also interact with other soil fauna and these interactions result in complex food webs [2]. Nematodes are small organisms (ca. 1 mm long at the adult stage) abundant in soil (several million m^{-2} soil), they present a high species diversity (about 11,000 species have already been described). Nematodes live in the film of water between soil particles and present various feeding behaviours. During the last twenty years, many studies have been conducted on these microfaunal organisms because they can be an efficient tool to assess soil quality and soil biological functioning [2–4]. Because they are present at different levels of the soil food web and present variable tolerance toward stress, nematofauna provide information about OM decomposition pathways and soil pollution status [3, 5–7]. Nematodes interact with other soil organisms including earthworms, which also play an important role in soil biological functioning and OM dynamics [8, 9]. Until now, studies on interactions between nematodes and earthworms have focused on the contribution of earthworm burrowing and casting activity to nematofauna abundance, composition, and activity [10–14]. These studies were mainly conducted in temperate regions or under specific conditions such as vermicomposting. Results show that interactions between nematodes and earthworms are site and species specific.

In Madagascar, like in other tropical countries, alternative cropping systems are being developed in order to decrease soil erosion and environmental impacts and to

increase crop productivity or sustainability. No-tillage systems with living or dead (mulch) cover plants have beneficial effects both on environment and crop biomass [15]. In Madagascar, it has been shown that these cropping systems increase the carbon sequestration in soil and decrease soil erosion [15]. Moreover, they increase the density, activity, and diversity of soil fauna and especially earthworms and soil microorganisms [16].

This study was part of a larger project aimed at determining the consequences of earthworm activity for soil aggregation, OM dynamics, and soil biological activity in no-tillage systems in Madagascar [16]. In the present experiment, we focused on the interaction between the tropical endogeic peregrine earthworm *P. corethrurus*, and soil nematofauna. This earthworm was chosen to be inoculated in the experiment because it was dominant in the study area, feeding and living within the soil although. It is an invasive earthworm, originated from South America.

The abundance and diversity of soil organisms, including nematodes, depend on cropping practices [17]. Indeed, OM amendments like manure generally have a positive effect on microbial biomass and consequently on nematode density [18, 19]. The quality of the organic resource applied (particularly the carbon to nitrogen ratio (C/N), lignin, phenolic compounds, and cellulose content) influences the trophic structure of the nematofauna [3]. OM incorporation by tillage can also lead to modifications in the nematode density [20, 21].

In this study, we tested the interaction between earthworms and nematofauna under applications of different organic matter (rice residues or soybean residues) brought as mulch or buried in the soil.

2. Materials and Methods

2.1. Study Site. The study was conducted in Madagascar in the region of Antsirabe (19°52 S; 47°04 E) at an altitude of 1500 m above sea level. Mean annual temperature is 16°C and mean annual rainfall 1300 mm. The climate is subtropical humid with hot and humid summers (Oct-Apr) and cold and dry winters (May-Sep). The soil at the sampling site is highly desaturated red ferrallitic (andic dystrustept), with 62% clay mainly as 1 : 1 minerals, but presents andic characteristics. In the upper 10 cm of soil, carbon content was 45.6 g kg^{-1} dry soil, bulk density was 0.76 g cm^{-3}, pH_{H_2O} was 5.7, the C/N ratio was 14.8, and exchangeable cation capacity was 17.3 cmol kg^{-1} dry soil [15].

The experiment was conducted in field mesocosms on a 100 m^2 plot and lasted 5 months (from January 2005 to June 2005, during the wet season) (see [16] for more details). Fifty plastic buckets (20 cm deep) with a diameter of 25 cm were filled with 8 kg of soil previously sieved at 2 mm and homogenised, and were then introduced into the soil so that surface level was similar inside and outside the buckets. Before the buckets were filled with soil, their bottoms were drilled (8 holes 1 cm diameter) and covered with a mosquito net so that water could flow but earthworms could not escape.

2.2. Experimental Design. Ten different treatments were tested: five treatments inoculated with earthworms and five without earthworms. Among the five treatments (with or without earthworms), two received soybean residues, two others rice residues, and one did not receive any residue. When residues were applied, they were placed either on the soil surface or buried. Each treatment was replicated five times, which led to a total of fifty mesocosms in the field experiment. In each bucket with earthworms (E+), six adult or subadult earthworms of the species *Pontoscolex corethrurus* Müller 1856 (Glossoscoloscidae), sampled near the study site, were inoculated in January 2005. This endogeic geophagous earthworm is a tropical peregrine earthworm [22] abundant in the study area, feeding and living in the soil. Soybean (*Glycine max L.*) and rice (*Oryza sativa L.*) residues were added at the same amounts as those measured in cropped fields in local no-tillage systems, that is, 293 g m^{-2} (18.3 g per bucket) for soybean residues and 365 g m^{-2} (14.6 g per bucket) for rice residues. Soybean residues were mostly composed of woody twigs with a C/N ranging between 16 and 23 while rice residues were mainly composed of straw with a C/N ranging between 45 and 64 [23, 24]. Residues were cut into 2-3 cm long pieces. Mesocosms were randomly located in the experimental plot. Buckets were watered during the experiment by natural rainfalls.

2.3. Analysis. After 5 months in the field, mesocosms were removed, and their content was separated into a 0–10 and a 10–20 cm soil layer. The soil was roughly disaggregated to check that earthworms were still alive. The earthworms were all alive, and since they were mostly present in the 10–20 cm layer, all analyses were performed in this layer.

Nematodes were extracted from an average of 93 g of humidified, and homogenized soil (min. 90 g, max. 95 g) using the Cobb method. It consists of mixing the soil with a large volume of water allowing a brief time for heavy particles to settle, and then pouring the mixture through several sieves of a mesh size from 500 μm to 50 μm to retain large debris or nematodes; the second step is used to further clean up the sample (48 hours on a 40 μm sieve) [25]. Nematodes were counted under a binocular microscope and fixed at 65°C with 4% formalin and subsequently mounted for mass-slide identification (5 cm × 7.5 cm slides). An average of 175 nematodes was identified on each slide. Nematodes were identified to family or genus level and assigned to seven trophic groups according to Yeates et al. [26]: bacterial-feeders, fungal-feeders, entomopathogenics, plant-feeders, root hair-feeders, omnivores, and predators. Microbivorous nematode taxa were also allocated to c-p groups following T. Bongers and H. Bongers [5]. Colonizers (c) and persisters (p) are the two extremes on a scale from 1 to 5, respectively. The c-p value takes into account nematode ecological characteristics, that is, their demography and their life-history strategies [5].

2.4. Statistical Analysis. Differences in taxa and trophic group densities were assessed by ANOVA (Xlstat 2006 Addinsoft) after $\log(n + 1)$ transformation of the data.

A two-way ANOVA was first performed on the 50 samples to test the effects of the addition of earthworms and residues. The 10 samples that did not receive any residues were excluded before performing a three-way ANOVA to test not only the effects of the addition of earthworms and the quality of the residue applied, but also the effect of the location of the residues. A nonparametric multidimensional scaling analysis (MDS) was performed using Primer software (PRIMER-E ltd) to compare the structure of the nematofauna between treatments. Statistical significant differences in community composition between treatments were assessed by analysis of similarities: ANOSIM (which uses permutation/randomisation methods on the similarity matrix).

3. Results

In our experiment, 31 nematode taxa were identified. Bacterial-feeders represented 74.9% of total nematofauna including 12 taxa (Table 1). Fungal-feeders (5 taxa) represented 13.7% of nematode abundance. An entomopathogenic nematode (*Steinernema)* was present in some samples and represented 0.3% of the total density. Plant-feeders represented 6.1% of total density; 9 genera were identifed *(Pratylenchus*, *Paratylenchus*, *Aorolaimus*, *Rotylenchus*, *Helicotylenchus*, *Tylenchorhynchus*, *Meloidogyne*, *Paratrichodorus*, *Xiphinema*). Root hair-feeders (only members of the Tylenchidae family) represented 3.9% of the total nematode abundance, whereas omnivores and predators (mainly diverse Dorylaimoidea, *Aporcellaimellus*, *Discolaimus*) together represented 1.2%. The ANOVA results on abundance of the different taxa are summarized in Table 1. There was only one statistically significant interaction in the 2-way ANOVA between earthworm and addition of residues; there were more interactions in the 3-way ANOVA between the three factors (earthworm, addition of residues, location of residues) (Table 1).

3.1. Effect of Earthworms on the Density of the Different Nematode Taxa. Total nematode density was significantly higher in the treatments with *P. corethrurus* than in the non-inoculated treatments (Table 1). The density of fungal-feeders was significantly higher in the presence of earthworms. Three nematode taxa were significantly more abundant in inoculated treatments: two bacterial-feeders, *Acrobeloides* and Prismatolaimidae, and one fungal-feeder, *Aphelenchus*. For the fungal-feeders, all taxa showed an increasing trend in the presence of earthworms (statistically significant only for *Aphelenchus* whereas, for bacterial feeders none of the taxa other than *Acrobeloides* and Prismatolaimidae showed any sign of response). None of the taxa were significantly reduced in numbers due to the presence of the earthworms.

3.2. Effect of Residue Addition on the Density of the Different Nematode Taxa. The addition of residues did not lead to any statistically significant differences between the three treatments (soybean, rice, or no residues) with regard to total nematode density (Table 1), but led to significant differences for the fungal-feeders, which were more abundant in the treatments with residues, whatever the residue quality, than in the treatments without residues. Among the bacterial-feeders, one group (Panagrolaimidae) was more abundant in presence of soybean residues than in absence of residues, whereas several groups (Rhabditidae, *Alaimus* and *Amphidelus*) were more abundant when rice residues were added. The abundances of *Acrobeles* and *Cervidellus* were reduced due to rice and soybean residues, respectively. Among the fungal-feeders, *Aphelenchus* density was significantly higher with soybean residues, whereas *Ditylenchus* density was higher with rice and soybean residues.

Nematode density was significantly higher in the mesocosms with buried residues than with mulched ones (Table 1). Moreover, bacterial-feeders density was significantly higher in the buried residue treatments with significant increases in Rhabditidae, *Acrobeloides*, Plectidae, *Rhabdolaimus,* and *Amphidelus* without any interactions, whereas density of Panagrolaimidae, Prismatolaimidae, and *Rhabdolaimus* was increased with an interaction between earthworms and localization and *Cervidellus*, Prismatolaimidae, and Alaimidae with an interaction between residues and localization.

A fungal-feeder group, Tylencholaimoidea, was significantly more abundant in the treatments with buried residues than with mulched ones with no interaction with the others treatments (earthworms and residue), whereas *Ditylenchus* showed a significant effect of localisation of residues but also an interaction between residue and localization. Entomopathogenics and root hair-feeders were also significantly more abundant in the treatments with buried residues (with no interaction).

3.3. Effect of Addition of Earthworms and Residue on the Structure of the Nematofauna. There was no significant difference in the structure of the nematofauna (abundance of the 31 taxa) between treatments with earthworms and treatments without earthworms (similarity analysis: ANOSIM, $P < .15$). In contrast, there was a significant difference in nematofauna structure between treatments with rice residues and treatments with soybean residues (ANOSIM, $P < .05$) (Figure 1). The outlier is a replicate of the treatment with earthworm, with rice residue addition, mulched residue where an unexplained proliferation of Aphelenchoididae occurred.

There was no significant difference in the structure of the nematofauna between treatments without residues and the two treatments with residues (soybean or rice) (ANOSIM, $P > .05$). Moreover, mixing the residues with soil led to a significantly different structure of the community than that measured in treatments without residues or with residues placed on the soil surface (ANOSIM, $P < .05$).

4. Discussion

Previous research showed that by grazing and ingestion of nematodes, earthworms could directly affect nematode community structure leading to a decrease in nematode

Table 1: Abundance (individuals kg^{-1} of dry soil) of nematodes in treatments: Earthworm—No earthworms, No residues added—with rice residue—with soybean residues, and in treatments with buried residue—mulch residue. Significant differences are indicated in bold by ∗ or different letters (Newman-Keuls test, $P < .05$). Results of analysis of effects of earthworms and residues were obtained by a 2-way anova ($n = 50$) including all the samples, whereas the effect of localisation was assessed by a 3-way anova ($n = 40$) excluding samples with no residue inputs. Significant interactions of the 2-way anova (earthworm (E) × residues (R) and of the 3-way anova (E × R, Localisation (L) × E, L × R, E × R × L) are indicated in the table by ∗ ($P < .05$).

	Earthworm	**Residues**														
	c-p	Earthworm	No earthworm		No residue addition		With rice residue addition		With soybean residue		*Interaction anova2*	Buried residue	Mulched residue	*Interaction anova3*			
											E × R			E × R	E × L	R × L	E × R × L
Nematodes trophic groups																	
Bacterial-feeders																	
Monhysteridae	1	20	25		4		33		21			28	26				
Panagrolaimidae	1	2054	2587		**1203**	**b**	**2123**	**ab**	**3075**	**a**		***4127***	***1071***	∗		∗	
Rhabditidae	1	944	652		**60**	**b**	**1451**	**a**	**514**	**b**		**1370**	**595**	∗			
Acrobeles	2	147	291		**383**	**a**	**104**	**b**	**252**	**c**		230	126				
Acrobeloides [a]	2	**7001**	**2840**	∗	4162		3054		7166			**6513**	**3707**	∗			
Cervidellus	2	196	229		**236**	**a**	**267**	**a**	**147**	**b**		***330***	***84***	∗			∗
Drilocephalobus	2	592	613		**598**	**ab**	**388**	**b**	**818**	**a**		608	598				
Plectidae	2	24	34		14		50		17			**52**	**15**	∗			
Prismatolaimidae	3	**628**	**427**	∗	491		475		599			***672***	***402***	∗		∗	∗
Rhabdolaimus	3	*345*	*322*		*292*		*346*		*343*		∗	***484***	***205***	∗	∗	∗	
Alaimus	4	447	481		**173**	**b**	**549**	**a**	**525**	**ab**		*697*	*377*			∗	∗
Amphidelus	4	41	30		**12**	**b**	**71**	**a**	**12**	**b**		**52**	**31**	∗			
Total		12 439	8531		7628		8911		13 489			**15 163**	**7237**	∗			
[-.7pt] Fungal-feeders																	
Aphelenchoididae	2	1108[z]	297		135		1317[z]		372			567	1121[z]				
Aphelenchus	2	**1133**	**793**	∗	**750**	**b**	**798**	**b**	**1235**	**a**		*991*	*1042*			∗	
Ditylenehus	2	286	139		**16**	**b**	**141**	**a**	**382**	**a**		***473***	***50***	∗			∗
Diphterophoridae	3	44	27		33		41		31			29	43				
Tylencholaimoidea	4	46	34		13		29		64			**80**	**13**	∗			
Total		**2617**	**1289**	∗	**946**	**b**	**2326**	**a**	**2084**	**a**		2140	2270				
Entomopathogenic		235	175		338		235		110			**268**	**77**	∗			
Plant-feeders		1000	722		983		912		749			*602*	*1059*			∗	
Root hair-feeders		547	553		564		617		476			**845**	**247**	∗			
Omnivores and predators		155	189		140		200		160			*203*	*158*			∗	∗
Nematode density		**16 994**	**11 460**	∗	10 600		13 200		17 068			**19 220**	**11 047**	∗			

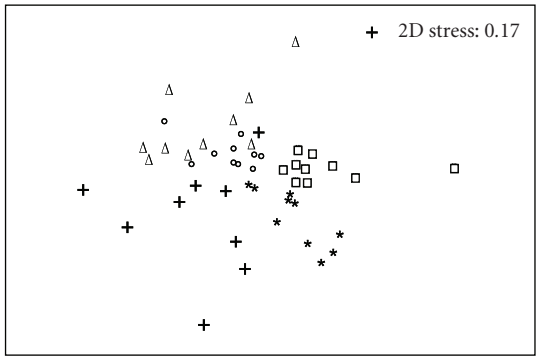

Figure 1: Multidimensional scaling analysis (MDS) of nematofauna structure for the different organic matter treatments ($n = 10$ in each category).

density [13, 27]. Contrary to these results, we found that nematode density was significantly higher in the treatments inoculated with earthworms. These differences may be due to earthworm ecology. Indeed, earthworms modify soil ecosystem properties by casting activity [28] and may thus have an indirect effect on soil microorganisms. Earthworms increase soil OM mineralization by fragmentation but also by the activation of microorganisms during passage through the gut as they secrete intestinal mucus that stimulates microbial activity [29]. This may lead to a priming effect allowing digestion of more complex compounds. The products of this digestion are excreted into casts which are enriched in partially digested OM and in mineral nitrogen [30]. These casts represent a directly available food resource for microorganisms that develop on them. The dominant group of bacterial-feeders, *Acrobeloides*, was significantly more abundant in the presence of *P. corethrurus*. Moreover, fungal-feeders (and specially *Aphelenchus*) were more abundant in the treatments inoculated with earthworms. These results indicate that earthworm activity stimulated the microbial community resulting in an increase in the density of microbivorous nematodes. Hyvönen et al. [12] suggested that the reduction of nematode density that occurred in their experiment in presence of earthworms was due to ingestion by earthworms and death during the digestive transit; however, most bacterial-feeding nematode taxa were unaffected by the presence of earthworms (*Acrobeloides*, *Alaimus*, Plectidae) in their study.

It was especially fungal feeders that responded positively to the presence of earthworms. As there was no interaction with the residue type, this increase must be mainly controlled by the activity of earthworms themselves (modification of physical and chemical properties). But this interesting issue would need further development to be better understood.

The predominant nematodes in this experiment (*Acrobeloides*, Panagrolaimidae, *Aphelenchus* and Aphelenchoididae) are characterized as opportunistic nematodes with high colonisation and reproduction capacities. These nematodes are r-strategists and respond rapidly to a disturbance. They have a c-p value equal to 1 or 2. Soil sieving, manual homogenization, and soil storage before the experiment led to a simplification of the nematofauna present in the mesocosms compared to a natural ecosystem as the weakest (most fragile) taxa probably died during preparation of the mesocosms. In natural conditions, earthworm activity may also influence these "fragile taxa". Some nematodes with c-p 3 and 4 also responded positively to the buried residues.

Incorporation of OM generally leads to an increase in microbial biomass [31]. Our results showed that buried residues treatment had a positive effect on total nematode density, mainly through an increase in the density of bacterial-feeders. This result could be explained by the improved availability of buried residues for microorganisms compared to mulched residues. The increase in the abundance of bacterial-feeder nematodes (mainly c-p 1 and c-p 2) reflects an increased microbial growth [32] and maybe also an increased microbial biomass [33]. Thus, when residues are buried, microorganism activity may increase and the predominant nematodes might be r-strategists. Our results, obtained under tropical conditions, are consistent with previous studies conducted in temperate regions [21, 34, 35].

In this experiment, nematode structure differed according to the two types of residues; this difference was not linked to a difference in abundance of the different trophic group but rather to differences in the composition of the bacterial-feeding community. The proportion of bacterial-feeding nematode taxa was very different from one treatment to another, suggesting that the microbial community that degraded these residues differed depending on the quality of the residues applied. We found a significant increase in some bacterial-feeders in the rice residue treatments, including Rhabditidae, an opportunistic c-p 1 nematode. The soybean treatment was responsible for an increase of two opportunistic c-p 1 and 2 bacterial-feeders, Panagrolaimidae and *Drilocephalobus*, but also one fungal-feeding taxon (*Aphelenchus)*, a c-p 2 opportunistic nematode. The nematode community presents different patterns depending on the resources available in the soil [36, 37]. After a fresh organic matter input in the soil, usually bacterial-feeding c-p 1 opportunistic nematodes like Rhabditidae develop first and are progressively replaced by fungal-feeding c-p 2 opportunistic nematodes. The higher density of Rhabditidae with residues in our experiment demonstrates both that the OM was highly processed by the bacteria, whereas the development of fungal-feeding c-p 2 nematodes like *Aphelenchus* populations showed that the conditions were favourable for the development of fungi [3, 31].

The density of fungal-feeders with rice residues was highly overestimated as in a replicate; there was a unexplained proliferation of Aphelenchoididae. Taking this into account, there was a trend of higher abundance of fungal-feeding nematodes with soybean than with rice (1541 versus 2084 individuals kg^{-1} dry soil, resp.). Actually, in

this experiment, there were more twigs in the soybean treatment than in the rice treatment, thus explaining the development of fungi which are able to digest complex compounds like lignin [31, 37, 38]. When correcting the value of Aphelenchoididae in the treatment "mulch residue", omitting the outlier replicate, we found that fungal-feeding nematodes (*Ditylenchus* and Tylencholaimoidea) were significantly more abundant with buried residues than with mulched residues.

5. Conclusion

Our results showed that the earthworm *P. corethrurus* had a positive effect on total nematode densities mainly by increasing the density of dominant bacterial-feeding (*Acrobeloides*) and fungal-feeding (*Aphelenchus*) nematodes. The structure of the nematode community indicated that the decomposition of soybean residues was more fungal-based than that of the rice residues. Changes in the composition of the nematode fauna were greater when organic matter was buried in the soil than when it was left on the surface. Buried residues were responsible for the development of bacterial-feeder nematode populations, reflecting a stimulation of the bacterial compartment.

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Review Article

Effects of Pesticides on the Growth and Reproduction of Earthworm: A Review

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Scientific literature addressing the influence of pesticides on the growth and reproduction of earthworm is reviewed. Earthworms are considered as important bioindicators of chemical toxicity in the soil ecosystem. Studies on this aspect are important because earthworms are the common prey of many terrestrial vertebrate species such as birds and small mammals, and thus they play a key role in the biomagnification process of several soil pollutants. Majority of the studies have used mortality as an endpoint rather than subtler endpoints such as reproductive output. It is now emphasized that, whereas higher concentrations of a pollutant can easily be assessed with the acute (mortality) test, contaminated soils with lower (sublethal) pollutant concentrations require more sensitive test methods such as reproduction test in their risk assessment.

1. Introduction

A greater proportion (>80%) of biomass of terrestrial invertebrates is represented by earthworms which play an important role in structuring and increasing the nutrient content of the soil. Therefore, they can be suitable bioindicators of chemical contamination of the soil in terrestrial ecosystems providing an early warning of deterioration in soil quality [1–3]. This is important for protecting the health of natural environments and is of increasing interest in the context of protecting human health [4] as well as other terrestrial vertebrates which prey upon earthworms [5]. The suitability of earthworms as bioindicators in soil toxicity is largely due to the fact that they ingest large quantity of the decomposed litter, manure, and other organic matter deposited on soil, helping to convert it into rich topsoil [6, 7]. Moreover, studies have shown that earthworm skin is a significant route of contaminant uptake [8] and thus investigation of earthworm biomarkers in the ecological risk assessment (ERA) can be helpful [9] .

Eisenia fetida is the standard test organism used in terrestrial ecotoxicology, because it can be easily bred on a variety of organic wastes with short generation times [10–13]. Its susceptibility to chemicals resembles that of true soil organisms. Sensitivity tests of multiple earthworm species have revealed that *Eisenia fetida* is comparatively less sensitive [14–16]. Although, earthworm species vary in their tolerance, reports have shown a decline in earthworm populations in response to large amounts of organic chemical deposition [17].

Mortality has been the most frequently used parameter to evaluate the chemical toxicity in earthworms [18–20]. It is postulated, however, that survival is less sensitive from an ecotoxicological point of view [21] and acute mortality tests would not provide the most sensitive risk estimate for earthworms in the majority (95%) of cases [22]. Amorim et al. [23] tested with herbicide Phenmedipham and found reproduction to be a more sensitive endpoint than mortality in *Enchytraeus albidus* and *Enchytraeus luxuriosus*. It is suggested that the chronic test, aiming at sublethal effects, is more sensitive and is a more realistic approach for the prediction of environmental effects because in the field, the exposure concentrations of pesticides are usually quite low [24]. Moreover, the lethal effect of a chemical is not a necessary consequence in intoxication and sublethal effects

may be produced. According to Riepert et al. [25] the acute earthworm test is part of the basic test set, but the earthworm reproduction test is considered ecologically more relevant. Therefore, growth and reproduction have been recommended as useful sub lethal criteria [26, 27]. This article reviews in short the available scientific literature on the effects of pesticides on the key biological processes, that is growth and reproduction of earthworms.

2. Sublethal Toxicity Testing Method

The earthworm reproduction test with *Eisenia fetida*/*Eisenia andrei* aims to assess the impact of soil contaminants on sublethal parameters in earthworms. Endpoints include reproductive parameters (cocoon production per adult per week, juveniles hatching per adult per week and cocoon viability) and weight change of adults. During the test, adult mature worms are exposed to different concentrations of a substance (pollutant) in a standard test soil; when field soils are used, homogenised and air-dried soil samples are sieved and added to the test chamber and brought to a given moisture content. Ten acclimatized individuals are added to each vessel containing 500 g dry weight of the selected soil. Growth effects and mortality are determined after four weeks and effects on reproduction are assessed after eight weeks of exposure. The assay has been used to measure the effects of a wide range of chemicals such as metals [28] and pesticides [29]. In addition use of a suitable control soil is essential. This test is standardized at the international level, being recognized and promoted by international organizations (OECD—Organization of Economical Cooperation and Development, and ISO—International Organization of Standardization), aiming to elaborate international guidelines on environment quality assessment.

3. Effects on Growth

A number of studies have been conducted on the standard worm *Eisenia fetida/andrei.* Some of the responses of earthworms to sublethal concentrations of pesticides is shown in Table 1. Zhou et al. [30] have reported that the weight of the earthworms was a more sensitive index compared to the mortality in indicating toxic effects of acetochlor and methamidophos. Espinoza-Navarro and Bustos-Obregón [31] treated *Eisenia fetida* with organophosphate insecticide malathion and Bustos-Obregón and Goicochea [3] explored the effect of exposure to commercial parathion on *Eisenia fetida;* both observed decrease in the body weight of treated worms . Weight loss has also been reported for organochlorine pesticides intoxication [18, 32, 33] and for the effects of fungicides and herbicides in *Eisenia fetida* and *Lumbricus terrestris* [34–36]. Choo and Baker [37] found endosulfan did significantly reduce the weight of juvenile *Aporrectodea trapezoides* within 5 weeks when applied to soil at normal application rate in both the field and laboratory while fenamiphos did so at normal application rate in the field only. Both fenamiphos and methiocarb reduced earthworm weight in the laboratory when applied at 10× normal rate. Weight loss appears to be a valuable indicator of physiological stress, related to the degree of intoxication and time of exposure [22, 38]. Coiling, another symptom seen in 100% of the Parathion treated worms, is related with weight loss and is regarded as the consequence of alteration in muscular function elicited by organophosphoric pesticides which may explain the difficulties for locomotion of the intoxicated worms and their relative inability to feed themselves [3].

Negative impact of pesticides on earthworm growth has been reported by various researchers. Xiao et al. [39] suggested that growth can be regarded as sensitive parameters to evaluate the toxicity of acetochlor on earthworms. Helling et al. [36] tested in laboratory the effect of copper oxychloride, while Yasmin and D'Souza [40] investigated the impact of carbendazim, glyphosate and dimethoate on *Eisenia fetida* and found a significant reduction in the earthworm growth in a dose-dependent manner. According to Van Gestel et al. [27] parathion affects the growth of *Eisenia andrei*. Booth et al. [41] studied the effect of two organophosphates, chlorpyrifos and diazinon, while Mosleh et al. [42] investigated the toxicity of aldicarb, cypermethrin, profenofos, chlorfluazuron, atrazine, and metalaxyl in the earthworm *Aporrectodea caliginosa* and observed a reduction in growth rate in all pesticide-treated worms. Mosleh et al. [43, 44] studied the effects of endosulfan and aldicarb on *Lumbricus terrestris* and have suggested growth rate as important biomarkers for contamination by endosulfan and aldicarb. Zhou et al. [45] assessed and found chlorpyrifos had adverse effect on growth in earthworm exposed to 5 mg/kg chlorpyrifos after eight weeks. Some studies have shown that growth of earthworms appeared to be more severely affected at juvenile stage than at adult stage [46, 47].

4. Effects on Reproduction

Numerous reproductive parameters have been studied in earthworms exposed to various xenobiotics: cocoon and hatchling production, viability of the worms produced [18, 20, 48–54], and sexual maturation [50]. Cocoon production was found to be the most sensitive parameter for paraquat, fentin, benomyl, phenmedipham, carbaryl, copper oxychloride, dieldrin [36, 55–57], while cocoon hatchability was most sensitive for pentachlorophenol, parathion and carbendazim, copper oxychloride [36, 55, 56]. Bustos-Obregón and Goicochea [3] explored the effect of exposure to commercial parathion on the reproductive parameters such as sperm and cocoon production and genotoxicity on male germ cells of *Eisenia fetida* and reported that alterations in reproductive parameters were conspicuous in regard to the number of sperm, cocoons, and worms born. Numbers of juveniles per cocoon can be regarded as sensitive parameters to evaluate the toxicity of acetochlor on earthworms as reported by Xiao et al. [39]. Choo and Baker [37] also found that cocoon production in *Aporrectodea trapezoides* was inhibited by endosulfan and fenamiphos at normal application rates and methiocarb at 10× normal rate.

Table 1: Laboratory experiments on responses of earthworm to sublethal concentrations of pesticides.

Pesticide	Concentration of pesticide/exposure	Test conditions	Species	Responses	Reference
Copper oxychloride (pure)	8.92, 15.92, 39.47, 108.72, 346.85 mg Cu/ kg substrate 56 days	Substrate = Dried, ground, finely sieved cattle manure pH = 7.1 ± 0.2–6.1 ± 0.3 Moisture = 77.6 ± 0.7–78.8 ± 1.1 Temp = 25°C	*Eisenia fetida* (Freshly hatched earthworms)	Earthworm growth and cocoon production were significantly reduced	[36]
Malathion (pure)	80.150, 300, 600 mg/kg soil 1, 5, 15, 30 days	Soil like substrate pH = 6.5, Temp = 21-22°C, Moisture = 50%,	*Eisenia fetida* (Adults)	Significant reduction in body weight decreased spermatic viability	[31]
Acetochlor	5, 10, 20, 40 and 80 mg/kg soil 7, 15, 30, 45 and 60 days (growth) 28 days (reproduction)	OECD artificial soil pH = 6.5, Moisture = 50% Temp = 20 ± 1°C	*Eisenia fetida* (Adults)	At higher concentrations of acetochlor (20–80 mg/kg growth and numbers of juveniles per cocoon were affected significantly	[39]
Chlorpyrifos (pure)	5, 20, 40, 60, 80 mg/kg soil 4 and 8 weeks	OECD artificial soil pH = 6.0 ± 0.5. Moisture = 50% Temp = 20°C	*Eisenia fetida* (Adults)	Adverse impact on growth and reproduction	[45]
Cypermethrin (pure)	5, 10, 20, 40, 60 mg/kg soil 4 and 8 weeks	OECD artificial soil pH = 6.0 ± 0.5, Moisture = 50% Temp = 20°C	*Eisenia fetida* (Juveniles)	Significant reduction in cocoon production Juveniles more sensitive than adults	[47]
Benomyl (pure)	0.32, 1.0, 3.2, 10, 32 mg/kg soil 56 days	OECD artificial soil Temp = 20 ± 2°C pH = 6.1 Moisture = 56% LUFA 2.2 pH = 6.1 Temp = 20 ± 2°C Moisture = 50% Tropical artificial soil Temp = 28 ± 2°C pH = 6.6 Moisture = 47% Tropical natural soil, Brazil pH = 3.9 Temp = 28 ± 2°C Moisture = 40%	*Eisenia fetida* (Adults)	Toxicity of benomyl was lower in tropical than temperate artificial soils No reproduction in tropical natural soil due to low pH	[24]

Table 1: Continued.

Pesticide	Concentration of pesticide/exposure	Test conditions	Species	Responses	Reference
Chlorpyrifos (pure) Carbofuran (pure) carbendazim, formulated as Derosal (AgrEvo, 360 g/L)	1, 3, 10, 30, 100, 300, 900 mg/kg soil 0.5, 1, 2, 4, 8, 16, 32 mg/kg soil 0.1, 0.3, 1, 3, 10, 30, 90 mg/kg soil 28 days, 56 days	OECD artificial soil Moisture = 50% Temp = 20 ± 2∘C pH = 6 LUFA 2.2 soil Moisture = 50% Temp= 20 ± 2∘C pH = 5.9–6.1 OECD artificial soil Moisture = 50% Temp = 26 ± 2°C pH = 6 Natural soil Moisture = 45% Temp = 26 ± 2°C pH = 6.2	*Eisenia Andrei* (Adults)	Toxicity of chlorpyrifos and carbofuran on growth and reproduction in artificial soil was higher at 26°C. in the natural soil carbendazim toxicity was lower at 26°C in both the soil types	[48]
Carbaryl (pure) Dieldrin (pure)	0, 25, 50, 100, 150, 200, 250, 500 mg/kg soil 0, 25, 50, 100, 150, 200, 250, 500 mg/kg soil 4, 6, 8 weeks	Horse manure, sand and deionized water Moisture = 75 ± 5% Temp = 25°C	*Eisenia fetida* (Juveniles)	Inhibition of growth and cocoon production	[49]
Paraquat (pure), Parathion (pure) Fentin (pure) Benomyl (pure) Pentachlorophenol (pure) Carbendazim (formulated as Derosal 60%) Phenmedipham (formulated as Betanal 16.2%)	20, 45, 100, 200, 450, 1000 mg/kg soil 10, 18, 32, 56, 100, 180 mg/kg soil 0.32, 1, 3.2, 10, 32 mg/kg soil 0.1, 0.32, 1,3.2 mg/kg soil 5, 10, 20, 40, 60 mg/kg soil 0.6, 1.92, 6 mg/kg soil 1.62, 5.18, 16.2, 51.8, 162 mg/kg soil	OECD artificial soil pH = 6.0–7.3, Temp = 20 ± 5°C Moisture = 35%	*Eisenia Andrei* (Adults)	Reduction in growth rate reduction in number of juveniles produced per worm	[27]
Dieldrin (pure)	10, 30, 50, 100 mg/kg Every 15 days, 90 days	Washed cow manure Moisture = 60% Temp = 20°C	*Eisenia fetida* (Juveniles)	Growth was retarded even at agricultural doze of 5 kg/ha Clitellum development retarded, influencing reproduction	[50]
Diazinon (formulated as Basudin 600 EW), Chlorpyrifos (formulated as Lorsban 40 EC)	High = 60 mg/kg; Low = 12 mg/kg High = 28 mg/kg; Low = 4 mg/kg	Natural soil pH = 6.5–7, Temp = 20°C Moisture = 20–25%	*Aporrectodea caliginosa* (Adults and juveniles)	Significant effect on growth of juveniles and adults cocoon production significantly reduced	[46]

Table 1: Continued.

Pesticide	Concentration of pesticide/exposure	Test conditions	Species	Responses	Reference
Endosulfan formulated as END 35 (endosulfan concentration of 350 g/L) Aldicarb formulated as Temik 10 G	Different concentrations used; LC10 and LC25 for aldicarb; LC10, LC25, and LC50 for endosulfan 2,7 and 15 days	Natural soil Temp = 14 ± 1°C, R.H = 70–90% pH = 8.16	*Lumbricus terrestris* (Adults)	Loss in weight Reduction in the growth rate Aldicarb was more toxic than endosulfan	[44]
Aldicarb (formulated as aldicarb; granular mix, 10% active ingredient) Cypermethrin (formulated as Cypermethrin emulsifiable concentrate 5%) Profenofos (formulated as Curacron, 50% EC) Chlorfluazuron (formulated as Atabron emulsifiable concentrate 50%) Atrazine (formulated as Gesaprim®, 80% WP) Metalaxyl Mn-Zn (formulated as Ridomil, 72% WP)	Different concentrations used 1, 2, 3, and 4 weeks	Artificial soil Temp = 23 ± 1°C R.H = 70–90%	*Aporrectodea caliginosa* (Adults)	Reduction in growth rate chlorfluazuron, atrazine, and metalaxyl caused the highest reduction in worm growth rate.	[42]
Chlorpyrifos (pure) Chlorpyrifos formulated as Judo 40 EC Carbofuran (pure) Carbofuran formulated as Curater (3% a.i.G) Mancozeb (pure)	1, 3, 10, 30, 100, 300 and 900 mg/kg soil 0.5, 1, 2, 4, 8, 16 and 32 mg/kg soil 1, 3, 10, 30, 100, 300, 900 and 1200 mg/kg soil	OECD artificial soil Moisture = 50% Temp = 26 ± 2°C pH = 6.5–6.8	*Perionyx excavates* (Adults)	Toxicity decreased in the order of carbofuran > chlorpyrifos > mancozeb for both the pure compounds and the formulations Chlorpyrifos, carbofuran and mancozeb are more toxic to P. excavatus than to the standard test species E. andrei at temperatures representative of tropical conditions Formulated compounds depressed earthworm reproduction more than the pure compounds.	[51]

Espinoza-Navarro and Bustos-Obregón [31] treated *Eisenia fetida* with organophosphate insecticide malathion and found that malathion decreased the spermatic viability in spermatheca, altering the cell proliferation and modifying the DNA structure of spermatogonia. Sperm count also seems to be a very sensitive marker [42, 50], malathion could affect the sperm count, but in addition, its metabolites could affect sperm quality [58].

Several scientists have reported that pesticides influence the reproduction (cocoon production, a reduced mean and maximum number of hatchlings per cocoon, and a longer incubation time) of worms in a dose-dependent manner, with greater impact at higher concentration of chemical [35, 40, 41, 56]. Gupta and Saxena [56] studied the effects of carbaryl, an N-methyl carbamate insecticide, on the reproductive profiles of the earthworm, *Metaphire posthuma* and found sperm head abnormalities even at the lowest test concentration of 0.125 mg/kg. Wavy head abnormalities were observed at 0.125 mg/kg carbaryl, whereas at 0.25 mg/kg and 0.5 mg/kg, the sperm heads became amorphous and the head nucleus was turned into granules deposited within the wavy head.

Xiao et al. [39] showed that acetochlor had no long-term effect on the reproduction of *Eisenia fetida* at field dose (5–10 mg/kg^{-1}). At higher concentrations, acetochlor (20–80 mg/kg) revealed sublethal toxicity to *Eisenia fetida*. Zhou et al. [45] assessed and found chlorpyrifos had adverse effect on fecundity in earthworm exposed to 5 mg/kg chlorpyrifos after eight weeks. According to Zhou et al. [47] reproduction of earthworms appeared to be more severely affected by cypermethrin at juvenile stage than at adult stage. Application of 20 mg/kg, cypermethrin caused significant toxic effects in reproduction of worms.

Coiling, seen in the parathion treated worms, interferes with the reproduction too since worms find their partner less easily and copulation is abnormal in terms of mating posture. Ejection of sperm seems also to be hindered and therefore a large number of spermatozoa are found in intoxicated worms in spite of a clear effect on sperm production under parathion treatment as discussed by Bustos-Obregón and Goicochea [3]. According to Espinoza-Navarro and Bustos-Obregon [58] malathion also has a direct cytotoxic effect causing coiling of the tail, with increase of metachromasia of the chromatin of the spermatozoa and altering the sperm count

5. Confounding Variables

The results of earthworm ecotoxicological tests may be confounded with different properties of soils such as organic matter, water holding capacity, pH, cation exchange capacity, Carbon/Nitrogen ratio, and clay content and its interaction with chemical substances and different species of earthworm chosen as test species [23]. Soil pH may affect the survival of adults and thus production of juveniles [23, 59]. Low reproduction of earthworm was seen in finely sieved soil as compared to sandy soil [23] indicating that porosity of soil may influence earthworm mobility and gaseous exchange, thus affecting its life cycle. Further, the effects of a pesticide can differ strongly when tested under tropical and temperate conditions [24]. This may be because the physicochemical variables affecting the biotic processes as well as the fate of pesticides in the tropics are different from those in temperate regions [60, 61]. The high temperature and humidity, found in the tropics, seem to favor degradation and volatilization of the chemical in the soil [62, 63]. On the other hand, humid and warmer conditions might enhance the toxicity of some pesticides by increasing the penetration through the skin of animals, and these might be taken up more quickly by tropical biota [64].

Furthermore, information on the side effects of pesticides in the tropics is scarce [65] and a risk assessment based on temperate data could be less appropriate for tropical conditions. Some of the studies have been conducted in this direction, for example, Garcia [66] attempted to compare the toxicity of selected pesticides on different strains of *Eisenia fetida* in temperate and tropical conditions, whereas, Helling et al. [36], Römbke et al. [24], and Garcia et al. [67] applied standardized protocols to determine pesticide effects to soil invertebrates under tropical conditions. De Silva et al. [48] found that sublethal effects (reproduction and growth) varied inconsistently with temperature and soil types. All these researchers suggested that toxicity of pesticides in tropics cannot be predicted from data generated under temperate conditions, even within the same species [48]. Furthermore, it is suggested that tropical risk assessment may be more realistic when conducted on ecologically relevant earthworm species, rather than standard *Eisenia* sp [51]. De Silva [68] suggests that *Eisenia* being temperate compost worms is less ecologically relevant and *Perionyx excavatus* may be used as standard test species for tropical soils.

An important aim in earthworm ecotoxicology is to be able to predict the effects of harmful chemicals in the field on the basis of laboratory experiments. Holmstrup [69] estimated the *in situ* cocoon production in grassland of two earthworm species, *Aporrectodea longa* and *Aporrectodea rosea,* in relation to application dose of benomyl. The results obtained in this field study were compared with results from laboratory reproduction tests with other earthworm species. There was good agreement between effects of benomyl on reproduction in the laboratory and in the field. These results therefore suggest that standardized laboratory tests provide a reasonable prediction of the effect in the field. However, according to Van Gestel [55], results of field studies on the earthworm toxicity of pesticides are in agreement with those of laboratory studies when a homogeneous distribution of the pesticide dosage over the top 2.5-cm soil layer is chosen as a starting point. In field situations, earthworm exposure is strongly dependent on the degree of deposition of pesticides on the soil surface, on the behavior of the pesticide in the soil, and on the vertical distribution of earthworms in the soil. The soil ecosystem is very complex, where interaction occurs between abiotic and biotic factors. Therefore, extrapolation of effects of pesticides observed in laboratory studies to effects in the field studies may be impeded by various environmental variables (especially the soil characteristics and weather conditions) influencing

exposure of earthworms to chemical [15]. Neuhauser and Callahan [49] suggested that more consideration should be given to evaluation of sublethal effects under field conditions. Ecotoxicological studies on soil fauna in laboratories usually involve single or a few species. For proper environmental risk assessment, three tiered studies should be conducted [70], that is (1) basic laboratory tests (mainly acute); (2) extended laboratory tests (mainly chronic); (3) tests using microcosms (model ecosystem tests) or even field tests. Although, the highest tier is most important for an ecotoxicological risk assessment, it is rarely performed due to its high complexity, costs and time needed [71]. De Silva [68] also indicates that linking of laboratory data to field may be possible and successful, but more research is required (especially w.r.t tropical conditions) on this aspect to state conclusively [15, 55, 69, 72, 73].

In conclusion, growth and reproductive parameters of earthworms exposed to agropesticides seem to be useful bioindicators of soil pollution. Such studies are simple to do and do not require great technical expertise. However, the studies conducted so far have focused on a few species of earthworms. Additional studies with different species of earthworm, including different endpoints, temperature regimes and soil types, are required. Research should be extended to ecologically relevant species of earthworms, as stated earlier [51], and also to other soil fauna to get a comprehensive knowledge on the malfunction in the soil biological processes due to pesticide pollution. All of the above-mentioned studies indicate negative impact of pesticides on earthworm growth and reproduction. Some studies also indicate that microorganisms in the soil help degrade the chemicals [74, 75]. So, there is a need to acquire more knowledge on the chemical nature, mode of action, and means of degradation of pesticides in soil, so that harm caused to soil fauna as well as to organisms higher up in the food chain can be minimized.

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Research Article

Effect of Butachlor Herbicide on Earthworm *Eisenia fetida*—Its Histological Perspicuity

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With the advent of the Green Revolution, there has been a quantum leap in the use of synthetic herbicides and pesticides throughout the world to sustain high yielding crop varieties. Continuous use of these synthetic chemicals leads to loss of soil fertility and soil organisms. To explore the effect of exposure to commercial herbicide (Butachlor) on the life history parameters (biomass, clitellum development, and cocoon production) and the histological changes in the earthworm *Eisenia fetida* over 60 days, the dried cow dung was contaminated with 0.2575 mg kg^{-1}, 0.5150 mg kg^{-1}, and 2.5750 mg kg^{-1} of butachlor based on the LC_{50} value, and a control was maintained. The mean earthworm biomass was found to be decreased with increasing herbicide concentration. Similarly, cocoon production was also reduced by the increasing herbicide concentration. A possible explanation is an increased demand for energy, needed for the regulation and detoxification of herbicide. All earthworms in the exposed group were found to have glandular cell enlargement and to be vacuolated.

1. Introduction

Earthworms are often used as test organisms because of their important function, for example, as decomposer [1] and their sensitive reactions towards environmental influences. Earthworms are ecologically very important because there may be a risk secondary poisoning through feeding on worms contaminated by toxic substances. This could occur whether or not the worms themselves suffer any adverse effects. However, for most chemicals doses, which are toxic to birds or mammals and toxic to worms too, in such poisoning incidents, dead worms are found with affected predators [2, 3]. For that reason, earthworms are regarded as a reference compartment to observe soil contaminant bioavailability [4]. They are used to evaluate the lethal and sublethal effects of chemical contaminants and pollutants. Therefore they are useful to assess the contaminant fractions which may act on all organisms getting in touch with soil. Earthworms have been shown to be affected by the fate of herbicides in soil. Earthworms directly influence the persistence of herbicides in soil by metabolizing a parent compound in their gut [5, 6], by transporting herbicides to depth and increasing the soil bound (non extractable herbicides) fraction in soil or by absorbing herbicide residues in their tissues. The OECD earthworm toxicity test number 207 is widely used for terrestrial ecotoxicological test which is applied both in prospective and increasingly in retrospective ecotoxicological research. The present work reveals clearly that sublethal effect of herbicide butachlor on earthworm *Eisenia fetida* that provide fresh impetus concerning the environmental biology of its histology sheds light on the damage done to the intestinal region of earthworm.

2. Materials and Methods

2.1. Chemical. A commercially available Butachlor (2-Chloro-2′, 6′-diethyl-N-(butoxymethyl) acetanilide) (label content Butachlor) was used. Butachlor is used for the control of undesirable grasses and broadleaf weeds in transplanted direct seeded paddy and barley fields. It is a member of the chloroacetanilide class of chemistry.

2.2. Acute Toxicity Test. Tests were conducted using laboratory-bred adult earthworms of *Eisenia fetida*. The LC_{50} value of a commercially formulated butachlor was determined using the method recommended by OECD guideline

Table 1: Influence of different concentrations of herbicide on the growth of *E. fetida* over 60 days.

Days	Control	0.2575 mg kg^{-1}	0.5150 mg kg^{-1}	2.5750 mg kg^{-1}
1st day	0.020 ± 0.0003	0.019 ± 0.0004	0.0193 ± 0.0002	0.019 ± 0.0004
15th day	0.094 ± 0.0026	0.080 ± 0.0035	0.097 ± 0.0050	0.094 ± 0.0039
30th day	0.324 ± 0.0141	0.314 ± 0.0234	0.357 ± 0.0453	0.339 ± 0.0284
45th day	0.318 ± 0.0212	0.349 ± 0.0267	0.339 ± 0.0269	0.265 ± 0.0164
60th day	0.365 ± 0.0194	0.3534 ± 0.0278	0.3626 ± 0.0404	0.342 ± 0.0203

Mean ± SE; Significant at $P < .001$; Tukey test confirms Significant at $P < .05$ between days and treatments.

number 207 [7]. Different amounts of test substances had been mixed homogenously. The selected earthworm species for toxicity test were exposed to different concentrations of herbicides (equivalent to 100 g dry weight) by amendment method for 96 hours. Each concentration level was tested where five replicates in lieu of level was tested with five replicates. Finney's [8] probit method using graphical analysis was followed to calculate the LC_{50} value. In this study, the LC_{50} value was 0.515 mg kg^{-1} dry weight of medium.

2.3. Sublethal Toxicity Test. The substrate used was urine-free cattle manure that was sun dried, ground and sieved to a particle size of 500–1000 μm. Butachlor was diluted in solvent and mixed into the substrate to give moisture content of 75%. Prepared substrate was left for 24 hours to evaporate the excess solvents mixed in test chemicals. One group served as control, and three groups were exposed to a concentration of 0.2575 mg kg^{-1}, 0.5150 mg kg^{-1}, and 2.5750 mg kg^{-1}, respectively. Three replicates of each concentration, each vessel containing ten animals, were set up, and ten-day-old earthworms were inoculated. Biomass was determined over 60 days by removing the worms from substrate, washing them with distilled water and drying them on paper towels. They were then weighed fortnightly in a preweighed water-filled boats. This was done to prevent the worms from drying out and dying.

2.4. Clitellum Development and Cocoon Production. Worms were observed closely every two days starting from four weeks after they hatched. Worms were classified as juvenile, preclitellate, and clitellate using the rather subjective criterium clitellum and absence, partial development of the clitellum, and the presence of a fully developed clitellum. The culture medium of troughs was thoroughly searched for cocoons every second day, starting from 35th day. Cocoons were transferred to multicell containers to be incubated in distilled water. The containers were kept in the dark place and the water contents were replaced weekly to prevent bacterial growth.

2.5. Histological Study. After completion of life cycle, earthworms from each concentration were taken and washed with distilled water after which 50 mL jars were filled with 30 mL of 1.5% agar gel prepared with deionized water. After getting cooled and solidified, this gel in the jars was taken out and cut into small pieces. The earthworms were transferred separately into jars containing agar pieces and kept for 96 hours to remove all the soil from their gut. After removing the gut contents, earthworms were narcotized and cut into pieces and transferred to Zenkar's fixative for 12 hours and washed with running tap water for 12–24 hours. The worm samples were mounted weekly and stained with Iron Haemtoxylin stain for histological observation.

3. Results and Discussion

3.1. Influence of Herbicide on the Growth of E. fetida. The worms gained weight up to the last day of the experiment (see Table 1). The earthworm (15th day) in the control group had a mean biomass of 0.094 ± 0.0026 g and those in the exposure group 0.3534 ± 0.0278 g in 0.2575 mg kg^{-1}, 0.3626 ± 0.0404 g in 0.5150 mg kg^{-1}, and 0.342 ± 0.0203 g in 2.5750 mg kg^{-1}. On the termination of experiment, earthworms (60 days old) in the control group had mean biomass of 0.365 ± 0.0194 g. But in the exposure group it was 0.3534 ± 0.0278 g in 0.2575 mg kg^{-1}, 0.3626 ± 0.0404 g in 0.5150 mg kg^{-1}, and 0.342 ± 0.0203 g in 2.5750 mg kg^{-1}; the difference was significant at $P < .001$ levels. Further, Tukey test confirms the significant deference between days and treatments. At the end of experiment there was no difference between the mean biomass of control group and the exposure group.

3.2. Influence of Herbicide on the Clitellum Development of E. fetida. The rate at which the worms attained maturity differed in *E. fetida* exposed to different concentrations of butachlor. The percentage of mature specimens (expressed in terms of clitellate specimens) is shown in Figure 1. All specimens of *E. fetida* started developing clitellum from 29th day and on 33rd day clitellum development completed. The percentage of clitellum development decreased with increasing concentration of butachlor. The maximum clitellum development was observed in control on 30th day (40%), 43.3% for 0.2575 mg kg^{-1} at 29th day, 33.3% for 0.5150 mg kg^{-1}, and 26.6% for 2.5750 mg kg^{-1} concentration. Analysis of Variance (ANOVA) shows that the percentage of clitellum development of *E. fetida* differed significantly at ($P < .01$) in between days and it was not significant between treatment.

3.3. Influence of Herbicide on the Cocoon Production of E. fetida. The mean number of *E. fetida*'s cocoon production in different concentrations of butachlor is listed in Table 2. The maximum number of cocoons laid by control worm was 79.66 ± 2.603 on the 65th day. The worms exposed

Table 2: Cumulative cocoon production of earthworm *E. fetida* in different concentrations of herbicide.

Days	Control	0.2575 mg kg^{-1}	0.5150 mg kg^{-1}	2.5750 mg kg^{-1}
45th	28.33 ± 1.763	26.00 ± 2.081	22.00 ± 1.154	18.00 ± 1.732
55th	51.00 ± 2.081	44.00 ± 1.000	41.66 ± 2.333	35.00 ± 3.055
60th	79.66 ± 2.603	73.00 ± 2.309	62.33 ± 1.763	48.33 ± 3.480

Mean ± SE; Significant at $P < .001$; Tukey test confirms Significant at $P < .05$ between days and treatments.

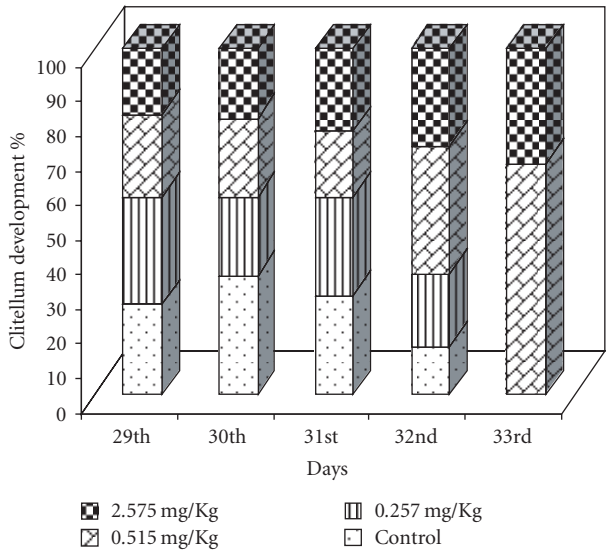

Figure 1: Percentage of clitellum development in *E. fetida* after exposure to different concentrations of herbicide.

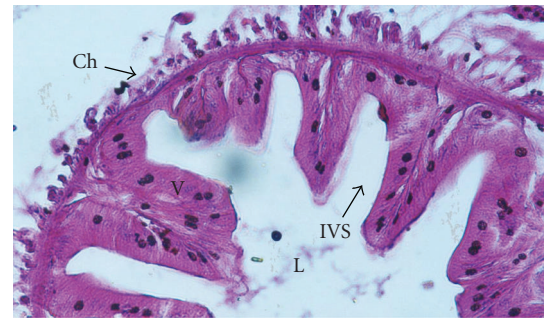

Figure 2: Cross section of earthworm *E. fetida* normal intestine and chloragogen tissue at control. L: Lumen; V: Villi; Ch: chloragogen tissue; IVS: Inter villious space.

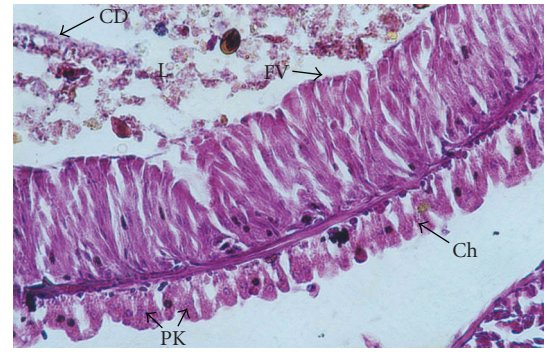

Figure 3: Cross section of earthworm *E. fetida* showing intestine and chloragogen tissue at 0.2575 mg kg^{-1} concentration of herbicide. PK: pyknotic nuclei; CD: Cellular Debris; L: Lumen; FV: Fused Villi; Ch: chloragogen tissue.

to 0.2575 mg kg^{-1} concentration exhibited the second peak of cocoon production over this period, which amounted to 73.00 ± 2.309 on 65th day. The minimum number of cocoon production was recorded in the 2.5750 mg kg^{-1} concentration (18.00 ± 1.732) on 45th day and maximum amount was 48.33 ± 3.480 on 65th day. The mean number of cocoon produced in the remaining concentration (0.5150 mg kg^{-1}) was found to be 62.33 ± 1.763 on 65th day. An ANOVA on the cocoon production shows significance ($P < .001$) between growth and treatment. Further, Tukey test confirms the significant ($P < .05$) difference between days and treatments.

3.4. Histopathological Changes on the Earthworm E. fetida. The intestine in control worm of *E. fetida* (Figure 2) consists of normal epithelial layer, the intermediate layer of longitudinal and circular muscle and blood vessels, and the other chloragogenous layer. Figure 3 revealed the histological changes of *E. fetida* at 2.5750 mg kg^{-1} concentration. The epithelial layer structure was grossly destroyed; fused and extra villous growth was pertained. Cell debris originated due to necrotic cell rupture and was found disseminated. Pyknotic nuclei had expressed. The chloragogen tissue was completely devastated with weak reserve inclusion.

In 0.5150 mg kg^{-1} concentration (Figure 4), the epithelial layer of villi was fused. A distinct cavitation was developed and pyknotic nuclei were observed in epithelial layer. The vaculoation could be seen inside the testis, testis sac, and vas deferens. The size of chloragogen tissue was reduced. However, 0.2575 mg kg^{-1} concentration (Figure 5) also shows the fused villous growth in the epithelial layer. There was no intervillous space between villi. In contrast, the pyknotic nuclei were found in many cells and cavitation was seen in chloragogen tissue layer.

4. Discussion

The biomass results of *E. fetida* revealed that earthworms had no inhibitory effect on the biomass. This result on growth was contradictory to that of Muthukaruppan et al. [9] who exposed *Perionyx sansibaricus* to the same herbicide butchlor and found that there was significant biomass difference. This is due to species sensitivity of earthworm.

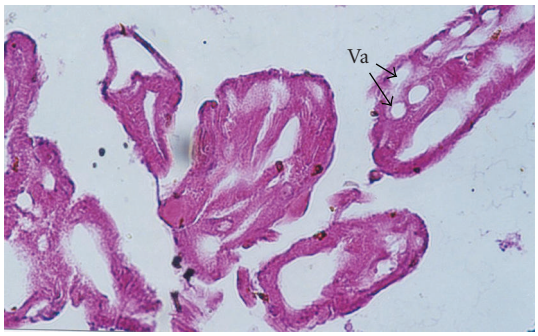

Figure 4: Cross section of earthworm *E. fetida* showing ovary at 0.5150 mg kg^{-1} concentration of herbicide. Va: Vacuoles.

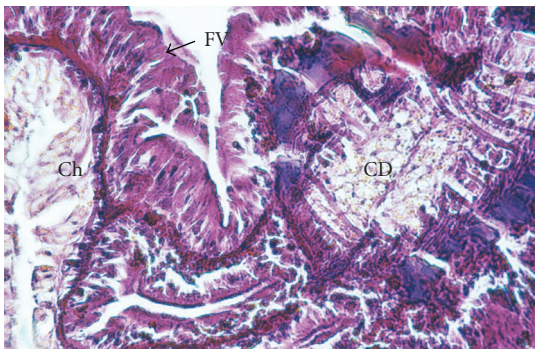

Figure 5: Cross section of earthworm *E. fetida* showing intestine and chloragogen tissue at 2.5750 mg kg^{-1} concentration of herbicide. Va: Vacuoles.

The maturation rate could not, therefore, be considered as a sensitive parameter to evaluate the effect of herbicide butachlor on this species.

In the present study, earthworm *E. fetida* produced cocoons which showed decreasing trend when the concentrations of butachlor were increased. Similarly [10] observed that the fungicide copper oxychloride reduced cocoon production with increased concentration of fungicide in *Eisenia fetida*. The present study confirms that the ability to resist a toxicant physiologically may be expensive interms of energy and other resources. This could involve a diminution of the ability to invest in other processes; for example, the energy available for reproduction is reduced. In the present study, epithelial tissue of earthworm *E. fetida*, exposed to butachlor, was severely affected. The present study confirms to the findings [11] that extreme nuclear swelling resulting in more than 2-fold volume increase of the average minimum could yet be observed only on the effect of sublethal paraquat toxication.

5. Conclusion

Earthworms are useful as test organisms to assess the toxicity of herbicidal contaminated soils, because of their sensitive changes occurred in biomass and cocoon production, and histological changes in tissues. The results clearly indicate that herbicide butachlor can retard growth and cocoon production and cause damage to epithelial tissue. The histological findings throw light on epithelial tissue damage especially fusion of intestinal villi that leads to the reduction of nutrient absorption area from food. Simultaneously, intoxication process consumes reserve energy from chloragogen tissue. This leads to reduced production of biomass and cocoon production The described methods and endpoints might help to understand the histopathological changes of earthworms towards herbicides and lead to an adapted test methodology. So histological-based end point provides more information about earthworm toxicology.

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Review Article

Earthworm Protease

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The alimentary tract of earthworm secretes a group of proteases with a relative wide substrate specificity. In 1983, six isozymes were isolated from earthworm with fibrinolytic activities and called fibriniolytic enzymes. So far, more isozymes have been found from different earthworm species such as *Lumbricus rubellus* and *Eisenia fetida*. For convenience, the proteases are named on the basis of the earthworm species and the protein function, for instance, *Eisenia fetida* protease (*Ef*P). The proteases have the abilities not only to hydrolyze fibrin and other protein, but also activate proenzymes such as plasminogen and prothrombin. In the light of recent studies, eight of the *Ef*Ps contain oligosaccharides chains which are thought to support the enzyme structure. Interestingly, *Ef*P-II has a broader substrate specificity presenting alkaline trypsin, chymotrypsin and elastase activities, but *Ef*P-III-1 has a stricter specificity. The protein crystal structures show the characteristics in their specificities. Earthworm proteases have been applied in several areas such as clinical treatment of clotting diseases, anti-tumor study, environmental protection and nutritional production. The current clinical utilizations and some potential new applications of the earthworm protease will be discussed in this paper.

1. Introduction

Earthworm has been recorded with a long history. Five hundred years ago, Shizhen Li compiled the famous medical book *Compendium of Material,* in which the earthworm (Earth dragon) was recorded as a drug prescribed for antipyretic and diuretic purposes in the form of dried powder in clinic. Now the remedy is still used in the folk.

In the end of 19th century, Frédéricq [1] discovered one enzyme secreted from the alimentary tract of earthworm. Then several proteases were separated from the earthworm in 1920 [2]. They could dissolve casein, gelatin, and albumin. This was the preliminary research about the earthworm proteases. Large-scale research about earthworm protease began in 1980. Mihara *et al.* [3] isolated a group of proteases with fibrinolytic activity from the earthworm *Lumbricus rubellus.* Subsequently different purification methods were applied to isolate the enzymes, including gel filtration, affinity chromatography, ion exchanging chromatography, and high-pressure liquid chromatography (HPLC). More proteases have been obtained from different species, such as lumbrokinase [4], earthworm-tissue plasminogen activator [5], earthworm plasminogen activator [6–11], component A of EFE (EFEa) [12, 13], and biologically active glycolipoprotein complex (G-90) [14–19].

2. Isozymes from Different Species

Earthworms are scientifically classified as animals belonging to the order Oligochaeta, class Chaetopoda, phylum Annelida. *Lumbricidae* is one of the main families in taxonomy [20]. Their native areas are in Europe, America, and Western Asia. *L. rubellus* (humus earthworm) and *E. fetida* (common brandling worm) can be raised and cultivated in large amount.

The earthworm proteases are multicomponent. Because of various living environments, different species of earthworms have different resultant isozymes. The proteases are independently studied in research groups, [3, 21, 22]. Thus, one isozyme may have multiple names. Here, we name the protease after the formal name of earthworm species and the protein function, for example, the protease from *L. rubellus* is called *L. rubellus* protease (*Lr*P).

Table 1: The molecule mass of the eight isozymes.

Isozyme	*Ef*P-0-1	*Ef*P-0-2	*Ef*P-I-1	*Ef*P-I-2	*Ef*P-II-1	*Ef*P-II-2	*Ef*P-III-1	*Ef*P-III-2
MW (kDa)	22.5	22.4	28.8	28.1	30.6	29.1	34.8	35.0

See Wu *et al.* [26].

2.1. Isozymes from Lumbricus rubellus. Six proteases (*Lr*P-I-0, *Lr*P-I-1, *Lr*P-I-2, *Lr*P-II, *Lr*P-III-1, and *Lr*P-III-2) of fibrinolytic enzymes were isolated from *L. rubellus* [3, 4, 23]. The molecular masses of the isozymes measured by ion-spray mass spectrometry are 23,013; 24,196; 24,220; 24,664; 29,667; and 29,662, respectively. They are single peptide chains having more asparagine and aspartic acid residues but less lysine. They have a wide functional acidic range (pH 1.0–11.0) and do not inactivate until 60°C. The enzyme activity (*Lr*P-II and *Lr*P-III-1) is maximally exhibited around pH 9.0 at 50°C [24].

In 2005, Nakajima and colleagues purified an enzyme that catalyzes the hydrolysis of triacylglycerol [25]. The N-terminal amino acid sequence and the catalytic function of the purified enzyme were identical to those of *Lr*P-II. The isozyme might act on the hydrolysis of triacylglycerol as well as the protein decomposition.

2.2. Isozymes from Eisenia fetida. In 1988, Zhou and coworkers [22] separated at least seven components with fibrinolytic activity from earthworm *E. fetida*. They are stable at pH 5.0–9.0 and denaturated below pH 2.6. After that, a plasminogen activator (e-PA) with two subunits was separated from *E. fetida* [10], similar to the results reported previously [6]. This enzyme is considered a serine protease and its molecule mass is 45,000 Da. The two constituting subunits (26,000 Da and 18,000 Da) with different fibrinolytic activities are bound by hydrophobic interaction. Wu and colleagues isolated eight fibrinolytic enzymes (*Ef*P-0-1, *Ef*P-0-2, *Ef*P-I-1, *Ef*P-I-2, *Ef*P-II-1, *Ef*P-II-2, *Ef*P-III-1, and *Ef*P-III-2) through a stepwise-purification procedure in 2007 [26]. They are all glycoproteins (Table 1). Two of them (*Ef*P-0-2 and *Ef*P-II-2) are new isozymes and the other six in their primary structures are similar to those purified by Mihara and coworkers.

In 2008, another serine protease was purified from the coelomic fluid of the earthworm *E. fetida* [27]. It has strong antiviral activities against cucumber mosaic virus and tomato mosaic virus. The protease (27,000 Da) is the most active at pH 9.5 and 40–50°C.

2.3. Isozymes from Lumbricus bimastus. Three proteins have been isolated from the extraction of earthworm *L. bimastus* by Xu and coworkers [28]. The apparent molecular masses of the proteins are about 30,000, 29,000, and 28,000 Da exhibited on SDS-PAGE, respectively. The fragment encodes a 242-amino-acid protein called PV_{242}.

2.4. Isozymes from Eisenia andrei. Lee and colleagues have isolated a protease fraction (SPP-501) from the earthworm *E. andrei* [29]. The antithrombotic activity has been investigated in a thrombosis model. SPP-501 shows both antithrombotic and fibrinolytic activities during oral administration.

Although several groups of isozymes have been studied in the species above, the total number is still not clear. The molecular weights of the proteases are in a relative narrow range (20–35 kDa) and they have activities in a wide pH scope.

3. Localization of the Protease in an Earthworm

*Ef*Ps are expressed and synthesized in the epithelial cells and mainly localized in the crop and gizzard, particularly in the anterior alimentary regions (Figures 1 and 2) [30]. In these regions, the proteases maybe contribute to digest protein and peptide in food.

4. Activity Assays

There are three methods to measure the activity of the isozymes: fibrin plate, chromophoric procedure, and light scattering (Table 2). Initially, fibrinolytic activity is measured by both plasminogen-rich and plasminogen-free fibrin plates [31, 32]. Individual earthworm is cut into pieces and placed on a plasminogen-rich fibrin plate. The fibrinolytic activity is determined by measuring the diameter of the plaque. Later, an assay using chromophoric substrate has been developed [24]. When the chromophoric substrate Chromozym TH reacts with the proteases, the absorbance at 405 nm will increase. The linear section from 15 to 30 seconds is used to calculate the activity of the protease. The unit is defined as the specific activity required converting 1 μM substrate/minutes/mg of enzyme.

Another method records the changes of the scattering intensity during the conversion of fibrinogen to fibrin [33]. Changes of the scattering intensity of the thrombin-fibrin colloid follow a sigmoid curve with a relaxation phase and the maximum at 480 nm. The intercept at the maximum slope is directly proportional to the concentration of thrombin when fibrinogen is at a constant concentration. Thus, the intercept is employed to calculate the thrombin activity. The earthworm protease blocks the increase in the light scattering intensity, because the enzyme hydrolyzes both fibrinogen and fibrin. The amount of the protease is inversely proportional to the intensity.

5. Substrates of the Earthworm Protease

The earthworm protease shows different activities in the presence of different substrates. *Lr*P-I (*Ef*P-I) is considered a chymotrypsin-like protease [4]. *Ef*P-II is capable of recognizing the six substrates N-α-benzoyl-L-Arginine ethyl

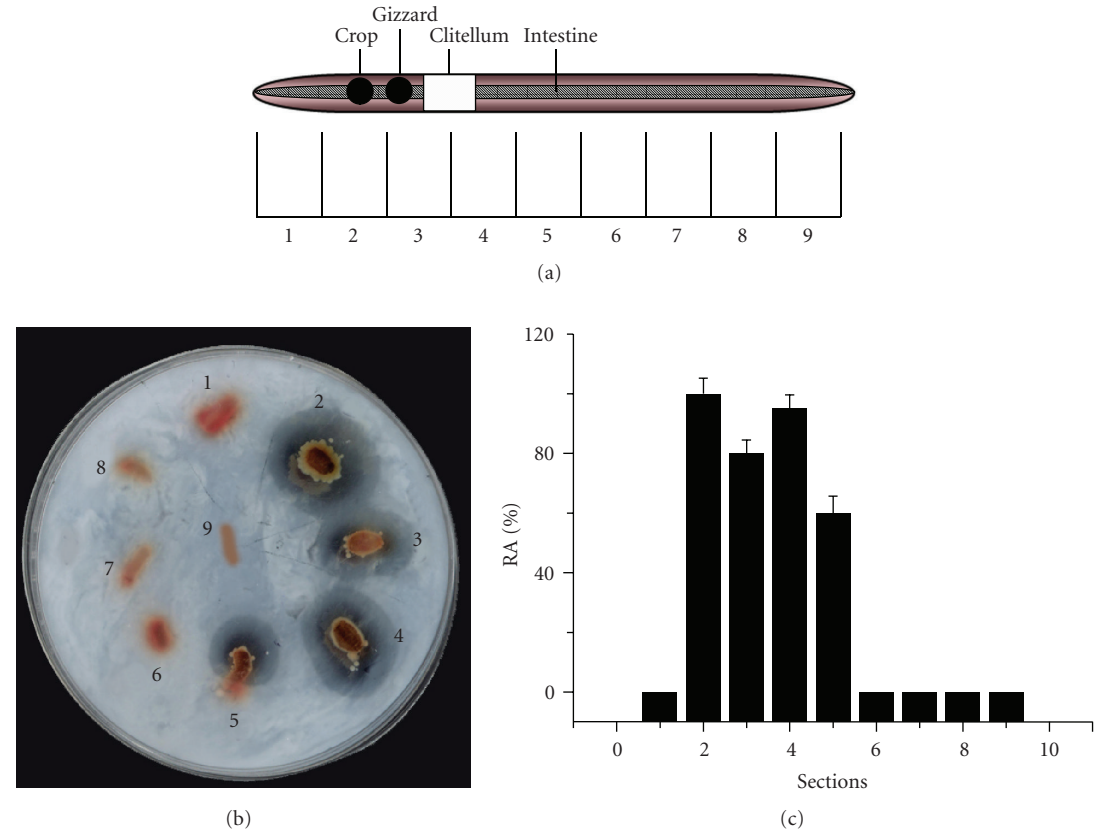

Figure 1: Localization and total relative activity of the earthworm segments: (a) nine pieces of the earthworm *E. fetida* (b) fibrin plate assay (c) enzymic activity of each segment (see [30]).

Table 2: Three assays of the earthworm protease.

Assays	Fibrin plate	Chromophoric procedure	Light scattering
Materials	Fibrinogen, thrombin, and agar	chromophoric substrates	Fibrinogen, thrombin
Principle	fibrinolytic activity	absorbance at 405 nm	Light scattering at 480 nm
Time	18 ~ 24 hours	~1 minute	~10 minutes
Feature	Fibrinolysis specificity, time consuming	Rapid, reproducible, lower specificity	Rapid, reproducible, lower specificity

ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), Chromozym TH (Ch-TH, Car-Val-Gly-Arg-4-NA), Chromozym TRY (Ch-TRY, Tos-Gly-Pro-Arg-4-NA), Chromozym U (Ch-U, Ben-β-Ala-Gly-Arg-4-NA), and Chromozy ELA (Ch-ELA, Suc-Ala-Ala-Ala- pNA) and gives relative K_m values as follows: $[K_{mTH}] < [K_{mU}] < [K_{mELA}] < [K_{mBAEE}] < [K_{mTRY}] < [K_{mATEE}]$. This sequence indicates that *Ef*P-II acts as a strong thrombin-like, moderate elastase-like, and weak chymotrypsin-like serine protease. On the other hand, *Ef*P-III-1 reacts with neither Ch-ELA nor ATEE, but reacts with BAEE, Ch-TRY, Ch-U, and Ch-TH, giving relative K_m values as follows: $[K_{mBAEE}] < [K_{mTRY}] < [K_{mU}] < [K_{mTH}]$, characteristic of a trypsin-like protease. Note that the K_m values for these substrates are approximately of the same order of magnitude (10^{-5} M), suggesting a higher substrate specificity for *Ef*P-III-1 than that for *Ef*P-II [34]. The earthworm proteases have the abilities to degrade and digest various substrates, which may make for their living in a vile environment.

6. Inhibitors of the Earthworm Protease

The activity of the earthworm protease is inhibited by several inhibitors [4, 9, 23, 35, 36]. Diisopropyl fluorophosphate (DFP) completely inhibits the activity of all the isozymes (pH 7.2) at room temperature. *Lr*P-I-0, *Lr*P-I-1, and *Lr*P-I-2 are partially inhibited while *Lr*P-III-1 and *Lr*P-II are strongly inhibited by SBTI and aprotinin. However, the activity of the protease is not significantly affected by tosyl-lysyl-chloromethylketose, Tosyl-phenylalanyl chloromethylketose, elastatinal, ε-amino caproic acid, EDTA, or various metal ions [23]. The specificity of substrates and inhibitors gives the evidence that the isozymes are alkaline serine-like proteases.

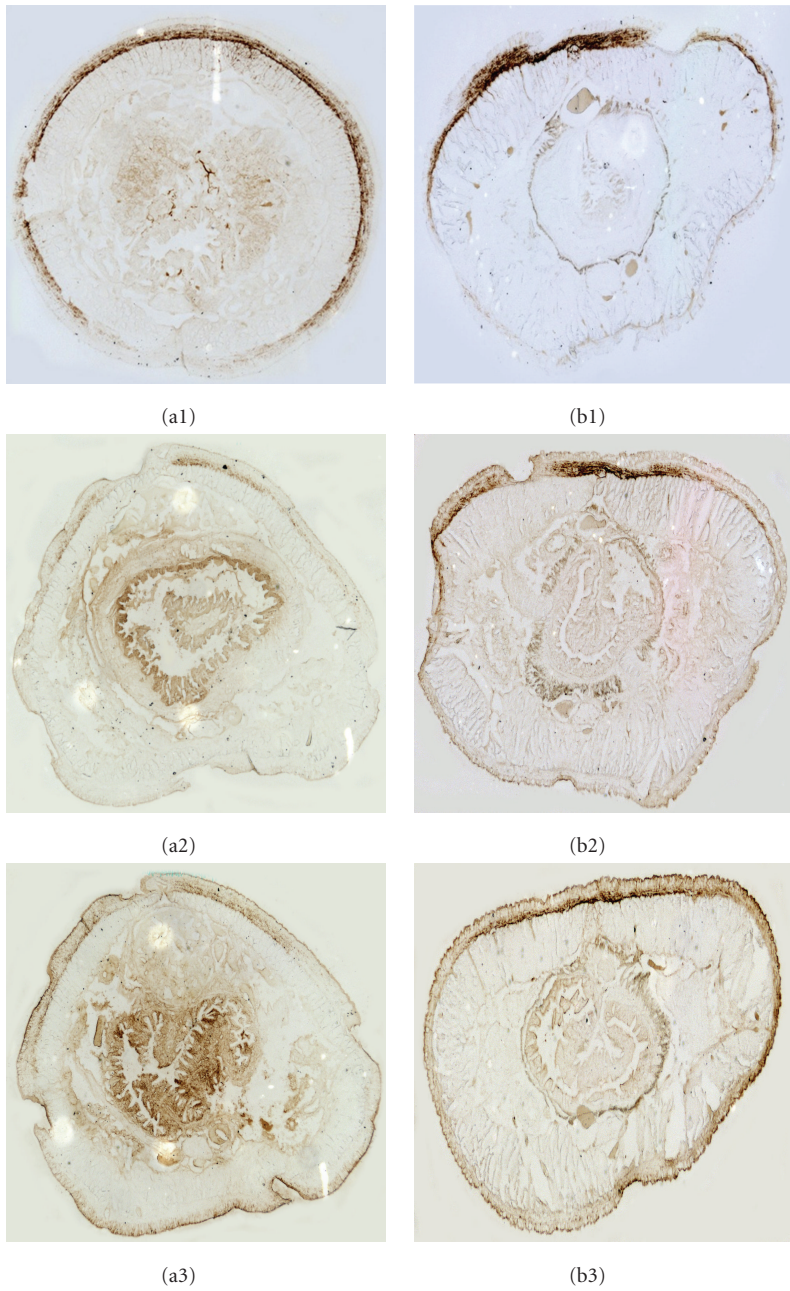

Figure 2: *In situ* localization of *Ef*P-II and *Ef*P-III-1 in the intestine of *Eisenia fetida* (a) segment 2 as shown in Figure 1. Anti-*Ef*P-II (panel a2) or anti-*Ef*P-III-1 (panel a3) as primary antibodies was added, without adding the primary antibodies as control (panel a1). (b) Segment 5 (see [30]).

α_2-Macroglobulin (α_2M), at a high concentration (approx. 2.0 μM) in blood plasma, is an important endogenous inhibitor with the ability to inhibit all four classes of (cysteine, serine, aspartate, and metallo) proteases [37, 38]. The fibrinolytic activity of *Lr*P-III-1 decreased to 65% when incubated with α_2M, while it decreases to 30% in the plasma under the same conditions [39]. After *Lr*P-III-1 goes into blood, the enzyme may be firstly inhibited by α_2M because the kinetics of inactivation of *Lr*P-III-1 with α_2M is similar to that of the initial phase with plasma. α_2M binds to the enzyme mole by mole equivalently and the interaction may undergo a chelate irreversible inhibition.

7. Protein Structural Features

7.1. Primary Structure. As shown in Table 3, the N-terminal sequences of the isozymes from *L. rubellus* and *E. fetida* have been analyzed [26]. The sequences of the isozymes from *L. rubellus* and *E. fetida* have a lot of identical residues.

Table 3: The N-terminal sequences of the isozyme.

*Ef*P	N-Terminal squence	*Lr*P	N-Terminal squence
EfP -0-1	VVGGSDTTIGQYPHQIL	*Lr*P-I-0	VVGGSDTTIGQYPHLSLRVT
*Ef*P -0-2	VVGGSDTTIGQ	—	—
*Ef*P-I-2	IIGGSNASPGEFPWQL	*Lr*P-I-1	IIGGSNASPGEFPWQLSQTRG
*Ef*P -I-2	IIGGSNASPGEFPWQL	*Lr*P-I-2	IIGGSNASPGEFPWQLSQTRG
*Ef*P -II-2	VIGGTNASPGEFPPQ	*Lr*P-II	VIGGTNASPGEIPWQLSQQRX
*Ef*P -II-2	VIGGTNASPGEFFP	—	—
*Ef*P -III-2	IVGGIEARPYEFP	*Lr*P-III-1	IVGGIEARPYEFPWQVSVRRK
*Ef*P -III-2	IVGXIEARPYEFPWQVSVRRK	*Lr*P-III-2	IVGGIEARPYEFPWQVSVRRK

See Wu *et al.* [26].

The N-terminal sequences of *Lr*P-III-1 and *Lr*P-III-2 are identical, and so are those of *Lr*P-I-1 and *Lr*P-I-2; *Ef*P-0-1 and *Ef*P-0-2; *Ef*P-I-1 and *Ef*P-I-2; *Ef*P-II-1 and *Ef*P-II-2; *Ef*P -III-1and *Ef*P -III-2, respectively. As shown in Figure 3, so far, they all are thought to belong to the serine protease family, which could be further divided into three groups according to the following sequences: earthworm protease-1 (*Ef*P-0), earthworm protease-2 (*Ef*P-I, EFEa, and *Ef*P-II), and earthworm protease-3 (*Ef*P-III-1, EFEb, *Lr*P-III-1, and *Lr*P-III-2), as shown in the phylogenetic tree.

The glycan chains play a role in the stability, the spatial conformation, and the antigenicity of the protein. Recent studies show that the earthworm proteases are glycosylated [26]. The result of staining on the native-SDS gel with Schiff's reagent shows that the eight isozymes isolated from *Eisenia fetida* by Wu and colleagues are all glycoproteins. In addition, the contents of sugar have been determined with sodium metaperiodate and glycoprotein-test reagent, shown in Table 4. The proteases have different glycan contents and the oligosaccharides are composed of mannose residues.

7.2. Secondary Structure. The secondary structures of *Lr*P-III-1 [40], *Lr*P-III-2 [41], EFEa [42], EFE-b [43], *Ef*P-0 [44], *Ef*P-I [45], *Ef*P-II [46], and *Ef*P-III-1 [47] are predicted on the basis of their primary structures. The proteins have distinct predicted secondary structures, for example, β-sheet, α-helix, turn, and coil, as shown in Table 5. The sequence of *Ef*P-II (EFEa) is highly similar to some related serine proteases with known structures [48–54] or other earthworm serine proteases [55] (Table 6).

7.3. Tertiary Structure. The catalytic characterization of the earthworm protease is influenced directly by their tertiary structures. The crystal structural study shows that *Ef*P-III-1 (EFE-b) is a trypsin-like protease with two chains (an N-terminal, pyroglutamated light chain and an N-glycosylated heavy chain) [56]. The structural features (Figure 4) probably endow *Ef*P-III-1 with high level of stability in resistance to heat, organic solvents, and proteases [57].

*Ef*P-II is not only a chymotrypsin-like serine protease but also has an essential S1 pocket of elastase (Figure 4). The S1 specificity pocket is preferable for elastase-specific small hydrophobic substrate, while its accommodation of long and/or bulky substrate is also feasible if enhanced binding of the substrate and induced fit of the S1 pocket are achieved. Compared with the stable active site of *Ef*P-III-1, that of *Ef*P-II (EFEa) is more flexible, resulting in a broader substrate specificity [13].

8. Fibrinolytic Mechanism and Cleavage Sites

The earthworm proteases have relatively broad substrate specificities, such as trypsin (cleaving the carboxylic sites of Arg and Lys) and chymotrypsin (cleaving the carboxylic sites of Phe, Trp, Tyr, and Leu) [34, 58]. Furthermore, *Ef*P-III-1 specifically recognizes the carboxylic sites of arginine and lysine. The protease cleaves the α chain of fibrinogen at R_{252}-G_{253}, R_{19}-V_{20}, and K_{429}-V_{430}, respectively. According to the densities of the protein bands on the SDS-PAGE, hydrolysis of α chain is the fastest, and hydrolysis of β chain is faster than that of γ chain. This indicates that *Ef*P-III-1 possesses strong α-fibrinogenase, moderate β-fibrinogenase, and weak γ-fibrinogenase activities. *Ef*P-III-1 activates plasminogen cleaving at R_{557}-I_{558}. This cleavage site is also recognized by tPA. Besides, *Ef*P-III-1 has Xa-like function. *Ef*P-III-1 recognized peptidyl bonds at R_3-A_4, R_{158}-S_{159}, R_{274}-T_{275}, R_{396}-N_{397}, and R_{287}-T_{288}. *Ef*P-III-1 cleaves prothrombin at R_{274}-T_{275}, thereby releasing the intermediates fragment 1.2 and prethrombin-2. As mentioned above, *Ef*P-III-1 cleaves at R_{287}-T_{288} and releases an α-thrombin-like product with a 13-residue deletion at the N-terminus of a chain. Similar to the preference for residue N_{397} by thrombin, which produces the β-thrombin-like fragments [59], *Ef*P-III-1 cleaves at residue R_{396}. That is to say, *Ef*P-III-1 has the ability to hydrolyze fibrinogen and to activate plasminogen and prothrombin, playing a part not only in fibrinogenolysis but also in fibrogenesis. Based on this, the roles of *Ef*P-III-1 in procoagulation and anticoagulation can be summarized as follows (Figure 5). The function in both activating prothrombin and catalyzing fibrinogenolysis suggests that *Ef*P-III-1 plays a role in the balance between procoagulation and anticoagulation.

9. Oral Administration

Usually the macromolecules cannot permeate the biological membranes. In particular, protein can be degraded by pepsin, trypsinase, and chymotrypsin. The gastric juice has a low pH value and denatures the ordinary proteins.

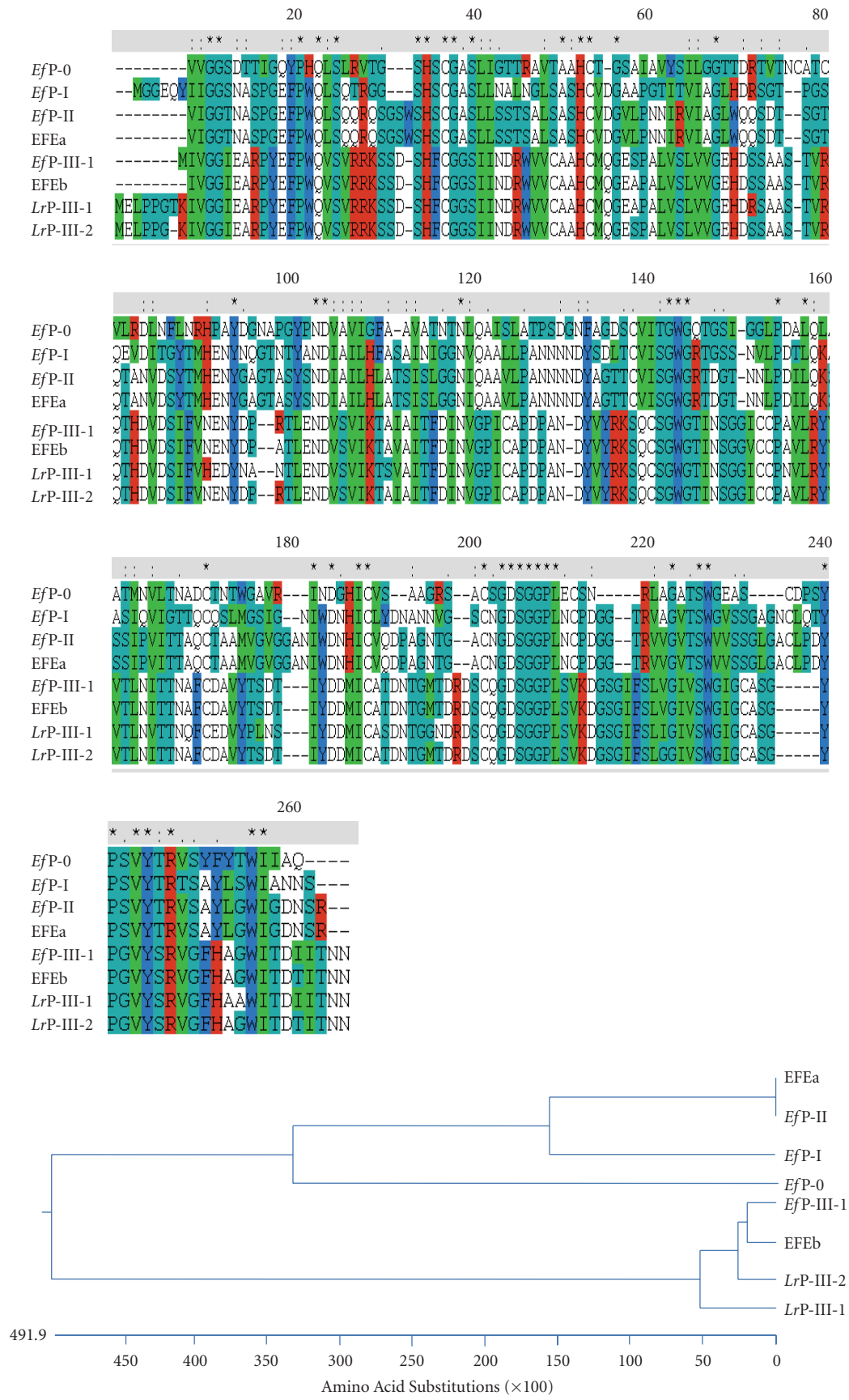

FIGURE 3: Multiple sequence alignment of some earthworm proteases. The amino acid sequences were from GenBank and PDB (see [40–47]).

Table 4: The Carbohydrate content of the eight isozymes.

Number	Earthworm protease	Carbohydrate content (%)[a]	Relative activity (%)[c]
1	*Ef*P-0-1	6.13	6.2
2	*Ef*P -0-2	4.30	12.8
3	*Ef*P-II-1	6.18	25.8
4	*Ef*P-I-2	1.38	31.6
5	*Ef*P-II-1	1.82	8.8
6	*Ef*P-II-2	7.40	2.1
7	*Ef*P-III-1	6.55	12.5
8	*Ef*P -III-2	4.41	2.3
9	Mixture of isozymes[b]	4.40	100

[a] Mass ratio between the carbohydrate content and the glycoprotein,
[b] Estimated on the fibrin-PAGE by Band-scanned software,
[c] *Ef*Ps purified by the SBTI-Sepharose-4B affinity chromatography column. See Wu *et al.* [26].

Table 5: Prediction of the secondary structures of the isozymes.

	*Lr*P-III-1	*Lr*P-III-2	EFEa	EFEb	*Ef*P-0	*Ef*P-I	*Ef*P-II	*Ef*P-III-1
Helix (%)	7	4	6	5	7	6	6	4
Sheet (%)	40	41	30	42	35	29	30	44
Turn (%)	15	14	18	13	12	19	17	14
Coil (%)	37	41	46	40	45	46	46	39

By Double Prediction Method (DPM), Antheprot software.

Table 6: Comparison of homologous sequences with some serine proteases.

Proteases	Identity (%)
Pocine pancreatic elastase (PPE)	36
Human leukocyte elastase (HLE)	28
Trypsin	35
Chymotrypsin	34
U(t)-PA, Plasmin	32
F-III-1(2)	35
Earthworm chymotrypsinogen	31
Lugworm chymotrypsinogen	44

See Tang *et al.* [13].

Whereas, some therapeutic proteins with specific properties can be absorbed with the intact and active form before being degraded in the alimentary tract, such as β-lactoglobulin, hepatitis-B surface antigen, bromelain, and epoxy-β-carotenes [60].

Furthermore, the earthworm protease could also be transported into blood through intestinal epithelium and perform its biological functions in the blood [61]. The *in vitro* experimental data show that 15% intact *Lr*P-III-1 is absorbed through intestinal epithelium. About 10% full-size enzyme is transported through the peritoneum after the intraperitoneal injection in the rat. The maximum activity in blood is detected around 60 minutes after the injection.

The N-terminal sequences of *Lr*P-III-1 and *Lr*P-III-2 are similar to protein transduction domain [62]. The sequences are rich in hydrophobic amino acid residues, which may play a role in the process of the membrane transportation of biological macromolecules.

10. Clinical Application and Medical Research

10.1. The Earthworm Protease as a Fibrinolytic Agent. The formation of thrombus in the blood causes many devastating diseases such as stroke and myocardial infarction. Several enzymes have been used as the thrombolytic agents including urokinase (UK), streptokinase, recombinant tissue-type plasminogen activator, staphylokinase, and recombinant prourokinase [63, 64]. These agents are administered via intravenous injection generally. Some of them are effective, but they also have some limitations such as fast clearance, lack of resistance to reocclusion, bleeding complications, and other adverse effects [63].

The earthworm protease functions in the fibrinolysis and plasminogen activation, distinct from those enzymes (UK, tissue-type plasminogen activator, etc.) [65–67]. Therefore they have been used to treat the thrombosis. The proteases during orally experiments both in animals and clinics show significant fibrinolytic efficacy. A distinct amelioration is observed in the treatment of blood high-viscosity syndrome and thrombocytosis [68]. In addition, the proteases are stable during a long-term storage at room temperature [69], in the form of oral capsule. Earthworm is easily raised, which renders the isozymes into a relatively inexpensive thrombolytic agent. So far, the earthworm proteases have been used as an orally administered fibrinolytic agent to prevent and treat clotting diseases, such as myocardial infarction and cerebral thrombus [70].

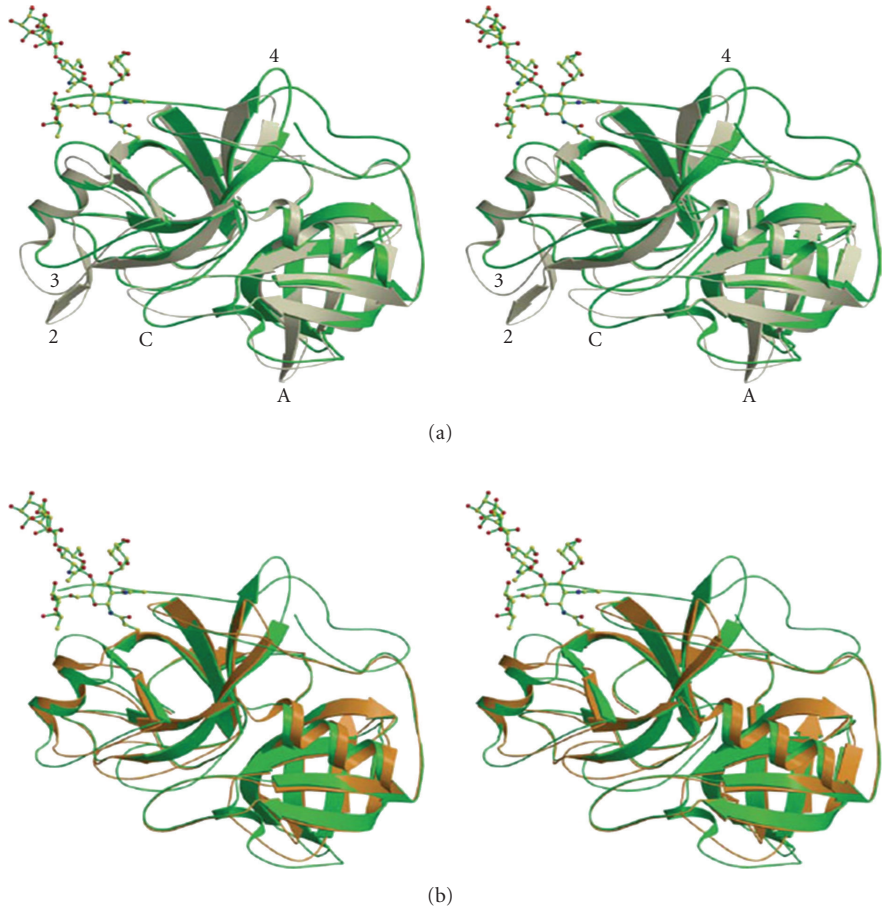

Figure 4: Superposition of EFE-b with EFE-a and trypsin. (a) Stereoview of the superposition of EFE-b with EFE-a is illustrated as follows: EFE-b is represented by green and EFE-a by light goldenrod yellow. The glycan is represented by a ball-and-stick model (carbon atoms, green). Some loops in which EFE-b greatly differs from EFE-a are indicated: loop A (34–41); loop C (97–103); loop 2 (217–225); loop 3 (169–174); loop 4 (201–208). (b) Stereoview of the superposition of EFE-b with trypsin is illustrated as follows: EFE-b is represented by green and trypsin (Protein Data Bank, accession number, 1PPE) by orange. The glycan is represented by a ball-and-stick model (carbon atoms, green) (see [56]).

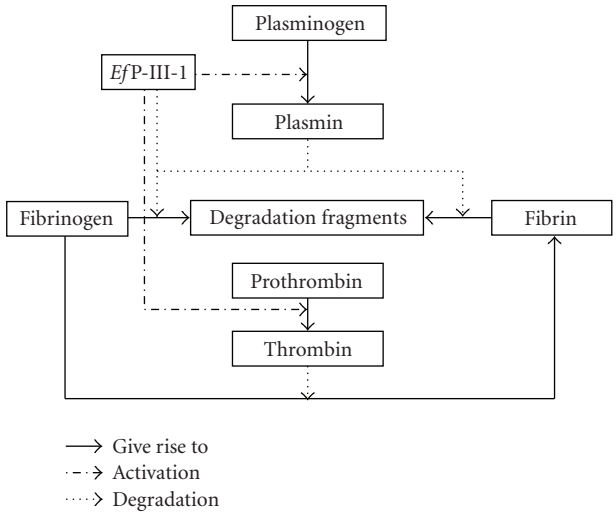

Figure 5: Roles of *Ef* P-III-1 in procoagulation and anticoagulation Activation is indicated by dashed lines, and degradation is indicated by dotted lines (see [34]).

10.2. Antitumor. Cancer has a reputation of being an incurable disease. Although some methods such as surgery, chemotherapy, radiation therapy, and immunotherapy are available, they are far from reaching the goal of complete removal of the cancer cells without damage to the rest of the body. It is demonstrated that the earthworm crude extract has the ability to kill the cancer cells directly in vitro [71, 72] and inhibit the occurrence and development of tumor in vivo [73]. Furthermore, it has been proved that the earthworm proteases enhance the curative effects by both radiation therapy and chemotherapy [74, 75].

The most malignant tumors secrete urokinase-type plasminogen activator (u-PA). In order to inhibit the hyperactivity of the u-PA, inhibitors of plasminogen activators are synthesized by the surrounding cells for tissue protection, resulting in a high concentration of fibrin locally. The glycolipoprotein mixture (G-90) was isolated from the homogenate of *E. fetida* [15–17, 19], which is assayed in a euglobulinic test applied to fibrin clot from blood plasma of patients who suffered from malignant tumors. The effect

of G-90 on the fibrinolysis rate is related to not only its concentration, but also to histological type where the malignant tumors invade. The blood with the fibrin clots derived from the dogs with cardiopathies and the dogs with malignant tumors was examined for the time of coagulation and fibrinolysis by adding different substances including G-90. The clotting time in the presence of G-90 shows dogs with malignant tumors > healthy dogs > dogs with cardiopathies [14].

Recently, a glycosylated component is separated from the earthworm *E. fetida* by Xie and coworkers [76], which has relations with apoptosis of tumor cells. It is highly homologous to *Lr*P-I-1 and *Lr*P-I-2. It is identified to be a plasmin and also a plasminogen activator. From the results of the phase-contrast microscopy observation of apoptotic cells and the localization of fluorescent antibodies in cell nucleus, the antitumor activity is observed.

The earthworm protease possesses obvious anti-tumor activity in the hepatoma cells. The proliferation of the hepatoma cell treated with the proteases is inhabited in proportion to the concentration of the proteases. The growth of tumor xenograft in nude mice is significantly suppressed after being fed with the earthworm protease for four weeks. At the same time, it has been found that the earthworm protease can induce apoptosis of hepatoma cells and downregulated the expression of matrix metal protease-2. As described above [77], the earthworm protease is a potential candidate for treating some kind of tumors.

10.3. Assistant to Implantation. After an artificial organ is introduced into a living body, small thrombus is usually formed on the surface of the graft. Many approaches have been tried to improve the blood compatibility to biomaterial. However, the results, so far, are not satisfactory. In 1994, *Lr*P was immobilized on the surface of polyurethane using maleic anhydride methylvinyl ether copolymer as an enzyme carrier [78]. So the *Lr*P-immobilized polyurethane surface has highly antithrombogenic activity and can reduce surface-induced thrombus. *Lr*P-immobilized surface may minimize platelet adhesion and activation by preventing fibrinogen from adsorption or by altering the conformation of adsorbed fibrinogen at an early stage of blood contact.

*Lr*P has been immobilized in a Korean-type total artificial heart valve by photoreaction, and polyallylamine is used as a photoreactive linker [79–81]. The proteolytic activity on the azocasein of the treated valves is three times higher than that of untreated valves. The *Lr*P-treated polyurethane valve leads to decreasing thrombus formation in vivo and their biocompatibility is, therefore, greater than that of untreated valve. This method may be developed and may be useful in clinical application.

10.4. Anti-Ischemia. Recently, the effect of the earthworm protease against myocardial ischemia [82] has been investigated on a rat model with acute myocardial infarction. Meanwhile the L-type calcium current (ICa-L) and intracellular calcium concentration ($[Ca^{2+}]i$) have been measured. The results indicate that it has protective actions on myocardial infarction in rats. Decreasing of the ICa-L and $[Ca^{2+}]i$ in ventricular myocytes is the possible mechanism.

The study has been conducted with 10 patients who had coronary artery disease and stable angina. Stress technetium-99 m sestamibi myocardial perfusion imaging has been performed before and at the end of the treatment period. As a result, the angina symptom is ameliorated in 6 out of 10 patients. No adverse reaction such as major or minor bleeding has been observed. That is to say, oral *Lr*P improves regional myocardial perfusion in patients with stable angina [83]. In this research, some expectable results have been achieved on patients with coronary artery disease and stable angina. It is in favor of better application of earthworm proteases.

The mechanism of the anti-ischemia function of *Lr*P in brain has been also studied. The results show that the anti-ischemic activity of *Lr*P was due to its antiplatelet activity by elevating cAMP level and attenuating the calcium release from calcium stores, the antithrombosis action due to inhibiting of ICAM-1 expression, and the antiapoptotic effect due to the activation of JAK1/STAT1 pathway [84].

11. Problems and Potential Solutions of the Earthworm Protease as a Medicine

Though the earthworm protease has good pharmaceutical effect in clinic application, some limitations still exist as a clinical fibrinolytic agent. It hydrolyzes not only the fibrinogen and fibrin but also some other proteins in vivo. Besides, the half-life of the earthworm protease is short in circulation. An ideal fibrinolytic medicine should meet the qualifications such as strong fibrinolytic activity, specificity on fibrinogen and fibrin, low immunogenicity, long half-life in vivo, low reocclusion rate, and reasonable cost [85]. In order to increase the bioavailability and strengthen the drug action, different methods are under trials.

11.1. Drug Delivery. Recently, some other ways of drug delivery have been studied. In Cheng's research, a water-soluble earthworm protease was used in the delivery of the water-in-oil (w/o) microemulsions. The w/o microemulsion comprises of Labrafac CC, Labrasol, Plurol Oleique CC 497, and saline (54/18/18/10% w/w). The characters of conductivity, viscosity, particle size, and in vitro membrane permeability have been studied. The intraduodenal bioavailability of the microemulsion group was 208 folds higher than that of control group. Meanwhile, no tissue damage of the intestinal mucosa has been found after oral multiple-dose administration of the protease microemulsion to rats. Therefore, the w/o microemulsion is a promising oral delivery system for hydrophilic bioactivity macromolecules [86].

Besides, the effect of some absorption enhancers on the intestinal absorption of the earthworm protease has been studied including chitosan, sodium deoxycholate, Na_2EDTA, sodium dedocyl sulfate, sodium caprylate, poloxamer, and HP-beta-CD. The enzyme can be transported into blood

and kept its biological activity across intestinal endothelial membrane after administration via duodenum site, whereas with lower bioavailability. Some of the absorption enhancers have effects on intestinal absorption in vitro and in situ experiments [87]. So the safety enhancer with few side effects is a good choice for drug manufacturing enterprise.

11.2. Parental Routes of Administration. The oral administration of the earthworm proteases has a relatively slow absorption process; hence, it is unsuitable to treat the emergency thrombus such as acute myocardial infarction, acute cerebral thrombosis, peripheral limbs arteriovenous thrombus. and other acute diseases involved in thrombosis. Therefore the injection agent is another choice. In order to fulfill the goal, first, we should analyze all primary structures of the isozymes and identify essential groups and then search the relationship between structure and function that are related to the preparation of injection agent. Second, the antigenic features of the isozymes should be investigated [88]. Third, the structure of the earthworm proteases molecule should be optimized and modified chemically, so that the domains or groups leading to hostile responses could be removed or blocked. Finally the method of cloning and expressing of the recombinant earthworm proteases should be established to investigate the possibility of an injection agent produced by gene engineering.

11.3. Chemically Modified Structure. In order to enhance the efficacy and tolerability of thrombolytic agents, we should improve the specificity of the enzyme on fibrin to decrease the side effects and enhance the resistance to plasminogen activator inhibitor to elongate the half-life.

Chemical modification has been used to stabilize the native structure of the earthworm protease and decrease the antigenicity during administration. The stabilization of the protease is managed by chemical modification of the enzyme with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and phenylglyoxal to protect the activity from the autolytic inactivation. Stabilization is also possible under acidic conditions, in which the stability of the enzyme was rather low, by immobilization with folded sheet mesoporous material [89]. The strongest fibrinolytic protease *Lr*P-III-2 has been modified chemically with fragmented human serum albumin (MW, 10,000–30,000) [90]. The modified enzyme lost the antigenicity of the native enzyme. The enzyme is a nonhemorrhagic protein and does not induce platelet aggregation. The enzyme kept potent proteolytic activity for fibrin and fibrinogen than that of human plasmin. The enzyme easily solubilizes actual fibrin clots (thrombi) of whole blood induced by thrombin in a rat's vena cava.

12. Other Potential Utilizations

12.1. Degradation of Proteins in Waste. The proteolytic activity, except for the fibrinolytic activity, of the earthworm protease has been studied using various protein substrates. Both *Lr*P-III-2 and *Lr*P-II are more effective than trypsin in the production of amino acids from elastin, hemoglobin, casein, and collagen. Thus, the proteases are useful in the field of waste treatment of nondegradable proteins.

12.2. Hydrolyzation of Ester. The earthworm proteases exhibited ester-hydrolyzing ability as well as the proteolytic activity [69]. The earthworm proteases could be used as a biocatalyst for unmasking of the unnecessary acetyl moiety from the building blocks in organic synthesis. For example, the preparation of vinyl p-coumarate from the acetyl p-coumarate vinyl ester in ethanol is enabled using isozymes *Lr*P-III-1, *Lr*P-III-2, and *Lr*P-II. Polylactate film was decomposed to some extent by the enzyme.

12.3. Nutrition for the Microorganisms. The production of the autolysate is considered to be caused mainly by the action of the earthworm's own proteases without the involvement of microbial degradation [69]. Growth of the microorganisms in the medium with the autolysate in place of polypepton and in the original medium without changes in the other ingredients has been compared. The growth of bakers' yeast in the medium containing the same amount of earthworm autolysate as would have been used for polypepton is substantially better than that in the medium containing polypepton. *E. coli* XL1-blue as well as *Bacillus coagulans* IFO 12583 and *B. stearothermophilus* DSM 297 could grow in the media containing the autolysate as well as in those containing polypepton [69].

13. Conclusion

Earthworm proteases are getting more significant in our daily life nowadays. It is applicable in both experiment and production, such as medical usage, environmental protection, and nutritional production. In the near future, more products based on the earthworm protease will reach the market.

Acknowledgments

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Review Article

Heavy Metal-Induced Oxidative DNA Damage in Earthworms: A Review

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Earthworms can be used as a bio-indicator of metal contamination in soil, Earlier reports claimed the bioaccumulation of heavy metals in earthworm tissues, while the metal-induced mutagenicity reared in contaminated soils for long duration. But we examined the metal-induced mutagenicity in earthworms reared in metal containing culture beddings. In this experiment we observed the generation of 8-oxoguanine (8-oxo-Gua) in earthworms exposed to cadmium and nickel in soil. 8-oxo-Gua is a major premutagenic form of oxidative DNA damage that induces GC-to-TA point mutations, leading to carcinogenesis.

1. Introduction

Molecular oxygen is essential for the survival of all aerobic organisms, and reactive oxygen species (ROS), which are byproducts of oxygen metabolism, are harmful for living organisms. Thus, oxygen is a double-edged sword. In fact, ROS are known to directly attack vital cellular components, including proteins, lipids, and nucleic acids. The oxidation of these molecules is associated with cellular dysfunction, leading to various biological responses, such as inflammation and apoptosis. Since ROS, such as superoxide radical ($O_2{\cdot}^-$), H_2O_2, and hydroxyl radical (•OH), are constantly generated in vivo as byproducts of respiration, agent metabolism, or pathophysiological conditions [1–3], it is difficult to completely prevent their harmful effects on cellular components.

When ROS attack DNA, oxidized bases are frequently generated [4]. Among the various forms of oxidative DNA damage, 8-oxoguanine (7, 8-dihydro-8-oxoguanine, abbreviated as 8-oxo-Gua or 8-OH-Gua) has been most extensively investigated [5]. Since 8-oxo-Gua is premutagenic, it has been suggested to contribute to human diseases [6, 7]. On the other hand, living organisms have repair systems for oxidative DNA damage, to preserve genetic stability. Recent studies have revealed the complicated network of 8-oxo-Gua repair systems (termed as the "GO system") [8]. However, if unrepaired oxidative DNA damage remains in DNA, then it can induce point mutations. Therefore, the ability to repair the damage is critical in terms of genetic stability. Such measurements of 8-oxo-Gua and its repair ability may open new fields in the studies of risk assessment, molecular epidemiology, and health promotion.

Among the many kinds of organisms living in soil, the earthworm is a quite useful organism for the evaluation of metal contamination in soil, because significant positive correlations have been found between the metal concentrations in the earthworm and the cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) concentrations in soil [9]. This evidence prompted us to verify the utility of the earthworm as a biomonitor. Recently, we studied the 8-oxo-Gua generation in earthworms exposed to Cd or nickel (Ni), to examine the possibility that earthworms could be used as a biomonitor for DNA-damaging factors in soil.

This paper focuses on the possibility of using earthworms as a biomonitoring method for oxidative DNA damage-inducing factors in soil.

2. 8-Oxoguanine

Point mutations generated via oxidative DNA damage are involved in cancer development, because mutations are a

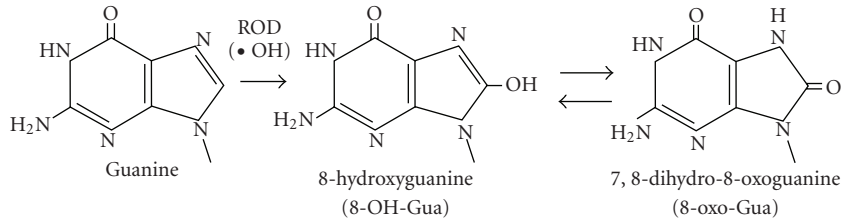

Figure 1: Structure of 8-oxo-Gua. 8-Oxo-Gua is formed by the hydroxylation of guanine at the C-8 position.

Table 1: Association of heavy metal exposure with 8-oxo-Gua repair.

Heavy metals	Animal organs or culture cells	Effects	Ref. no. (year)
As (Sodium arsenite)	Human lung carcinoma A 549 cells	Inhibition of 8-oxo-Gua base excision repair activity and hOGG1 expression	[21] (2002)
As (arsenic trioxide, sodium arsenite, sodium arsenate)	Mouse nonparenchymal hepatocyte NCTC	Fragmentation of mOGG1	[24] (2006)
Cd (Cadmium chloride)	Testis of Sprague-Dawley rat (♂, 8-week-old)	Inhibition of 8-oxo-Gua base excision repair activity	[20] (1997)
Cd (Cadmium acetate)	Testis of F344/NCr rat (♂, 6-7-week-old)	Inhibition of 8-oxo-dGTPase activity	[23] (1999)
Cd (Cd aerosol for rats, Cadmium chloride for cultured cells)	Lung of Lewis rat (♂, 185 ± 5 g), Adult rat lung epithelial cell line	Down-regulation of hOGG1	[30] (2003)
Cd (Cadmium chloride)	Human fibroblast GM00637, HeLa S3 cell	Down-regulation of hOGG1	[22] (2005)
Cd (Cadmium chloride)	TM3 cell (mouse testicular Leydig cell line)	Down-regulation of OGG1 and MUTYH	[25] (2009)
Cr (Sodium dichromate)	Human lung carcinoma A 549 cells	Down-regulation of hOGG1	[26] (2002)
Cr (Sodium dichromate)	White blood cells of healthy adult volunteers ($n = 72$)	Decrease in Ser326Cys OGG1 activity	[27] (2005)
Mn (Manganese chloride)	PC12-derived neuronal cells	Decrease in OGG1 activity	[28] (2004)
Pb (Lead acetate)	Brain of timed-pregnant Long-Evans rat	Decrease in OGG1 activity up to 12 months of age and increase between 12 and 20 months of age	[29] (2006)

common feature of human cancers. In this context, the studies of 8-oxo-Gua, which is an oxidized guanine, have significant implications for understanding the mechanisms of mutation-associated diseases, especially cancer [10]. 8-oxo-Gua is a mutagenic lesion formed spontaneously in the genomic DNA of aerobic organisms (Figure 1) and by the actions of exogenous factors, such as ionizing radiation, chemical pollutants, heavy metals, food, and bacteria. Although 8-oxo-Gua is not necessarily the most abundant form of oxidative DNA damage, it has been the most extensively studied, because it can be quantitated with high sensitivity by high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD), and it is quite easily measured in laboratories [5, 11]. 8-oxo-Gua and 8-oxoadenine (8-oxo-Ade) have been well studied in mutagenic oxidized DNA products, and their frequencies of generation in mammalian DNA and their degrees of mutagenicity are similar [12–15].

Since 8-oxo-Gua was discovered and reported in 1984 [16], this form of DNA damage and its repair systems

have been studied vigorously. 8-oxo-Gua induces GC-to-TA transversion-type point mutations [17], and thus it is believed to have a key role in cancer development. Moreover, 8-oxo-Gua is efficiently removed from DNA via the short-patch base excision repair (BER) pathway, initiated by 8-oxoguanine DNA glycosylase 1 (OGG1).

3. Heavy Metals and 8-Oxoguanine/8-Oxo-Gua Repair System

Heavy metal pollution of soil is widespread across the globe and has caused biological problems, leading to potential toxicity to living organisms. Recent research found that the atmospheric input of heavy metals to agricultural systems also significantly contributed to metal loading in soil [19]. These complicated pathways of contamination make it difficult to avoid the exposure to the metals existing in our surroundings.

We previously reported the relationship between 8-oxo-Gua/its repair ability and some heavy metals [20–23]. In the studies, we found that cadmium chloride and arsenic compounds increased the level of 8-oxo-Gua accumulation [20, 21, 24]. It is noteworthy that these heavy metals inhibited the 8-oxo-Gua repair activity. Other studies besides ours also generated similar data, as shown in Table 1. Some metals, such as hexavalent chromium (CrVI), manganese (Mn), and Pb, as well as Cd and arsenic (As), also reportedly inhibited the 8-oxo-Gua repair system [25–29]. Among heavy metals, the association of Cd with 8-oxo-Gua repair systems has been studied since the early stage of the research. In 1997, we first described an association between Cd exposure and the inhibition of 8-oxo-Gua excision repair activity in rat testes [20]. After the cloning of mammalian OGG1, it was demonstrated that Cd exposure down-regulated OGG1 expression in rat lung and alveolar epithelial cells [30]. Youn et al. suggested that Cd attenuated the removal of γ-ray-induced 8-oxo-Gua adducts, which in turn increased the mutation frequency, and that this effect might, at least in part, result from the suppression of hOGG1 transcription via the inactivation of the Spl transcription factor, as a result of Cd treatment [22]. These inhibitory effects of Cd on OGG1 activity are similar to the inhibition of 8-oxo-dGTPase activity induced by Cd treatment, which led to the accumulation of 8-oxo-Gua in DNA [23]. Although it is likely that Cd exposure might broadly disturb the 8-oxo-Gua repair system, the exact mechanism of the inhibition remains unclear.

4. Oxidative Stresses and Earthworms

Several oxygen radical studies using earthworms have been performed. The biochemical effects of tetrabromobisphenol A (TBBPA) on the earthworm *Eisenia fetida* (*E. fetida*) were examined to assess the ecological toxicity of TBBPA. The ROS generated in the earthworm was identified as ·OH. With increasing TBBPA concentrations, the levels of antioxidant enzymes, glutathione, and MDA varied significantly. The study indicated that TBBPA exerted its toxic effects on *E. fetida* by inducing the generation of ROS, resulting in oxidative damage [31].

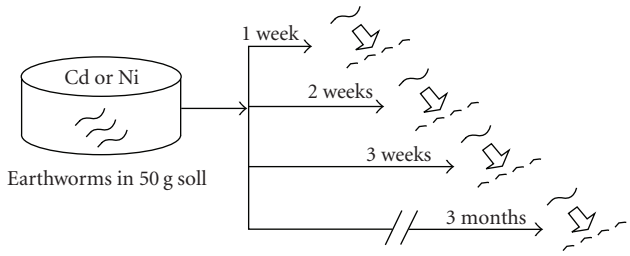

Figure 2: Experimental design. Metals (0, 0.5, or 10 mg as Cd or Ni) were added to 50 g soil in a 600 mL glass container. After 0-, 1-, 2-, 3-week, and 3 month exposures, E. fetida were cut into four rough segments (S1–S4). S1: head region, S2: anterior body region, S3: posterior body region, S4: tail region.

Since earthworm skin contains several molecules (tetraene and triene sterol) similar to those in human beings, it is considered to be useful as a biomonitor for environmental factors for human beings. For example, the phototoxic effects of UVR in sunlight and its possible mechanism of action were analyzed by using earthworms [32]. In the study, the generation of ROS, the photooxidation of lipids, and the histopathological changes in the earthworm integument were examined. The study indicated that the UVR-exposed earthworm skin homogenate produced a significant amount of ROS, such as singlet oxygen ($^1O_2$), $O_2\cdot^-$, •OH, and photooxidized lipids. The authors concluded that the earthworm could be a simple, sensitive, and cost-effective test organism for assessing the hazard potential of solar radiation and also for planning safety measures for humans.

Thus, it is likely that the biological features of the earthworm are useful as biomonitors to estimate the oxidative damage that could occur in humans.

5. 8-Oxoguanine and Earthworms

Recent research has indicated that the earthworm is a candidate organism as a biomonitor for soil contaminants, because it plays an important role in the soil macrofauna biomass. The species *E. fetida* is most commonly used in ecotoxicology, as a useful biomonitor for soil [33]. In particular, this species' proximity to the soil contaminants is a merit for the analysis [34, 35]. Among the many kinds of organisms living in soil, the earthworm is the most useful organism for the evaluation of metal contamination, because significant positive correlations have been found between the metal concentrations in the earthworm and the soil Cd, Cu, Pb, Zn, and Hg concentrations [9, 36–39].

We recently analyzed the 8-oxo-Gua accumulated in the DNA of *E. fetida* exposed to heavy metals, to determine if a method using earthworms as a biomonitor is useful for the assessment of soil mutagenicity [18]. We employed Cd and Ni as test metals, because the carcinogenic potentials of Cd and Ni have been established for humans and animals [40, 41], and these metals are known to generate 8-oxo-Gua in DNA [20, 42–44].

In the study, *E. fetida* were kept in a 20-liter stainless steel tank at an ambient temperature of 24°C, using a mold

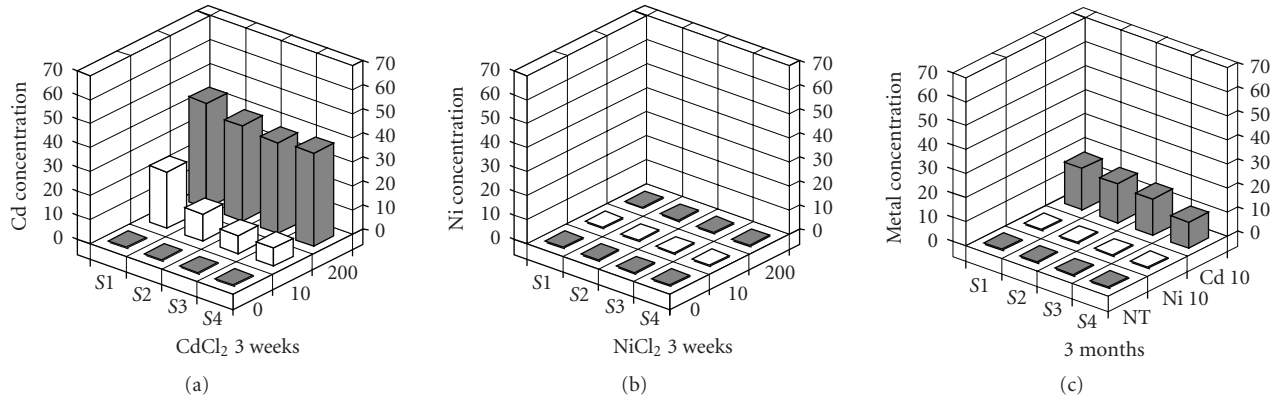

Figure 3: Heavy metal accumulation in *E. fetida*. Each data point represents the mean of three individuals. Heavy metal concentrations were measured by atomic absorption spectrometry, and are expressed as μg per body weight. (Data are modified by Nakashima et al. [18]).

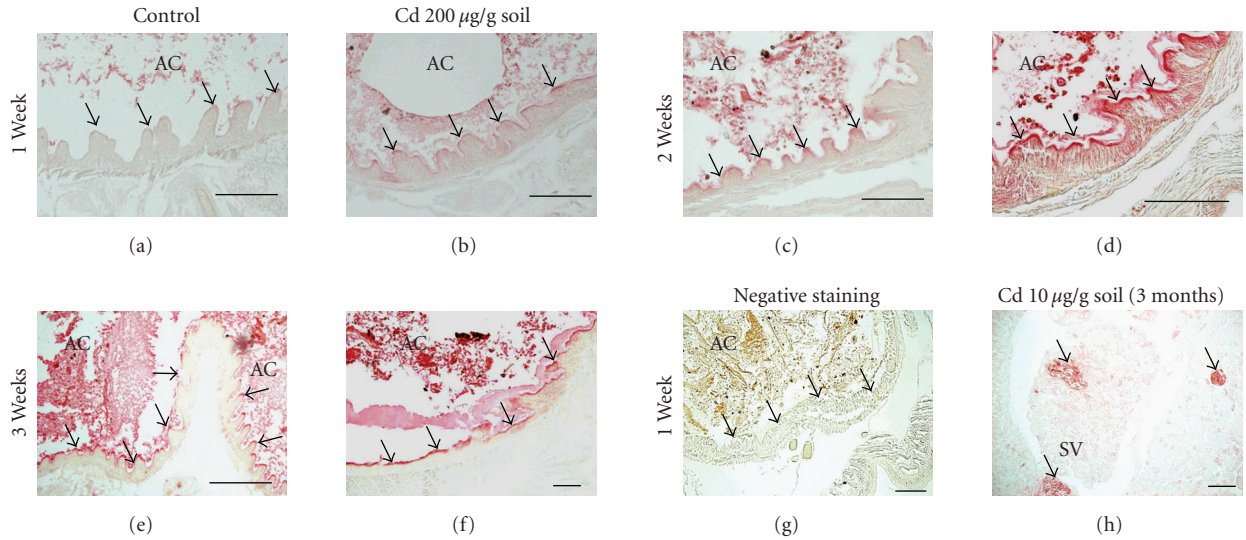

Figure 4: Immunohistochemical analyses of 8-oxo-dG accumulation in the gut epithelial layers and seminal vesicles (SV) of *E. fetida* (S1). Controls for the gut epithelial layer: 1 week (a), 2 weeks (c), 3 weeks (e). The gut epithelial layer of the Cd (200 μg/g soil) treatment group: 1 week (b), 2 weeks (d), 3 weeks (f). (g) Negative staining of Cd (200 μg/g soil, 1 week) with a 2% bovine serum albumin solution. Arrowheads show the gut epithelial layer. AC: alimentary canal. (h) The SV. Arrowheads show positive signals in SV. All scale bars are 100 μm. (Data of (h) are modified by Nakashima et al. [18]).

with skim milk as a food source until heavy metal exposure. Three to six individuals were kept in a 600 mL glass container containing 50 g of soil with/without heavy metal. They were exposed to 10 or 200 μg heavy metal/g soil for 1, 2, and 3 weeks or to 10 μg heavy metal/g soil for 3 months (Figure 2). As a result, we detected a high level of Cd accumulation in *E. fetida* (Figures 3(a) and 3(c)). On the other hand, no Ni accumulation was observed (Figures 3(b) and 3(c)).

In addition, we observed positive staining of 8-oxo-Gua in the gut epithelial layers in almost all samples (Figures 4(a)–4(f)). The metal absorption routes include the digestive system and the surface wall [45, 46], but the main route is the digestive system. Since gut epithelial layers are frequently exposed to ROS, 8-oxo-Gua accumulation was constantly detected. Although the 200 μg Cd-exposed *E. fetida* showed relatively stronger signals (P1+++ in Table 2) at 2 weeks in comparison to the others, this did not seem to be significant evidence, because almost all of the specimens showed positive signals in the gut epithelial layers and the difference in the signal strength was too small to conclude that Cd exposure increased 8-oxo-Gua accumulation in the organs. On the other hand, the positive signals in the seminal vesicles were clearly detected only in *E. fetida* treated with 10 μg of Cd for 3 months (Figure 4(h)). The seminal vesicles are considered as metallothionein-(MT-)poor organs. Therefore, it seems reasonable to speculate that a lower level of MT expression is involved in Cd-induced DNA damage accumulation.

6. Conclusions

In our recent study, we observed a high level of Cd accumulation and no Ni accumulation in *E. fetida*, accompanied with an increase in 8-OH-dG accumulation in the organs of Cd-exposed *E. fetida*. Based on these results, it is reasonable to

Table 2: Profiling of immunohistochemical analyses of 8-oxo-Gua accumulation in *E. fetida*.

		Segment no.			
		1	2	3	4
Control	-1 wk	P1++	P1+	NS	NS
	-2 wk	P1++	P1+	P1++	P1+
	-3 wk	P1++	P1++	P1+	NS
Cd 10	-1 wk	P1++	P1+	NS	NS
	-2 wk	P1++	P1++	P1+	NS
	-3 wk	P1+	P1+	P1+	P1+
Cd 200	-1 wk	P1++	P1++	P1+	NS
	-2 wk	P1+++	P1+++	P1++	P1+
	-3 wk	P1++	P1++	P1+	P1+
Ni 10	-1 wk	P1++	P1++	P1+	P1+
	-2 wk	P1++	P1++	P1+	P1+
	-3 wk	P1++	P1++	P1+	NS
Ni 200	-1 wk	P1++	P1++	P1+	P1+
	-2 wk	P1++	P1+	P1+	P1+
	-3 wk	P1++	P1++	P1+	NS
Control	-3 M	P1++	P1++	P1+	P1+
Cd 10	-3 M	P1++ / P2++	P1++	P1+	P1+
Ni 10	-3 M	P1++	P1++	P1+	P1+

Cd10: Cd 10 μg/g soil exposure, Cd200: Cd 200 μg/g soil exposure, Ni10: Ni 10 μg/g soil exposure, Ni200: Ni 200 μg/g soil exposure.
P1: positive signal in gut epithelial layers
P1+: weak signal
P1++: moderate signal
P1+++: strong signal
P2: positive signal in seminal vesicles
P1+: weak signal
P1++: moderate signal
P1+++: strong signal
NS: no signal.

conclude that the increase in 8-OH-dG accumulation is due to Cd accumulation.

Taken together, we demonstrated the possible utility of using earthworms as biomonitors, by measuring the oxidative DNA damage generated in the earthworms, as a biomonitoring method for assessing soil mutagenicity. However, many points remain unresolved. For example, this method could be reliable only for bioaccumulated metals, such as Cd, but not for non-bioaccumulated metals, such as Ni, even if they generate 8-oxo-Gua. To establish a broader biomonitoring method using earthworms to assess soil mutagenicity, further studies will be required.

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Research Article

Nutrient Status of Vermicompost of Urban Green Waste Processed by Three Earthworm Species—*Eisenia fetida, Eudrilus eugeniae,* and *Perionyx excavatus*

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Major nutrient status of vermicompost of vegetable market waste (MW) and floral waste (FW) processed by three species of earthworms namely, *Eudrilus eugeniae, Eisenia fetida*, and *Perionyx excavatus* and its simple compost were assessed across different periods in relation to their respective initiative substrates. Their physical parameters—temperature, moisture, pH, and electrical conductivity—were also recorded. The nutrients—nitrogen, phosphorus, potassium, calcium, and magnesium—increased in the vermicompost and compost while the organic carbon, C/N and C/P ratios decreased as the composting process progressed from 0 to 15, 30, 45, and 60 days. The nutrient statuses of vermicomposts of all earthworm species produced from both the wastes were more than that of the compost and that of their respective substrates. Moreover, the vermicompost produced by *E. eugeniae* possessed higher nutrient contents than that of *E. fetida, P. excavatus*, and compost. The MW showed higher nutrient contents than the FW. Thus, vermicomposting is the paramount approach of nutrient recovery of urban green waste.

1. Introduction

The urban green waste generally comprises of garden or park waste such as grass or flower cuttings and hedge trimmings, domestic and commercial food waste, and vegetable market waste, the later is generated in large quantities and accumulated in unhygienic way adjacent to vegetable markets emanating unbearable malodor due to lack of proper scientific disposal management particularly in developing countries like India. The vegetable market waste is the leftover and discarded rotten vegetables, fruits, and flowers in the market. This urban waste can be converted to a potential plantnutrient enriched resource—compost and vermicompost that can be utilized for sustainable land restoration practices [1]. Vermicomposting is a mesophilic process and is the process of ingestion, digestion, and absorption of organic waste carried out by earthworms followed by excretion of castings through the worm's metabolic system, during which their biological activities enhance the levels of plant-nutrients of organic waste [2]. Compost and vermicompost are the end products of aerobic composting process, the later with using earthworms. Vermicompost possessed higher and more soluble level of major nutrients—nitrogen, phosphorus, potassium, calcium and magnesium [3–5]—compared to the substrate or underlying soil, and normal compost. During the process, the nutrients locked up in the organic waste are changed to simple and more readily available and absorbable forms such as nitrate or ammonium nitrogen, exchangeable phosphorus and soluble potassium, calcium, magnesium in worm's gut [6, 7]. Vermicompost is often considered a supplement to fertilizers and it releases the major and minor nutrients slowly with significant reduction in C/N ratio, synchronizing with the requirement of plants [8].

The vegetable market waste (MW) as well as floral (*Peltophorum pterocarpum*) waste (FW) were collected and composted using three different earthworm species—*Eisenia fetida, Eudrilus eugeniae, and Perionyx excavatus* during the present study. These worms have been considered as key agents for organic waste management through the process

Table 1: Growth parameters of three earthworm species during the process of vermicomposting of MW and FW.

Earthworm growth parameters	*E. eugeniae*		*E. fetida*		*P. excavates*	
	MW	FW	MW	FW	MW	FW
Av. Individual length:						
Initial (cm)	15.0 ± 0.02	15.0 ± 0.02	8 ± 0.01	8 ± 0.01	4 ± 0.02	4 ± 0.02
Final (cm)	18.5 ± 0.04	19.4 ± 0.05	11.5 ± 0.05	12.3 ± 0.04	7.4 ± 0.06	8.9 ± 0.07
Av. Individual weight						
Initial (gm)	3.5 ± 0.02	3.5 ± 0.02	0.67 ± 0.01	0.67 ± 0.01	0.31 ± 0.02	0.31 ± 0.02
Final (gm)	10.5 ± 0.05	12.8 ± 0.1	2.42 ± 0.01	3.57 ± 0.02	1.94 ± 0.04	3.12 ± 0.03
Av. Total biomass						
Initial (gm)	175 ± 0.06	175 ± 0.06	33.5 ± 0.05	33.5 ± 0.05	15.5 ± 0.05	15.5 ± 0.05
Final (gm)	2724 ± 0.2	3975 ± 0.4	735.4 ± 0.06	1194.3 ± 0.0	682.6 ± 0.04	1142.7 ± 0.0
Av. Cocoon production rate	0.51 ± 0.006	0.51 ± 0.006	0.5 ± 0.003	0.5 ± 0.003	2.7 ± 0.001	2.7 ± 0.001
Av. Worm number per cocoon	2.7 ± 0.09	2.7 ± 0.09	3.8 ± 0.01	3.8 ± 0.01	1.1 ± 0.03	1.1 ± 0.03
Av. Cocoon number at the end	57 ± 0.3	76 ± 0.07	51 ± 0.05	74 ± 0.06	197 ± 0.05	218 ± 0.03
Av. Juvenile number at the end	78 ± 0.08	93 ± 0.06	95 ± 0.07	124 ± 0.05	143 ± 0.06	162 ± 0.06
Av. Adult number at the end	254 ± 0.04	310 ± 0.04	298 ± 0.08	331 ± 0.06	345 ± 0.04	362 ± 0.09
Av. Mortality rate	0.03 ± 0.002	0.05 ± 0.003	0.06 ± 0.004	0.08 ± 0.005	0.3 ± 0.02	0.6 ± 0.03

of vermicomposting [9–13]. The main aim of the present investigation was to know the extent to which vermicomposting and the normal composting of urban green waste may be combined in order to maximize the potentials of both the processes. Earlier, Graziano and Casalicchio [14] have proposed a combination of aerobic composting and vermicomposting to enhance the value of the final products. Frederickson and Knight [15] have showed that vermiculture and anaerobic systems can be combined to enhance organic matter stabilisation. The benefits of a combined system to process urban green waste could include effective sanitization and pathogen control due to an initial brief period of thermophilic composting, enhanced rates of stabilization, plus the production of earthworms and vermicompost [16]. Stabilization of green waste such as yard waste and vegetable waste through the process of composting and vermicomposting has been carried out earlier [16–18]. The present investigation attempted mainly to evaluate the nutrient status of different vermicomposts produced by the three earthworm species and that of compost of urban MW and FW in relation to the respective initial substrates, and also to obtain empirical information on the growth and productivity of the three species of earthworms cultured in the two substrates.

2. Materials and Methods

2.1. Methods of Waste Collection. The MW and FW samples each weighing about 125 kg were collected separately in random manner. The MW, both fresh and decomposed, was collected from the main vegetable market of Puducherry, which comprised of different leftover putrefied vegetables such as cabbage, tomato, potato, onion, carrot, turnip, brinjal, and leafy vegetables; the FW was obtained from the *P. pterocarpum* (Family-Fabaceae and Subfamily Caesalpinioideae), a widely appreciated shade tree and a reclamation plant with dense spreading crown, and planted along the roadsides in the Pondicherry University campus. These wastes were characterized by segregating and discarding the nonbiodegradable fraction, and the biodegradable component was used for the experiment. Five samples of each waste were taken for experimentation and analyses.

2.2. Sample Processing—Pre-Composting. The collected MW and FW were air dried separately spreading over a polythene sheet for 48 hours. The air dried samples were pre-composted for three weeks before putting into vermicomposting and composting process. Pre-composting is the pre processed and pretreated practice of raw waste. The waste materials, in the pre-composting process were decomposed aerobically by the active role of bacteria due to which temperature raised up to 60°C. As such a high temperature was lethal for earthworm survival, the thermal stabilization was done prior to introduction of earthworms into the substrate. When the temperature of the pre-composted substrate diminished to 25°C, adult earthworms with well-defined clitella belonging to the three species namely, *E. eugeniae, E. fetida,* and *P. excavatus* were introduced on the pre-composted material filled in each set of earthen pots (The earthworms were collected from a local vermiculture unit at Lake Estate of Auorbindo Ashram, Puducherry, India).

2.3. Experimental Design. In each pot five kg of the substrate mixed with cow dung in 3 : 1 ratio were taken for vermicomposting and composting. A total of four sets of earthen pots each set comprising six replicates was taken for each waste, of which three sets were used for vermicomposting with each set using one species of earthworm and the forth set was used

for normal composting that is, without using any earthworm. Three species of earthworms, each of fifty adult individuals, were introduced on the top of the pre-composted substrate in each of the three sets of pots keeping aside the fourth set for composting without earthworms. All the pots were covered on the top by jute cloth cover and wire mesh to prevent and protect the earthworms from the predators—centipedes, moles, and shrews. Small holes were drilled at the bottom of each pot which was filled with small stones up to a height of 5 cm for air circulation and good drainage. The processes of vermicomposting and composting were carried out for a period of 60 days. The temperature and moisture content were maintained by sprinkling adequate quantity of water at frequent intervals. The harvesting of vermicompost and compost, and total earthworm biomass, individual body weight, total numbers of juveniles, adults, and cocoons were carried out, and the mortality rates of the three earthworm species were calculated after 60 days, at the end of the experiment.

2.4. Physico-Chemical Analyses. The homogenized subsamples of each substrate material and their respective compost and vermicompost samples (on the basis of 100 g dry weight) were collected undestructively at 0 (i.e., substrate), 15, 30, 45, and 60 days from each replicate pot and compound samples were made, which were processed for analyses of organic carbon (OC) and major nutrients—total nitrogen (N), available phosphorus (P), exchangeable potassium (K), calcium (Ca), and magnesium (Mg). The temperature (°C), moisture (%), pH, and electrical conductivity (EC) were recorded for the substrate and during the vermicomposting and composting processes. Temperature was noted daily using a thermometer, and moisture content was measured gravimetrically. The pH and EC of samples were recorded by a digital pH meter and conductivity meter, respectively. The OC of the samples was measured by Walkey-Black method [19]; the N was estimated by the Kjeldahl method [20], and the P and K contents of the samples were analyzed by calorimetric method [21] and flame photometric method [22], respectively. The Ca and Mg contents of the samples were also analyzed using atomic absorption spectrophotometer (GBC make) [20]. The C : N ratio was calculated from the measured values of C and N.

2.5. Statistical Analysis. Two-way analysis of variance (ANOVA) was computed using SPSS (version No. 10) to test the level of significance of difference between the vermicomposts produced by the three species earthworms and compost samples with respect to nutrient parameters.

3. Result and Discussion

3.1. Growth and Productivity of Earthworms. The growth parameters of three earthworm species cultured in MW and FW showed that the length increased by 23.3% in *E. eugeniae,* 43.7% in *E. fetida,* and 85.0% in *P. excavatus* grown in MW, while it increased by 29.3% in *E. eugeniae,* 53.7% in *E. fetida,* and 122.5% in *P. excavatus* grown in FW, whereas the net individual weight gained by each of the three species was 200.0, 261.2, and 525.8% in MW and 265.7, 432.8, and 906.4% in FW respectively, at the end of the experiment (Table 1). The net individual weight gain and total biomass gain were higher in *P. excavatus* than that of *E. fetida,* and *E. Eugeniae*. The total biomass gain was found 1456.6 and 2171.4% by *E. eugeniae*, 2095.2 and 3465.1% by *E. fetida,* and 4303.9 and 7272.3% by *P. excavatus* in MW and FW respectively, at the end of the vermicomposting process. Cocoon production rate was higher in *P. excavatus* than that of *E. eugeniae* and *E. fetida*. The number of worms produced per cocoon was 28.9 and 71.0% higher in *E. fetida* than that of *E. eugeniae* and *P. excavatus,* respectively, while the number of cocoons collected at the end of the experiment was more in *P. excavatus* by 245.6% than that of *E. eugeniae* and 286.3% than that of *E. fetida* in MW; and by 186.8% and 194.6% than that of *E. eugeniae* and *E. fetida* in FW, respectively. The number of juveniles collected was 83.3% higher in *P. excavatus* than that of *E. eugeniae* and 50.5% than that of *E. fetida* in MW, whereas the increase was 74.2% in *E. eugeniae* and 30.6% in *E. fetida.* Adult earthworm number was higher in *P. excavatus* than that of *E eugeniae,* and *E. fetida* by 35.8 and 15.8% in MW, and 16.8 and 9.4% in FW, respectively. The production of cocoons, juveniles, and adults of all the three species was higher in FW than that of MW, which indicated the former waste material as a better substrate for the earthworms. The mortality rate of the *P. excavatus* was 900% higher than that of *E eugeniae* and 400% higher than that of *E. fetida* grown in MW, while it was higher by 1100 and 650% than *E eugeniae* and *E. fetida* grown in FW, respectively.

The mean individual length and live weight, mean growth rate of an individual (mg/day), individual and total biomass gain, reproduction rate (cocoon worm^{-1}day^{-1}), fecundity rate (worm cocoon $^{-1}$day–1), total cocoon, juveniles and adult numbers, and mortality rate in the present study varied across different treatments. The worms when introduced into wastes showed an increased growth rate and reproduction activities [1]. The increase in body weight of all three earthworm species was noted in both the substrates during vermicomposting process, which could be due to the substrate quality or could be related to fluctuating environmental conditions [23–25]. The readily available nutrients in MW and FW enhanced the feeding activity of the worms, showing their increase in biomass [1]. Interestingly, cocoon production rate was higher in *P. excavatus*, whereas the number of worms per cocoon was higher in *E. fetida* compared to other species. The indigenous species, *P. excavates,* exhibited better growth and reproduction performance compared to the other two exotic species [26]. The higher numbers of cocoons, juveniles, and adults collected from the vermicompost processed by *P. excavatus*, were probably because its indigenous nature being acclimatized to the abiotic environmental conditions extremely well compared to other species. The difference in worm mortality among the three species could be related to the species-specific composting behavior or to specific tolerance nature of earthworm according to the changing microenvironmental conditions in composting subsystem

Table 2: Weight and other different physical parameters of the substrates—MW and FW—and their respective compost and vermicompost of three earthworm species (Mean ± sd; $n = 3$).

Parameters	0 days		60 days							
			Vermicompost						Compost	
			E. eugeniae		*E. fetida*		*P. excavatus*			
	MW	FW	MW	FW	MW	FW	MW	FW	MW	FW
Weight (kg)	5.00 ± 0.005	5.00 ± 0.01	1.25 ± 0.03	0.85 ± 0.05	1.85 ± 0.04	1.65 ± 0.04	2.5 ± 0.03	2.2 ± 0.01	3.7 ± 0.009	3.5 ± 0.008
Temperature (0c)	29.8 ± 0.06	26.5 ± 0.05	24.1 ± 0.04	22.3 ± 0.03	24.2 ± 0.05	23.4 ± 0.03	24.4 ± 0.04	23.5 ± 0.05	24.7 ± 0.05	23.9 ± 0.02
Moisture content (%)	55.73 ± 0.08	34.62 ± 0.03	65.2 ± 0.03	60.8 ± 0.08	64.72 ± 0.03	59.67 ± 0.02	64.04 ± 0.01	58.68 ± 0.05	63.11 ± 0.04	57.49 ± 0.05
pH	6.31 ± 0.07	6.84 ± 0.04	7.12 ± 0.02	7.37 ± 0.02	7.08 ± 0.01	7.28 ± 0.03	6.95 ± 0.02	6.89 ± 0.04	6.87 ± 0.03	6.79 ± 0.04
Electric conductivity (mhos/cm)	495.5 ± 0.04	152.2 ± 0.02	3354.4± 0.02	532.5 ± 0.03	2716.7± 0.07	466.3 ± 0.03	1983.2± 0.06	415.7 ± 0.02	1789.3± 0..07	363.5 ± 0.01

[1]. Moreover, the growth rate difference between the three species was probably due to the species-specific growth patterns or could be related to the feed quality and preferences by individual species of earthworm [1].

3.2. Waste Stabilization. The reduction in bulk dry mass of both the substrates—MW and FW, the range of temperature, moisture content, pH, EC of the substrate, compost and vermicompost presented in Table 2. depicted that higher mass reduction of MW was recorded in the vermicompost processed by *E. eugeniae* (75%), followed by that of *E. fetida* (63%), *and P. excavatus* (50%) compared to that of compost (26%), whereas the mass reduction was higher 83% in vermicompost produced by *E. eugeniae,* 67% by that of *E. fetida*, 56% in that of *P. excavates,* and 30% in sole compost than that of FW. The marked stabilization of both the substrates due to vermicomposting process was higher in the vermicompost processed by *E. eugeniae* compared to that of other two and the compost. The FW and its vermicomposts and composts were found to be more stabilized than that of MW.

The pre-composting because of its thermophilic nature prior to vermicomposting helped in mass reduction and pathogen reduction [27]. It was found that the bulk (dry) mass reduction and stabilization of both the wastes during present study through vermicomposting process were significant [2, 27]; the vermicomposting may also be known as vermistabilization [28]. The cow dung used as the inoculant in the vermicomposting process enhanced the quality of feeding resource attracting the earthworms and accelerated the breakdown of wastes resulting in the reduction of C : N ratio by increasing certain nutrients [1, 29–31].

3.3. Physical State of MW and FW during Vermicomposting and Composting Processes. The physical characteristics recorded during the period of this study presented in Table 2 were conducive for vermicomposting process [6, 32]. The temperature ranged from 22.3 to 29.8°C and was lower by 19.1 and 15.8% in the vermicompost processed by *E. eugeniae,* by 18.8 and 11.7% in that of *E. fetida*, by 18.1 and 11.3% in that of *P. excavates,* and by 17.1 and 9.8% in compost than that of initial substrate of MW and FW, respectively. The moisture content of vermicompost of *E. eugeniae* varied by 17.0 and 75.6%, by 16.1 and 72.4% in that of *E. fetida,* by 14.9 and 69.5% in that of *P. excavates,* and by 13.2 and 66.1% in the compost than that of initial MW and FW, respectively. The pH ranged from 6.31 to 7.37 and increased by 12.8, 12.2, 10.1, and 8.9% than that of MW; and 7.7, 6.4, 2.1 and 0.7% than that of FW, in vermicompost of *E. eugeniae, E. fetida, P. excavatus,* and compost, respectively. The EC of vermicompost ranged from 152.2 to 3354.4 mhos/cm and increased EC noted in vermicompost processed by *E. eugeniae, E. fetida, P. excavatus* and in compost was 577.0, 448.3, 300.2, and 261.1% more than that of MW, and was 249.9, 206.4, 173.1, and 138.8% more than that of FW, respectively, at the end of composting process. Temperature, moisture content, and EC were more and pH was less in MW compared to that of FW.

3.4. Temperature. At the start of the experiment, the temperature of the substrate was high and then decreased gradually as the composting process progressed. The heat released by the oxidative action of intensive microbial activity on the organic matter resulted in the rise in temperature during the first mesophilic phase of composting process [33]. The temperature of the following thermophilic phase rose up above 40°C reaching about 60°C when most of the organic matter was degraded with the help of thermophilic bacteria and fungi, consequently depleting most of the oxygen. The thermophilic phase was followed by cooling phase, when compost maturation stage occurred and compost temperature dropped to that of the ambient [34]. Then, the decreasing trend of temperature with the progress of composting process occurred, which was probably due to the decreased bacterial activity. It may also be attributable to regular sprinkling of water.

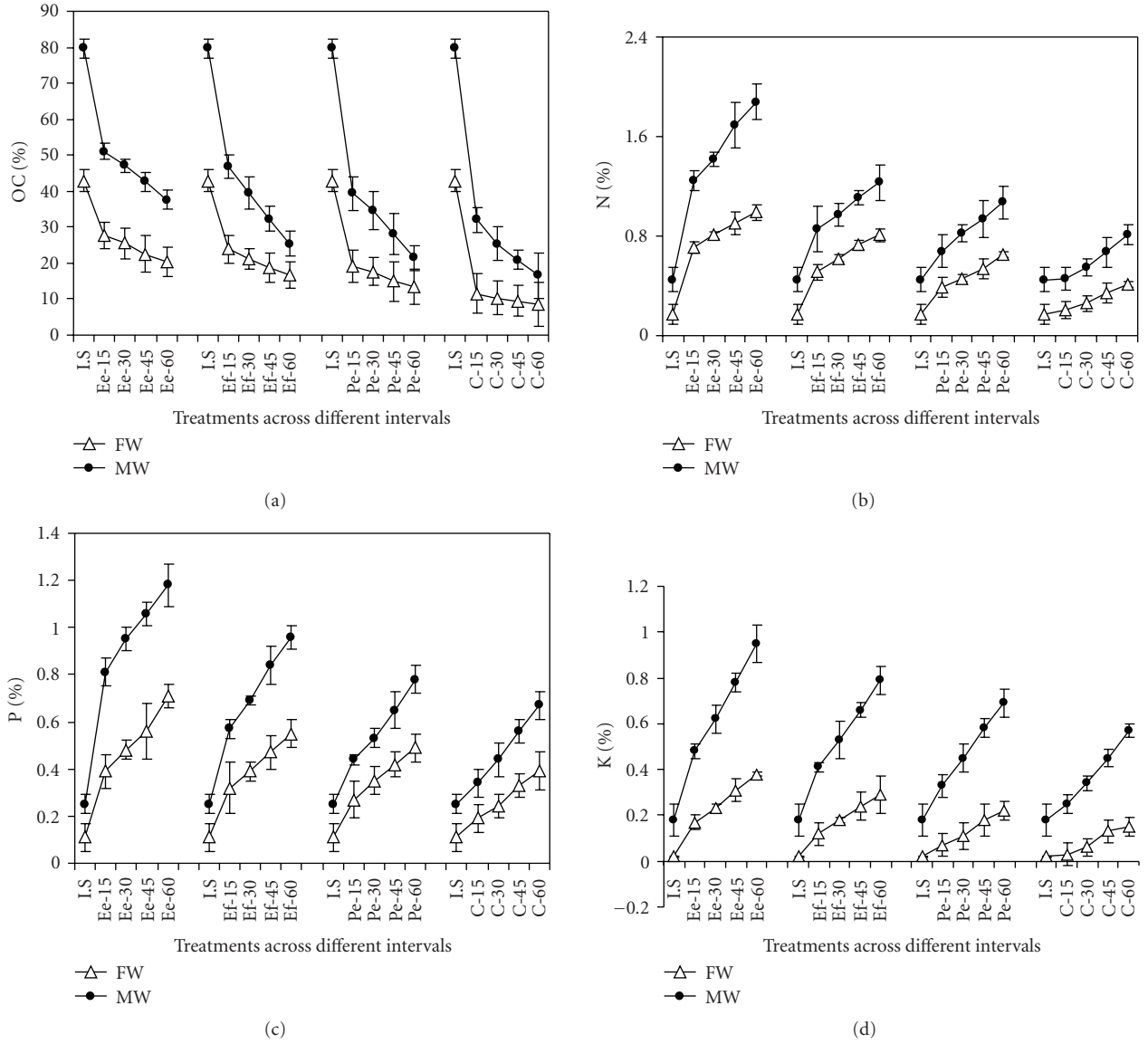

Figure 1: Major nutrients—OC, N, P, K (%) of vermicompost (VC) of three different species of earthworms—*Eudrilus eugeniae* (Ee) at 15 days (Ee-15), 30 days (Ee-30), 45 days (Ee-45), and 60 days (Ee-60); *Eisenia fetida* (Ef) at 15 days (Ef-15), 30 days (Ef-30), 45 days (Ef-45), and 60 days (Ef-60); *Perionyx excavatus* (Pe) at 15 days (Pe-15), 30 days (Pe-30), 45 days (Pe-45), and 60 days (Pe-60); and Compost (C) at 15 days (C-15), 30 days (C-30), 45 days (C-45), and 60 days (C-60) produced from FW and MW. (a) OC, (b) N, (c) P, (d) K.

3.5. Moisture Content. Moisture content ranged from 50–70% [35]. Edwards and Bater [36] reported that optimum moisture content for growth of earthworms—*E. fetida, E. eugeniae and P. excavatus*—was 85% in organic waste management. The rate of mineralization and decomposition becomes faster with the optimum moisture content [37]. According to Liang et al. [38], the moisture content of 60–70% was proved having maximal microbial activity, while 50% moisture content was the minimal requirement for rapid rise in microbial activity. Vermicompost samples during the present study showed higher moisture content than the compost and substrate, which may be due to their high absorption capacity, and may also be because of assimilation rate by microbial population indicating the higher rate of degradation of waste by earthworms. Relatively highest moisture content of vermicompost produced by *E. eugeniae* followed by that of *E. fetida* and *P. excavatus* implied greater palatability of the substrate by the species.

3.6. pH. It was neutral being around 7 and increased gradually from substrate to compost to vermicompost [35, 39]. The near-neutral pH of vermicompost may be attributed by the secretion of NH_4^+ ions that reduce the pool of H^+ ions [40] and the activity of calciferous glands in earthworms containing carbonic anhydrase that catalyzes the fixation of CO_2 as $CaCO_3$, thereby preventing the fall in pH [9]. The increased trend of pH in the vermicompost and compost samples is in consistence with the findings of Tripathi and

Table 3: ANOVA of different nutrients of vermicomposts produced by three species of earthworms and compost (Treatments) of Market Waste across different time intervals.

Source of Variation	SS	df	MS	F
OC				
Time Intervals	648.6706	3	216.2235	83.74185**
Treatments	923.0771	3	307.6924	119.1671**
Error	23.23823	9	2.582025	
N				
Time Intervals	0.431569	3	0.143856	38.0167**
Treatments	1.881169	3	0.627056	165.7113**
Error	0.034056	9	0.003784	
C/N Ratio				
Time Intervals	2834.197	3	944.7322	36.40393**
Treatments	301.5306	3	100.5102	3.87302**
Error	233.5624	9	25.95138	
P				
Time Intervals	0.286919	3	0.09564	418.6049**
Treatments	0.568369	3	0.189456	829.231**
Error	0.002056	9	0.000228	
C/P Ratio				
Time Intervals	6752.972	3	2250.991	39.35673**
Treatments	225.6022	3	75.20074	1.314823**
Error	514.751	9	57.19456	
K				
Time Intervals	0.32795	3	0.109317	141.5612**
Treatments	0.2005	3	0.066833	86.54676**
Error	0.00695	9	0.000772	
Ca				
Time Intervals	28.18897	3	9.396323	2027.679**
Treatments	8.064019	3	2.688006	580.0583**
Error	0.041706	9	0.004634	
Mg				
Time Intervals	0.30515	3	0.101717	1220.6**
Treatments	0.3242	3	0.108067	1296.8**
Error	0.00075	9	8.33E-05	

Level of significance: $^{**}P < .001$

Bhardwaj [41] and Loh et al. [42], which was due to higher mineralization, whereas the present findings are in contradiction to the findings of Suthar and Singh [1], Haimi and Huhta [40] and Ndegwa et al. [43] who reported lower pH. The increased pH during the process was probably due to the degradation of short-chained fatty acids and ammonification of organic N [44–46]. Fares et al. [47] found the increased pH at the end of the composting process, which was attributed to progressive utilization of organic acids and increase in mineral constituents of waste.

3.7. EC. The increased EC during the period of the composting and vermicomposting processes is in consistence with that of earlier workers [48, 49], which was probably due to the degradation of organic matter releasing minerals such as exchangeable Ca, Mg, K, and P in the available forms, that is, in the form of cations in the vermicompost and compost [44, 46].

3.8. Nutrients in MW and FW and Their Vermicompost and Compost. It was found that the N was 0.45% in MW and 0.17% in FW; P was 0.25% in MW and 0.11% in FW; K was 0.18% in MW and 0.02% in FW, Ca was 0.62% in MW and 0.07% in FW; Mg was 0.17% in MW and 0.04% in FW, while the content of OC was 79.6% in MW and 42.9% in FW (Figures 1 and 2).

The present study revealed that all vermicomposts prepared from their respective organic wastes possessed considerably higher levels of major nutrients—N, P, K, Ca, and Mg compared to that of the substrates [31, 50]. The increase in the nutrients and decrease in OC, C/N ratio and C/P ratios in the vermicompost, are in consistence

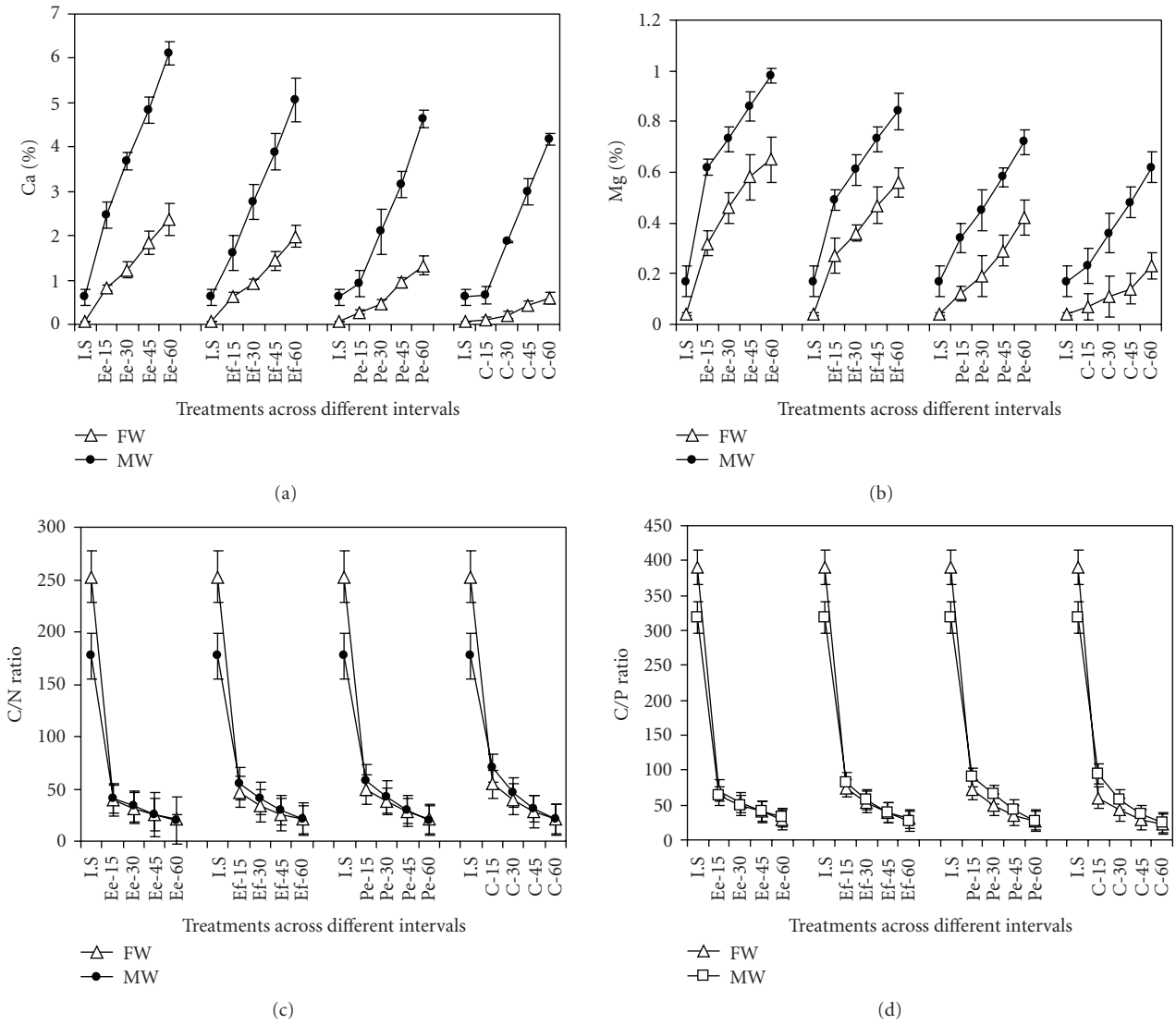

Figure 2: Major nutrients—Ca and Mg (%), C/N ratio and C/P ratio of vermicompost (VC) of three different species of earthworms—*Eudrilus eugeniae* (Ee) at 15 days (Ee-15), 30 days (Ee-30), 45 days (Ee-45), and 60 days (Ee-60); *Eisenia fetida* (Ef) at 15 days (Ef-15), 30 days (Ef-30), 45 days (Ef-45), and 60 days (Ef-60); *Perionyx excavatus* (Pe) at 15 days (Pe-15), 30 days (Pe-30), 45 days (Pe-45), and 60 days (Pe-60); and Compost (C) at 15 days (C-15), 30 days (C-30), 45 days (C-45), and 60 days (C-60) produced from FW and MW. (a) Ca, (b) Mg, (c) C/N ratio, (d) C/P ratio.

with the findings of earlier investigators [25, 26]. Moreover, comparing the nutrient contents of vermicompost with that of compost, vermicompost possessed significantly higher concentrations of nutrients than that of compost ($P < .05$), which was probably due to the coupled effect of earthworm activity as well as a shorter thermophilic phase [51, 52], making the plant-availability of most the nutrients higher in vermicomposting than that of composting process [3, 53, 54].

3.9. Temporal Variation in Nutrients. In the present study the percentage of OC decreased (Figure 1(a)) and that of N increased (Figure 1(b)), while the percentage of P (Figure 1(c)) and K (Figure 1(d)), and that of Ca (Figure 2(a)) and Mg (Figure 2(b)) also increased gradually in all the three vermicomposts and in the sole compost as the composting process progressed from 15 days to 60 days. Interestingly, the C/N ratio (Figure 2(c)) and C/P ratio (Figure 2(d)) in all the samples of vermicomposts and compost declined at the end of the experiment (i.e., after 60 days of processing). The nutrient contents showed significant temporal variation in vermicompost and compost of both the substrates, that is, MW (Table 3) and FW (Table 4) ($P < .001$).

The vermicompost of MW produced by *E. eugeniae* showed 177.8, 224.0, 166.7, 296.7, and 264.7% increase after 15 days of processing and 317.8, 372.0, 427.8, 887.1, and 476.5% increase after 60 days of processing in N, P, K, Ca, and Mg compared to that of the substrate, respectively, whereas it decreased by 35.9 and 52.8% after 15 and 60 days,

Table 4: ANOVA of different nutrients of vermicomposts produced by three species of earthworms and compost (Treatments) of Floral Waste across different time intervals.

Source of Variation	SS	df	MS	F
OC				
Time Intervals	76.11592	3	25.37197	28.39579**
Treatments	426.3413	3	142.1138	159.0508**
Error	8.041606	9	0.893512	
N				
Time Intervals	0.151425	3	0.050475	118.7647**
Treatments	0.649525	3	0.216508	509.4314**
Error	0.003825	9	0.000425	
C/N Ratio				
Time Intervals	1629.242	3	543.0806	67.49946**
Treatments	103.9289	3	34.64297	4.305774**
Error	72.41133	9	8.045703	
P				
Time Intervals	0.130719	3	0.043573	78.33333**
Treatments	0.127569	3	0.042523	76.44569**
Error	0.005006	9	0.000556	
C/P Ratio				
Time Intervals	4035.872	3	1345.291	636.1514**
Treatments	295.7819	3	98.59395	46.6224**
Error	19.0326	9	2.114733	
K				
Time Intervals	0.062619	3	0.020873	81.45528**
Treatments	0.072769	3	0.024256	94.65854**
Error	0.002306	9	0.000256	
Ca				
Time Intervals	2.90885	3	0.969617	23.82676**
Treatments	3.5505	3	1.1835	29.08259**
Error	0.36625	9	0.040694	
Mg				
Time Intervals	0.1621	3	0.054033	35.62637**
Treatments	0.31855	3	0.106183	70.01099**
Error	0.01365	9	0.001517	

Level of significance: $^{**}P < .001$

respectively in OC; whereas that of *E. fetida* increased by 91.1, 128.0, 127.8, 161.3, and 188.2%; and 173.3, 284.0, 338.9, 716.1, and 394.1% while decreased by 41.2% and 68.1% after 15 and 60 days of processing, respectively. The N, P, K, Ca, and Mg contents in vermicompost produced by *P. excavatus* increased by 51.1, 76.0, 83.3, 50.0 and 100.0%, respectively and the OC decreased by 50.5%, at 15 days of processing; whereas the increase was 137.8, 212.0, 283.3, 648.4 and 323.5% and the decrease was 73.1% at 60 days of processing, respectively. In compost, the increase was relatively less and was 2.2, 36.0, 38.9, 4.8, and 35.3% and 80.0, 168.0, 216.7, 572.6, and 264.7% in N, P, K, Ca, and Mg, respectively and its decrease in OC was 59.7, and 79.1% compared to that of substrate after 15 and 60 days of composting process, respectively. The C/N ratio reduction was 76.9, 69.2, 67.3, and 60.6% after 15 days of processing and 88.7, 88.4, 88.7, and 88.4% after 60 days while the C/P ratio reduction was respectively 80.2, 74.2, 71.9, and 70.4% at 15 days of processing and 90.0, 91.7, 91.2, and 92.2% at 60 days of processing in the vermicompost produced by *E. eugeniae, E. fetida, P. excavatus,* and in sole compost compared to that of the substrate.

The vermicompost of FW produced by *E. eugeniae* increased by 317.6, 254.5, 750.0, 1057.1, and 700.0% after 15 days of processing and 482.3, 545.4, 1800.0, 3285.7, and 1525.0% after 60 days of processing in N, P, K, Ca, and Mg, respectively compared to the substrate, whereas it decreased by 35.7 and 52.6% after 15 and 60 days, respectively in OC, while that of *E. fetida* increased by 200.0, 190.9, 500.0, 814.3 and 575.0% and 376.5, 400.0, 1350.0, 2728.6, and 1300%; while decreased by 44.3 and 60.9% after 15 and 60 days of processing, respectively. The N, P, K, Ca, and Mg contents in vermicompost produced by *P. excavatus* increased by 129.4, 145.4, 250.0, 285.7, and 200.0%, respectively, and the OC

decreased by 55.2% at 15 days of processing, whereas the increase was 282.3, 345.4, 1000.0, 1785.7, and 950.0% and the decrease was 68.5% at 60 days of processing, respectively. In compost, there was less increase and was 23.5, 72.7, 50.0, 28.6, and 75.0% and 141.2, 254.5, 650.0, 742.8, and 475.0% in N, P, K, Ca, and Mg, respectively, and its decrease in OC was 73.4, and 79.9% after 15 and 60 days, respectively of composting process compared to that of substrate. The C/N ratio reduction was 84.6, 81.4, 80.5 and 78.5% after 15 days of processing and 91.9, 91.8, 91.7, and 91.7% after 60 days while the C/P ratio reduction was respectively 81.9, 80.8, 81.7, and 84.6% at 15 days of processing and 92.6, 92.2, 92.9, and 94.3% at 60 days of processing in the vermicompost produced by *E. eugeniae, E. fetida, P. excavatus* and in sole compost more than that of the substrate.

The considerable enrichment of nutrients of the vermicomposts of the three species of earthworms—*E. eugeniae E. fetida* and *P. excavatus*—compared to that of composts of substrates, that is, MW and FW ($P < .01$) were in consistence with the findings of earlier reports [2, 25, 26, 30]. At the end of the experiment, the increase in OC, N, P, K, Ca, and Mg was 55.8, 56.9, 43.2, 40.0, 31.8, and 36.7% in the vermicompost of MW and 57.7, 58.6, 45.1, 60.5, 75.1, and 64.6% in that of FW produced by *E. eugeniae;* 34.5, 34.1, 30.2, 27.8, 17.6, and 26.2% in that of MW and 48.6, 49.4, 29.1, 48.3, 70.2, and 58.9% in that of FW produced by *E. fetida;* and 22.5, 24.3, 14.1, 17.4, 10.1, and 13.9% in that of MW and 36.3, 36.9, 20.4, 31.8, 55.3, and 45.2% in that of FW produced by *P. excavatus*, compared to that of sole compost, respectively.

The nutrients and OC were found higher in MW compared to that of FW, which was most probably because of mosaic nature of the MW. In all the vermicompost and compost of the present study the nutrients increased and OC, C/N ratios and C/P ratio decreased significantly with the passage of time (from 0 to 15, 30, 45, and 60 days), from the substrate (organic waste) to compost and vermicompost, respectively [2]. The present findings are in agreement with the findings of earlier workers: Nagavallemma et al. [35], Uthaiah [55], Muthukumarasamy et al. [56], Parthasarathi and Ranganathan [57], and Khwairakpam and Bhargava [58]. The waste materials ingested by the earthworms undergo physical decomposition and biochemical changes contributed by the enzymatic and enteric microbial activities while passing through the earthworm gut due to the grinding action of the muscular gizzard releasing the nutrients in the form of microbial metabolites enriching the feed residue with plant nutrients and growth promoting substances in an assimilated form, which is excreted in the form of vermi-cast [31, 59].

Comparing the nutrients of vermicompost produced by the three earthworm species (*E. Eugeniae, E. fetida,* and *P. excavatus),* it was found that the vermicompost of *E. eugeniae* possessed significantly higher concentrations of the nutrients followed by *E. fetida* and *P. excavates,* and the sole compost, in the order of *E. Eugeniae* > *E. fetida* > *P. excavatus* > compost, which may indicate that the earthworm is more efficient in recovering nutrients from the waste through vermicomposting process [2, 60]. However, the findings of Sangwan et al. [61], in contrast to the present findings, reported decrease in potassium content in the vermicompost produced by *E. fetida* compared to that of the substrate. Khwairakpam and Bhargava [58] compared the vermicompost of sewage sludge processed by these three earthworm species in order to report the suitability of worm species for composting. Reddy and Okhura [5] have assessed the vermicomposts produced by different earthworm species—*Perionyx excavatus, Octohaetona phillotti,* and *Octonachaeta rosea* using the rice straw as substrate and found that vermicompost produced *P. excavatus* possessed possessed higher concentration of nutrients than that of *O. rosea* and *O. phillotti*.

Further, it was found that the OC, N, P, K, Ca, Mg was 85.4, 164.7, 127.3, 800.0, 785.7, and 325.0%, respectively increased in MW than that of FW, and the nutrients were also significantly higher in the vermicompost and compost of MW than that of FW ($P < .05$). The vermicompost of MW produced by *E. eugeniae* showed 84.9, 76.0, 107.7, 182.3, 203.7, and 93.7% increase at 15 days and 84.8, 89.9, 66.2, 150.0, 158.2, and 50.8% increase at 60 days of processing in OC, N, P, K, Ca, Mg than that of FW; whereas the increase was 95.8, 68.6, 78.1, 241.7, 153.1, and 81.5% at 15 days of composting and 51.4, 51.8, 74.5, 172.4, 155.6 and 50.0% at 60 days in the vermicompost produced by *E. fetida*, and the increase of OC, N, P, K, Ca, and Mg (Figures 1 and 2) in vermicompost of MW produced by *P. excavatus* than that of FW was 104.7, 74.4, 62.9, 371.4, 244.4, and 183.3% and 58.5, 64.6, 59.2, 213.6, 251.5, and 71.4% after 15 and 60 days of processing, respectively. The compost of MW was higher by 181.1 and 92.9% in OC, 119.0 and 97.6% in N, 78.9 and 71.8% in P, 733.3 and 280.0% in K, 622.2 and 606.8% in Ca, and 228.6 and 169.6% in Mg after 15 and 60 days of processing, respectively compared to that of FW.

3.10. Total N. The total nitrogen content of vermicompost of the tree earthworm species was higher than that of compost and substrate. The increasing trend of N in the vermicomposts produced by the earthworm species in the present study corroborated with the findings of earlier reports [62, 63]. The enhancement of N in vermicompost was probably due to mineralization of the organic matter containing proteins [3, 8] and conversion of ammonium-nitrogen into nitrate [1, 64]. Earthworms can boost the nitrogen levels of the substrate during digestion in their gut adding their nitrogenous excretory products, mucus, body fluid, enzymes, and even through the decaying dead tissues of worms in vermicomposting subsystem [25]. The vermicompost prepared by all the three earthworm species showed a substantial difference in total N content ($P < .01$), which could be attributed directly to the species-specific feeding preference of individual earthworm species and indirectly to mutualistic relationship between ingested microorganisms and intestinal mucus [1].

3.11. OC. Total organic carbon decreased with the passage of time during vermicomposting and composting processes in both the substrates. These findings are in consistence

with those of earlier authors [12, 46]. The organic carbon is lost as carbon dioxide through microbial respiration and mineralization of organic matter causing increase in total N [65]. Part of the carbon in the decomposing residues released as CO_2 and a part was assimilated by the microbial biomass [11, 66, 67]; microorganisms used the carbon as a source of energy decomposing the organic matter. The reduction was higher in vermicomposting compared to the ordinary composting process, which may be due to the fact that earthworms have higher assimilating capacity. The difference between the carbon loss of the vermicompost processed by *E. eugeniae, E. fetida,* and *P. excavatus* could be due to the species-specific differences in their mineralization efficiency of OC.

3.12. C/N Ratio. The C/N ratios of vermicomposts of three earthworm species were around 20 : 1; such ratios make nutrients easily available to the plants. Plants cannot assimilate mineral N unless the C/N ratio is about 20 : 1, and this ratio is also an indicative of acceptable maturity of compost [68]. The C/N ratio of the substrate material reflects the organic waste mineralization and stabilization during the process of composting or vermicomposting. Higher C/N ratio indicates slow degradation of substrate [69], and the lower the C/N ratio, the higher is the efficiency level of mineralization by the species. Lower C/N ratio in vermicompost produced by *E. eugeniae* implied that this species enhanced the organic matter mineralization more efficiently than *E. fetida* and *P. excavatus* [1, 60]. The loss of carbon through microbial respiration and mineralization and simultaneous addition of nitrogen by worms in the form of mucus and nitrogenous excretory material lowered the C/N ratio of the substrate [25, 70–72].

3.13. P. The total P was higher in the vermicompost harvested at the end of the experiment compared to that of the initial substrate [8, 25, 73]. The enhanced P level in vermicompost suggests phosphorous mineralization during the process. The worms during vermicomposting converted the insoluble P into soluble forms with the help of P-solubilizing microorganisms through phosphatases present in the gut, making it more available to plants [1, 60, 74]. This was buttressed by increased trend of EC showing enhancement of exchangeable soluble salts in vermicompost of all the three earthworm species.

3.14. K. Vermicomposting proved to be an efficient process for recovering higher K from organic waste [1, 25, 73]. The present findings corroborated to those of Delgado et al. [75], who demonstrated that higher K concentration in the end product prepared from sewage sludge. The increase in K of the vermicompost in relation to that of the simple compost and substrate was probably because of physical decomposition of organic matter of waste due to biological grinding during passage through the gut, coupled with enzymatic activity in worm's gut, which may have caused its increase [76]. The microorganisms present in the worm's gut probably converted insoluble K into the soluble form by producing microbial enzymes [48].

3.15. Ca and Mg. The higher Ca content in vermicompost compared to that of compost and substrate is attributable to the catalytic activity of carbonic anhydrase present in calciferous glands of earthworms generating $CaCO_3$ on the fixation of CO_2 [60]. The higher concentration of Mg in vermicompost reported in present study was also in consistence with the findings of earlier workers [60, 77].

4. Conclusions

It is concluded that among the three species, the indigenous species, *P. excavates,* exhibited better growth and reproduction performance compared to the other two exotic species. *E. eugeniae* was more efficient in bioconversion of urban green waste into nutrient rich vermicompost compared to *E. fetida* and *P. excavatus*; the vermicompost produced by *E. eugeniae* possessed higher nutrients—N, P, K, Ca and Mg—compared to that of *E. fetida* and *P. excavatus*. Vermicomposts produced by all the earthworm species showed higher contents of nutrients compared to that of the sole compost as well as substrates—the green waste (vegetable market and floral waste). Moreover, the vermicompost and compost of vegetable market waste possessed higher nutrient contents probably because it comprised of a mosaic of materials compared to that of floral waste. Thus, vermicomposting was proved to be a better technology than that of sole composting and may be preferred for the management and nutrient recovery from the urban waste such as market waste and floral waste.

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