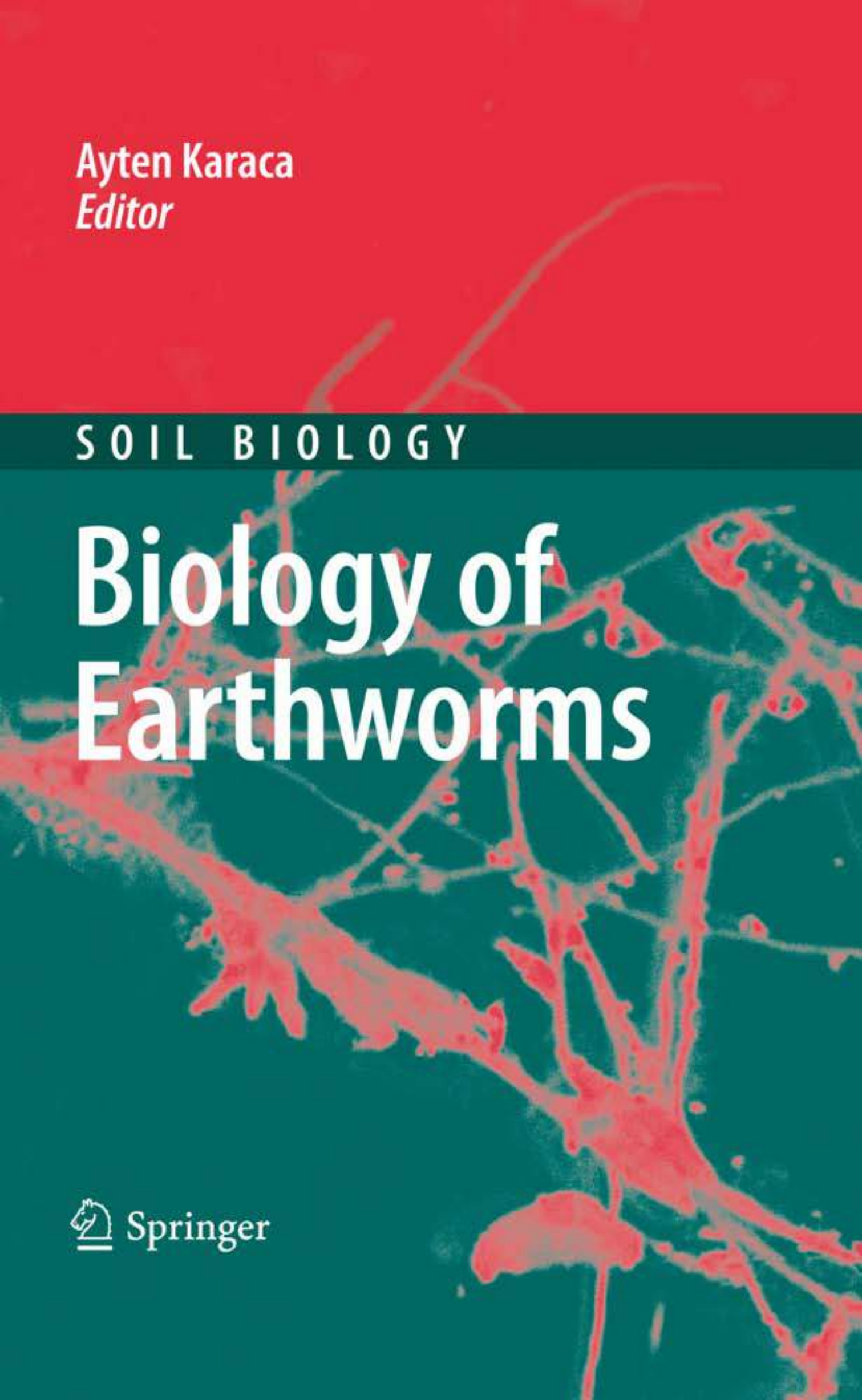
Ayten Karaca
Editor

SOIL BIOLOGY

Biology of Earthworms



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Biology of Earthworms

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Preface

Soils need energy for their biota. Most of this energy comes indirectly from the sun via the primary producers. These deliver energy-rich organic compounds into soils either in the form of litter or by direct exudation through roots. Besides microbes, earthworms are highly involved in energy and carbon cycling in soils. Finally, various compounds with biogeochemical and climatic relevance are realized from soils into atmosphere. The most important are carbon dioxide, methane, and diverse nitrous oxides such as N_2O, NO, and NO_2, often cited under the term NO_3. Organic matter is the second most important constituent in soils next to the mineral phase.

Traditionally, organic matter is subdivided into nonhumic substances and humic substances. The former encompass all nonaltered or weakly altered plant materials that are still morphologically identifiable and are composed of defined biomolecules. In contrast, humic substances represent strongly altered organic materials that do not show macroscopically identifiable structures. The process that leads to the formation of humic substances is called humification.

The soil food web is extraordinarily complex; its trophic structure and the relationships between components are poorly understood. By recycling plant material and mineralizing nutrients therein, the belowground decomposer system provides the basis for soil fertility and plant life.

Decomposition process is dominated by microorganisms with their vast array of enzymes for the breakdown of organic matter. However, the microbial environment and, therefore, the activity and composition of the microbial soil community are strongly structured and influenced by an exceptionally diverse community of soil-dwelling invertebrates. Interactions between microorganisms and soil invertebrates include not only direct predator–prey relationships but also indirect effects, such as competition for resources and habitat formation. Macrofauna species such as earthworms, millipedes, and isopods also ingest microorganisms, but in contrast to predator–prey interactions, they predominantly affect microbial life indirectly by forming their habitat. New techniques and methodological developments including molecular and stable isotope techniques provide the opportunity to analyze the

complex interactions between microorganisms and micro-and mesofauna grazers in unprecedented detail, promising fundamental progress in the near future.

The Soil Biology volume *Biology of Earthworms* has 18 chapters. Each chapter provides a general review and statement of current understanding, recent developments and advances, priorities for future research, and applications:

A general protocol of antibacterial vermipeptide preparation methods from earthworms, including crude peptide preparation and purification by ultrafiltration, ion-exchange chromatography, gel-filtration, and HPLC chromatography is discussed in Chap. 1 by Zhenjun Sun. The optimization of earthworm sampling in terms of how to sample, where to sample, and how many samples to take are expressed in detail by Jan Valckx, Gerard Govers, Martin Hermy, and Bart Muys in Chap. 2. Influences of earthworm on soil aggregate formation and soil structure is discussed in Chap. 3 by the author Yasemin Kavdir. In the next chapter (Chap. 4), Maria Jesus Iglesias Briones and Trevor George Piearce perform a comparative anatomical study of the gland of 30 earthworm species belonging to thirteen genera of the family Lumbricidae to identify the main morphotypes present and to unravel their taxonomical and ecological significance within the family.

Several issues regarding sexual selection such as the role of spermathecae, copulatory behavior, allohormone injection, or adjustment of the donated sperm volume are reviewed by Darío J. Díaz Cosín, Marta Novo, and Rosa Fernández in Chap. 5. In the following chapter, Kevin R. Butt focuses on a method to encourage the natural engineering qualities of soil-dwelling earthworms to assist soil improvement and examines one direction taken by a group of researchers who sought to develop a technique to maximize the possibility of successful soil-stimulation by the addition of earthworms.

Controlled cultivation of endogeic and anecic earthworms seek to describe methods that have been progressed to allow production of specific groups of (temperate) soil-dwelling earthworms and demonstrate how their beneficial activities can be harnessed by Kevin Richard Butt and Christopher Nathan Lowe in Chap. 7. In the next chapter (Chap. 8), Visa Nuutinen reviews the sporadic discussion, which has mainly occurred within evolutionary biology, recently within the niche construction theory, while it consists of diverse and partly opposed views.

In Chap. 9, the relationships between soil earthworms and enzymes in different extents are discussed by Ridvan Kizilkaya, Oguz Can Turgay, Sema Camci Cetin, and Ayten Karaca. They discuss Interactions in Microscale, Mesoscale, and Macroscale and also Effects of Agricultural Activities on Earthworm–Enzyme Interactions. Avril Rothwell, Keith Chaney, and Pat Haydock review the effects of conservation tillage and conventional tillage on earthworm populations in Chap. 10. The scope of present review "Assessing the Role of Earthworms in Biocontrol of Soil-borne Plant Fungal Diseases" is to evaluate the role of earthworms in controlling soil-borne plant fungal diseases by Mukesh K. Meghvansi, Lokendra Singh, Ravi B. Srivastava, and Ajit Varma in Chap. 11. Yurdagul Ersahin focuses on comprehensive utilization of vermicompost products, either solid or liquefied, for the inhibition of a variety of plant diseases and pest attacks in Chap. 12. Mohammadi Goltapeh, J. Tajbakhsh, and Ajit Varma discuss the

characteristics of vermicompost and the effect of worm castings on crop yields in Chap. 13.

Earthworm innate immune system is presented in Chap. 14 by Péter Engelmann, Edwin L. Cooper, Balázs Opper, and Péter Németh. In Chap. 15, "Earthworms A Potent Herbal Target for TCM (CAM) Research" is discussed by Yung-Ming Chang, Wei-Yi Chi, Edwin L. Cooper, Wei-Wen Kuo, and Chih-Yang Huang. In Chap. 16, Heinz-Christian Fründ, Ulfert Graefe, and Sabine Tischer give an overview of the use of earthworms as bioindicators and biomonitors. Fatma Lazrek, Velavan Thirumalaisamy Palanichamy, Jérôme Mathieu, and Lise Dupont discuss current molecular markers appropriate to address various issues of earthworm ecology in Chap. 17.

Finally, some key literature on earthworm population dynamics generally and the influences of organic farming systems in temperate climates specifically are reviewed by James Kotcon in Chap. 18.

In planning this volume, invitations for contributions were extended to leading international authorities working with Earthworms. The editors would like to express sincere appreciation to each contributor for his/her work and for their patience and attention to detail during entire production process. We sincerely hope that these eminent contributors will encourage us in the future as well, in the greatest interest of academia.

We are extremely grateful to the staff members of Springer Heidelberg, especially Hanna G. Hensler-Fritton, Editorial Director Life Sciences/Biomedicine Europe II, Dieter Czeschlik (now retired), and Jutta Lindenborn for their continued interest, critical evaluation, constructive criticism, and support. We wish to acknowledge the help and support given to us by our students, faculty colleagues, and family members for their constant encouragement.

Ankara, Turkey Ayten Karaca

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Chapter 1
Antimicrobial Vermipeptides: From Methods to Characteristics

Sun Zhenjun

1.1 Introduction

The earthworm is one of the typical saprozoic organisms, living in the environment replete with microorganisms; some of the microorganisms may be a threat to the earthworm's existence. To survive in such an environment, they have developed efficient defense mechanisms against invading microorganisms. Earthworms lack real antibodies and efficient innate immune systems to defend themselves against invading foreign materials such as vertebrates (Jay et al. 2005). Alternatively, it can be supposed that earthworms must have some active antibacterial proteins and peptides, which are different from immunoglobulin (Ig) in the defense mechanism against bacteria and other pathogens (Kauschke et al. 2007). The defense network of earthworms can be regarded as the concentric circles, whose core had the intrinsic resistance (Fig. 1.1). When microorganisms invade earthworm in an export-oriented fashion from one layer to another layer, the earthworm uses the defense mechanism to counteract the invader. In the defense mechanism of earthworms, only the antibiotic barrier and the natural immunity play the main roles. Hence, these two offer natural resistance. From Fig. 1.1, it could be also found that the antibiotic barrier of earthworms included physical elimination and mucilaginous elimination. So it could be supposed that there were some antibacterial peptides (ABP) in the skin secretions of earthworms (Wang 2005) (Fig. 1.1).

The immunity in invertebrates, such as that in vertebrates, has also been proved to involve both humoral and cellular defense mechanisms. From studies on insects, it was established that many ABP have been successfully extracted from insects. A novel antibacterial peptide was first purified from earthworms in Earthworm Biology and Ecology Laboratory of China Agricultural University in 1996

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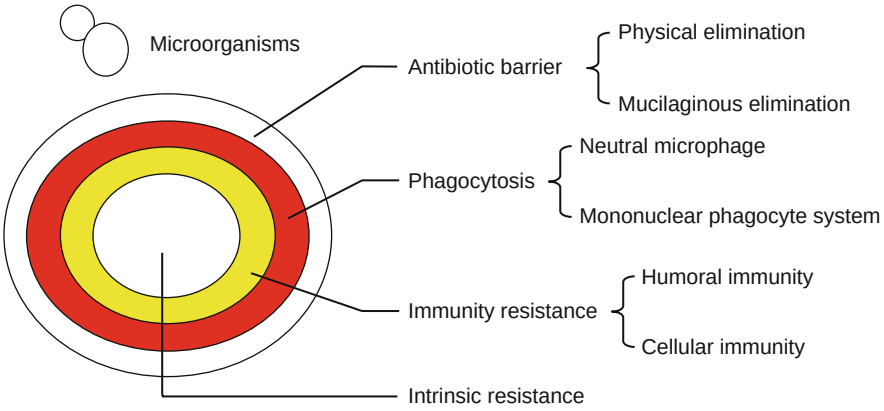

Fig. 1.1 Sketch map of earthworm immunity

(Sun 1997). Since then eight vermipeptides from the earthworm *Eisenia fetida* have been found in this laboratory. These works provided strong proof on:

- Supporting theoretical hypotheses that earthworm peptides in the coelomic fluid are the antibacterial components of the immune system and they can be induced by using different methods exhibiting nonspecific immune responses to diverse environments.
- Inducing the antibacterial peptide from the earthworm with a nonspecific immune response.
- Assessing physicochemical and antibacterial properties of earthworm ABP.
- Developing the technology and methods for preparation, isolation, and purification of ABP from coelomic fluid, tissue homogenate, and skin secretions of earthworm.

We mainly introduce those research works in this chapter.

1.2 Protocol of Antibacterial Vermipeptide Preparation and Activity Assays

How to get pure peptide from earthworm tissues is the first and important step. The following is a general protocol of antibacterial vermipeptide preparation and activity assays (see Fig. 1.2).

1.2.1 Animal and Bacterial Strains

The earthworm used in the experiment was of the species *E. fetida* and was obtained from a vermiculture farm near Beijing.

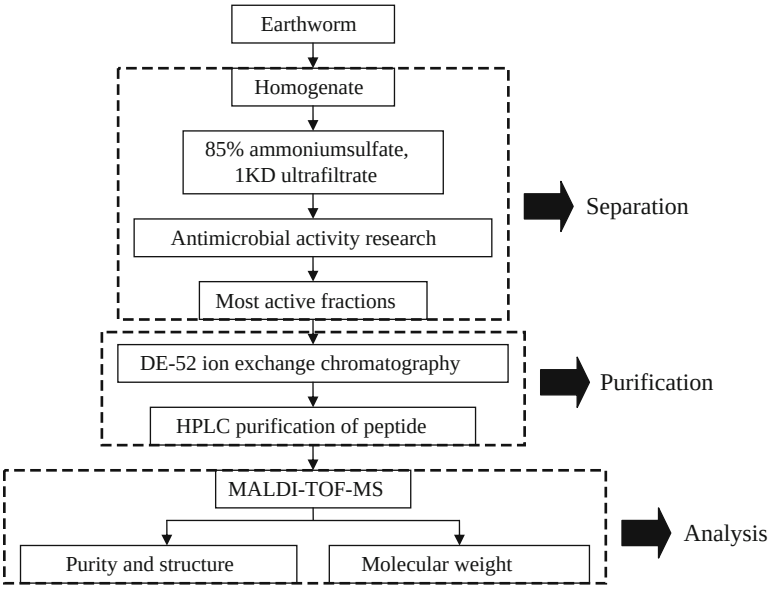

Fig. 1.2 Protocol of antibacterial vermipeptide preparation and activity analysis

Strains used for determining antimicrobial activity included *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Klebsiella terrigena*, *Enterococcus gallinarum*, *P. pyocyanea*, *Acinetobacter baumanii*, *Enterococcus faecalis*, *K. pneumoniae*, *Branhamella catarrhalis*, *Serratia marcescens*, and *K. oxytoca* obtained from the Microbial Research Lab of Veterinary Medicine College, China Agricultural University, and China–Japan Friendship Hospital in Beijing. The bacteria Luria–Bertani (LB) medium was used as the growth medium.

The ultrafiltration systems were provided from Shanghai Atomic Nucleus Institute, Shanghai Institute of Applied Physics, Chinese Academy of Sciences. The freezing drier used was FTS SYSYTEM^{TM}, TDS-3D-MP, Revco, Germany; AKTA Prime was from Pharmacia, Sweden. DE-52 was purchased from *Waterman*, Sephadex G-10 from Pharmacia, trifluoroacetic acid (TFA) from Merck-Schuchardt, and chromatography purity methanol and all the other purity chemicals from China.

1.2.2 Preparation of Crude Specimen

Earthworms were washed, dried in soft paper, and stimulated to induce them to extrude coelomic fluid through epidermal dorsal pores. Then the stimulated earthworms were washed with pH 6.8 PBS (5 mM) twice and dried in soft paper. Finally, the earthworms were homogenized and centrifuged at 10,000 g min^{-1} for 30 min. The collected supernatant was precipitated with ammonium sulfate at a final saturation

of 85% in a cooling bath on top of a magnetic stir plate and kept in a refrigerator overnight at 4°C followed by centrifugation at 10,000 r m^{-1} for 30 min at 4°C. The precipitate was collected and dissolved in pH 6.8 PBS (5 mM) then ultrafiltrated by 1 kDa molecular weight cut-off ultrafiltration membranes. The flow-through was freeze dried and dissolved with methanol, then centrifuged to collect the supernatant. The crude peptides under 1 kDa were obtained by evaporating the methanol in a vacuum condition. Finally, the antimicrobial activities of the obtained components were tested using a disk method.

1.2.3 Purification of the Peptide

1.2.3.1 DE-52 Ion Exchange Chromatography

The most active fractions were loaded on a DE-52 column (1.6 × 30 cm) previously equilibrated with a 5 mmol l^{-1} phosphate buffer solution (pH 8.0) containing 10 μmol l^{-1} EDTA. After washing with 5 mM phosphate buffer solution (pH 8.0) to the UV absorbance and returned to the baseline, the absorbed peptides were eluted with a linear gradient of NaCl (0–500 mM) in 5 mM PBS (pH 8.0) at a flow rate of 24 ml h^{-1}. The elution profile was monitored at 220 nm. The fractions from each peak were pooled and lyophilized to dry, then desalted with methanol as described earlier. The peptides in the methanol were dried with a vacuum pump and stored at −30°C. The antibacterial activity of the fractions of both the unabsorbed and eluted fractions by NaCl solution was evaluated using the disk method.

1.2.3.2 Gel Filtration

Fractions with antibacterial activity, after DE-52 ion exchange chromatography, were placed on a column of Sephadex G-10 (2 × 100 cm) in a 50% (v/v) methanol solvent. They were dissolved up to 250 mg of the active fraction in 10 ml of 50% (v/v) methanol. Then the active fraction was loaded gently onto the surface of the column. After absorption, 3 ml of 50% (v/v) methanol was added, by gently washing the walls without disturbing the column bed. Then it was eluted with 50% methanol at a flow rate of 18 ml h^{-1}, and the absorbance at 220 nm was monitored, then the fractions and lyophilized were collected to dry. Finally, the antibacterial activity of the fractions was tested again using the disk method.

1.2.3.3 HPLC Purification of Peptide

The active fraction after the Sephadex G-10 gel filtration was further purified by high-performance liquid chromatography (HPLC) on a 3.9 × 300 mm Delta Pak C18 column (Millipore) connected to a Agilent 1100 HPLC system with a simple

linear gradient from 0.1% (v/v) TFA to 70% (v/v) acetonitrile (+0.1% (v/v) TFA) at a flow rate of 0.8 ml min^{-1} at ambient temperature. The elution pattern was monitored at 220 nm. The biggest peaks were collected, lyophilized to dry, and assayed for antibacterial activity using the disk method. The purity of the active fraction after HPLC was assessed by C18 reversed phase HPLC and eluted with 70% ethanol, 30% water, at a flow rate of 0.8 ml min^{-1}, and detected at 220 nm.

1.2.4 Assessment of Peptide Characters

1.2.4.1 Molecular Weight Determination

The exact molecular mass of the purified peptide was determined by matrix-associated laser adsorption ionization (MALDI) mass spectroscopy. Approximately, 20 nmol of the lyophilized peptide was dissolved in 50% acetonitrile (v/v) containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removing the solvent in warm air, the peptide was adsorbed by the Pt probe, and was applied to a vacuum chamber and analyzed.

1.2.4.2 Amino Acid Sequence Determination of the Isolated Peptide

The amino acid sequencing of the purified peptide was performed by MS/MS at the Academy of Military Medical Sciences.

1.2.4.3 Antibacterial Assays

Disk method: bacterial cells were grown overnight in a LB media and inoculated into 5 ml of molten 0.6 g l^{-1} LB agar with a final concentration of 10^7 colony-forming units per ml, which was overlaid on a 90-mm Petri dish containing 10 ml solidified 2 g l^{-1} LB agar. After the top agar hardened, it was placed on a sterilized blotting paper (about 6 mm in diameter), which is free from any antibacterial activity, and was impregnated with 20 μl of the fractions to be tested and placed on agar dishes inoculated with one bacterial strain. The dishes were incubated overnight at 37°C. Control tests were performed with papers impregnated with PBS. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 6-mm papers.

Antimicrobial activities of the raw coelomic fluid of the earthworm both crude and purified peptides were obtained and tested.

1.2.4.4 Antitumor Activity Assays

Cancer cells, MGC803 and HeLa, were provided from China–Japan friendship Hospital, Beijing; RPMI 1640 and DMSO were from GIBCOBRL products; BIO-RAD 3550 was made in Sweden and the scanning electronic microscope was made in Japan.

The antitumor activity of peptides of AVPF were probed with methods of 3-(4,5 dimethylthiazol-2yl)-2,5 (diphenyltetrazoliumbromide) (MTT) and were observed using the scanning electronic microscopy. The cancer culture solution, having a living cell concentration of 0.5–1 $\times$ 10^5 ml^{-1}, was inoculated on cell culture board with 96 holes (100 μl $hole^{-1}$) and then cultured in a cell member of 10^6 ml^{-1}. A crude antibacterial peptide was dissolved and diluted with the solution and poured into the holes (25 μl $hole^{-1}$) and continuously calculated in conditions of 5% CO_2 and 37°C temperature for 24 and 48 h to observe and calculate abnormal cancer cell members under the scanning electronic microscope.

MTT staining method and AO/EB fluorescent staining method was used for the determination of the apoptotic effect of ABP on HeLa cells (Liu 2003).

1.2.4.5 Antiviral Activity Assays

Baby hamster kidney (BHK) cells, pseudorabies virus (PRV), and HeLa cells were provided and stored in wet virus lab of China Agricultural University. Minimum essential medium (MEM) was purchased from GIBCO Company, USA, Dulbecco's modified eagle medium (DMEM) was from Hecolyne, and MTT was from Sigma.

Normal BHK cells were inoculated on cell culture boards with 96 holes. When the cells developed well with a cell layer, a crude peptide was dissolved and diluted with 1% MEM and poured into the holes (200 μl $hole^{-1}$). The board was incubated in a 5% CO_2 incubator at 37°C to observe the cytopathic effect (CPE) under the microscope and to confirm the minimum drug concentration to make CPE and the maximum drug concentration without CPE.

The antiviral effect of antimicrobial peptides on PRV was detected using the CPE inhibition test (Yin and Liu 1997). Twenty microliters of PRV solution per hole (100 tissue culture infection dose 50) was used to poison BHK cells. The inhibition rate of CPE was recorded at 24, 48, 96 h, respectively.

1.3 Antibacterial Characteristics from Coelomic Fluid to Pure Peptide

1.3.1 Antibacterial Effects of Raw Coelomic Fluid of Earthworm

Antibacterial effects of raw coelomic fluid from earthworm were unstable on different strains of *E. coli*, *P. aeruginosa*, and *Bacillus subtilis*. The biggest antibacterial activity was found against *P. aeruginosa*, with a 1.53 cm diameter of inhibiting zone. But it had no effect on *E. coli* and *B. subtilis*. The minimal inhibitory concentration (MIC) of raw coelomic fluid to *P. aeruginosa* was 6.25 mg ml^{-1}. In addition, the protein compositions of raw coelomic fluid were studied using the SDS-PAGE method. The results showed that the main protein or

the abundant protein in raw coelomic fluid were those having a molecular weight of 43, 40, 33.8, 25.6, 22.1, 20.6, 14.2, and 6.6 kDa. Hemolytic assay showed that raw coelomic fluid had hemolytic activity (Sun 1997).

1.3.2 Antibacterial Effects of Earthworm Crude Antibacterial Peptides

The raw coelomic fluid was precipitated with ammonium sulfate at a final concentration of 85% (w/v), then was ultrafiltrated by 1 kDa molecular weight cut-off ultrafiltration membranes, and the obtained components were named earthworm crude antibacterial peptides (ECP). The antibacterial activity and MIC of ECP to *P. aeruginosa* was detected using the standard micro-dilution method. The diameter of the inhibiting zone of ECP was 16.8 mm. The MIC of ECP3 to *P. aeruginosa* was 0.018 mg ml^{-1}. Hemolytic assay showed that ECP had no significant hemolytic activity.

1.3.3 Antibacterial Effects of Pure Antibacterial Peptides

After ammonium sulfate precipitation and ultrafiltration (<1 kDa), crude ABP were isolated and purified step-by-step by a DE52 ion exchange, Sephadex G-10 column chromatography, and C-18 reversed phase HPLC. Six peptides from ECP were obtained from earthworm tissue, homogenate liquid, coelomic fluid, and skin secretions, which were characteristic of strong antimicrobial specialty (see Table 1.1). Among them there were at least three sorts of shot peptides composed of 5–7 amino acids, with the same or similar antimicrobial specialty and amino acids sequence, Ala-Met-Val-Ser-Gly. We concluded and named this shot peptide group as a novel antibacterial vermipeptides family (AVPF), according to the structural characteristics of these ABP (Wang et al. 2007).

Table 1.1 Antimicrobial peptides obtained from *Eisenia fetida*

Name	AA sequence	AA residues	Molecular weight (Da)	Origin from	Antimicrobial character
EP1	/	40	4,832	Whole body	Bacteria, fungi
EP2	*Ac-Ala-Met-Val-Ser-Ser*	6	535.27	Tissues	Bacteria
EP3	*Ac-Ala-Met-Val-Gly-Thr*	6	519.27	Tissues	Bacteria
EP4	/	/	ab.20000	Tissues	Bacteria
EP5-1	*Ala-Cys-Ser-Ala-Gly*	5	510.80	Coelomic fluid	Bacteria
EP6-1	/	50	5814.32	Skin secretions	Bacteria

1.3.4 Antimicrobial Chart of AVPF

Peptides of AVPF performed wide antibacterial activity not only toward bacteria but also toward fungi. The MIC of peptides against several Gram-positive and Gram-negative bacteria was determined by incubating approximately 10^4–10^5 CFU ml^{-1} of the cells with serial dilutions of peptides in a 96-well microtiter plate. The MIC of EP2 and EP3 against *E. gallinarum*, *P. pyocyanea*, *A. baumanii*, *K. terrigena* was 11.4 and 12.85 mg l^{-1}, respectively, and against *E. faecalis* was 22.8 and 25.68 mg l^{-1}, respectively, but there was no function against *C. albicans* (see Table 1.2)

1.3.5 Antitumor Activity of AVPF

The antitumor activity and mechanism of peptides of AVPF were probed with MTT and by the observation using the scanning electronic microscopy. Results showed that EP3 played a part in the antitumor activity of MGC803 cells and morphological changes were observed at the same time (see Fig. 1.3). Shot peptides with 5–7 amino acid residues could form multimers in the PB buffer, which was a more likely an explanation for the antibacterial mechanism of EP3.

In another experiment, the effect of crude antimicrobial peptide (EP5) specimens from earthworm coelomic fluid on HeLa cells was studied using the MTT staining method, AO/EB fluorescent staining method, and DNA agars electrophoresis. The results showed that the effects of EP5 on HeLa cells lead to the apoptosis and break down of the cancer cells (see Fig. 1.3, yellow color).

By investigating and taking count of each 100 HeLa cells, the apoptotic cell rate was found to be 60.20, 48.12, 9.65, and 8.97%, respectively, in the concentrations of 1.5, 0.75, 0.375, and 0.1875 mg ml^{-1} of EP5. The control was 1.34% in apoptotic rate of HeLa cells. The apoptotic cell rate in groups of 1.5 and 0.75 mg ml^{-1} was significantly higher than that of the control group $p < 0.01$.

Table 1.2 Antibacterial activity of EP2 and EP3

Item	*Candida albicans*	*Enterococcus gallinarum*	*Pseudomonas pyocyanea*	*Acinetobacter baumanii*	*Enterococcus faecalis*	*Klebsiella terrigena*
A	+	−	−	−	−	−
B	+	−	−	−	−	−
C	+	−	−	−	+	−
D	+	++	+	++	+	++
a	+	−	−	−	−	−
b	+	−	−	−	−	
c	+	−	−	−	+	−
d	+	++	+	++	+	++

Different concentration of EP2: A 46.7 μg ml^{-1}; B 22.8 μg ml^{-1}; C 11.4 μg ml^{-1}; D 5.7 μg ml^{-1}
Different concentration of EP3: a 52.6 μg ml^{-1}; b 25.68 μg ml^{-1}; c 12.58 μg ml^{-1}; d 6.42 μg ml^{-1}
+++ the same with that of contrast; ++ remarkably less than contrast; + growing little; − no growing

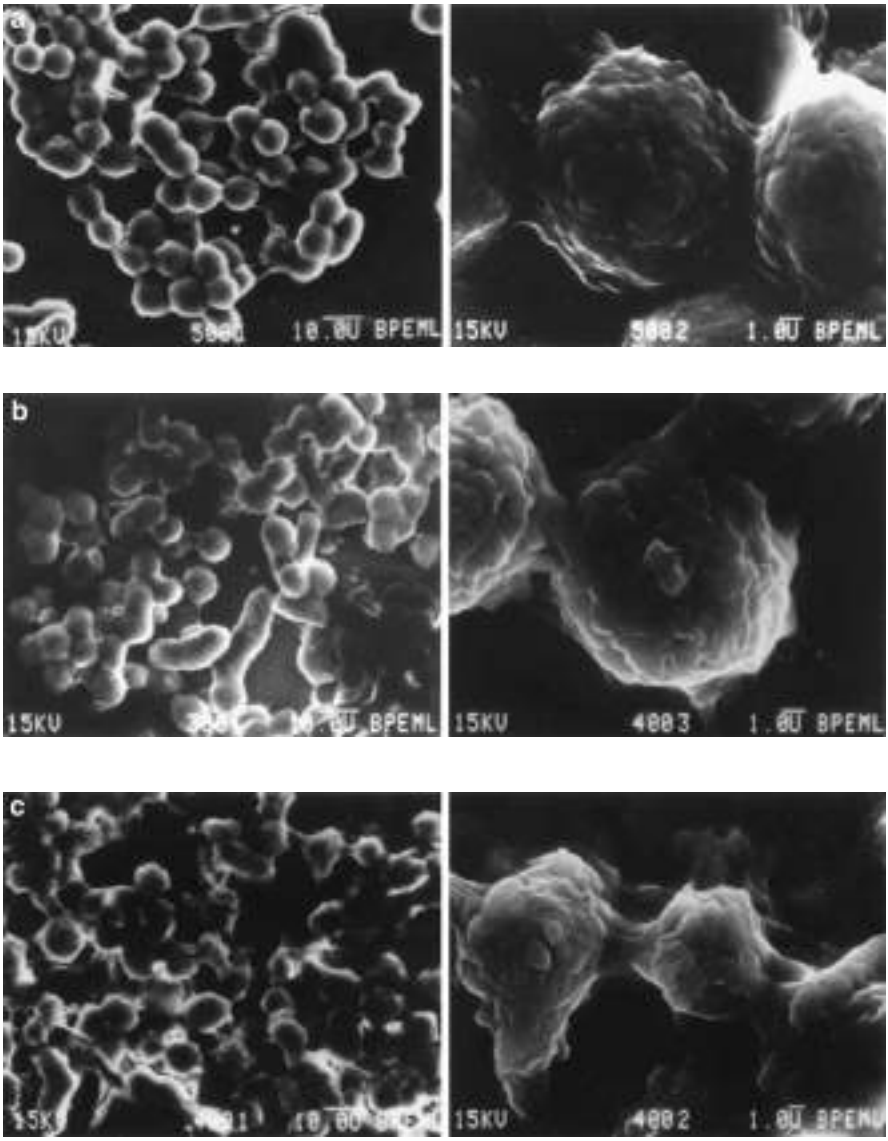

Fig. 1.3 (**a**) Scanning electron microscopically observation on MGC803 cells, which was treated without antibacterial peptides. (**b**) Scanning electron microscopically observation on MGC803 cells treated with EP3 for 24 h. (**c**) Scanning electron microscopically observation on MGC803 cells treated with EP3 for 48 h

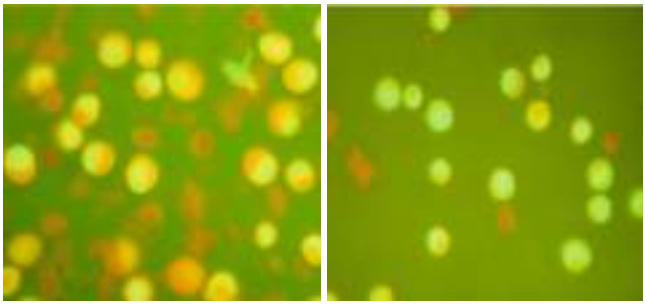

Fig. 1.4 Effects of EP5 to HeLa cell under fluorescence microscope

1.3.6 Antiviral (PRV) Activity

An antiviral effect of EP5 was detected using the CPE inhibition test (Fig. 1.4). The results showed that EP5 could inhibit the CPE leaded with PRV on the BHK cells. The antiviral effect was dose dependent and had a relationship to the treatment time. The inhibition rate of CPE was very high with a reaction in 96 h (Liu et al. 2005).

1.4 Inducement of Peptides

In organisms, ABP are an important defense component. Many ABP have been reported in various invertebrates and vertebrates (Caciancich et al. 1994; Lehrer et al. 1993; Nakamura et al. 1988; Wang et al. 2003). It has been reported that the level of ABP was determined by inducement and gene expression (Wang 2005; Ju et al. 1998; Sun 1997; Zheng et al. 2009). In recent year, some researches and developments on antibacterial peptide were focused on humans as a functional preparation (Naïma et al. 2008; Lee et al. 2010; Elbarbary et al. 2010). In this section, we introduced methods to induce peptide and discussed some characteristics of inducible peptides.

1.4.1 Inducement Methods and Effects of Vermipeptide

1.4.1.1 Bacterial Treatment

The strain employed for antimicrobial substance induction and assay was *E. coli*. D_{31} (antistreptomycin). *E. coli* culture in mid-logarithmic phase is referred to Sumida's methods. Induction methods: Live *E. coli* injection, exposure of earthworm to *E. coli* suspension, dead *E. coli* injection, and exposure of earthworm to suspension of dead *E. coli*.

1.4.1.2 Physical Treatment

These treatments were used including (1) γ-ray induction: radiation dosages are 10, 20, 50, 100 GY; (2) Ultrasonic wave induction: inducing powers are 50, 70, 100 W; (3) Electric vibration induction: inducing intensity is slow, medium, and fast speed vibration; (4) Mechanical injury induction: puncture on one side of the body wall, puncture on both sides of the body wall, cutting worm body into two pieces.

1.4.1.3 Chemical Treatment

Chemical treatment included the following: (1) Heavy metal induction: worm substrate mixed with $CdCl_2$, (concentration in substrate 20 and 200 mg kg^{-1}); worm substrate mixed with Cu_2SO_4, (concentration in substrate 50 and 400 mg kg^{-1}); (2) EDTA solution induction: substrate mixed with EDTA solution, (concentration in substrate 10^{-2} and 10^{-5} M); (3) L-Cys solution induction: substrate mixed with L-Cys solution, (concentration in substrate 10^{-2} and 10^{-5} M); and (4) DLMT solution induction: substrate mixed with DLMT solution, (concentration in substrate 10^{-6} and 10^{-9} g ml^{-1}).

Extraction of worm antimicrobial substance as per the method described above after 72 h by induction for comparison of their electrophoretic antimicrobial bands.

1.4.2 Characteristics of Induced Peptides

1.4.2.1 Antibacterial Vermipeptide Inducement

Because of good heat stability of antibacterial vermipeptides, mixed proteins were minimized by extraction of coelomic fluid by heat treatment and ultrafiltration. Protein concentration defected by Folin-phenolic method showed crude extract protein concentration of 185 mg ml^{-1} in coelomic fluid, and after heat treatment and ultrafiltration, its protein concentration was found to be 6.2 mg ml^{-1}. The elution profile monitored at 254 and 280 nm revealed five peaks, of which the peak four showed maximum antibacterial activities and was named $A_{3\text{-}4\text{-}2}$. On freeze-drying, $A_{3\text{-}4}$ 23 mg white powder was recovered and 15 mg was dissolved and applied to iron-exchange chromatography, DEAE-cellulose DE_{52} column. $A_{3\text{-}4}$ gave two fractions and the second fraction showing antibacterial activities was named $A_{3\text{-}4\text{-}2}$. Contents of tube numbers 27–33 were pooled and named $A_{3\text{-}4\text{-}2}$, and were subjected to desalting treatment by chromatography Sephadex G-10 column. It was found to be free from any salt contamination. $A_{3\text{-}4}$ was isolated into three peaks by HPLC and $A_{3\text{-}4\text{-}2}$ was isolated into two peaks. A control was run with 0.1 ml 0.01 M Tris in the same conditions, to get a peak whose profile and retention time is same as the second peak of $A_{3\text{-}4}$. The result suggested that the $A_{3\text{-}4\text{-}2}$ has been purified. $A_{3\text{-}4\text{-}2}$ is composed of 40 amino acid residues belonging to 11 kinds of

Table 1.3 Bacteriostatic activity comparison of vermi-antibiotics on various bacteria

The diameter of inhibitory ring (mm)	*E. coli*	*Staphylococcus aureus*	*Xanthomonas campestris*	*Erwinia carotovora*	*Agrobacterium tumefaciens*
Crude fluid extract (A)	4.4	4.3	4.1	4.9	3.8
A_3	4.3	3.5	4.5	3.3	–
$A_{3\text{-}4\text{-}2}$	4.32	3.21	4.43	3.4	–

amino acids, and among them acid amino acids constitutes 30% of the total amino acid and alkaline amino acids constitute only 5% of the total amino acid. Level of Gly was 25% and Val, Met, Pro, Arg, Ile, Tyr, Phe were absent in the total amino acid. It is estimated that the molecular is a unit of polypeptide for its 40 amino acid residues. Molecular weight of the $A_{3\text{-}4\text{-}2}$ was calculated on the amino acid residues composition to be 4,538 Da. Its isoelectric point is at pH 3 or 4 by isoelectrofocusing electrophoresis. It suggests that the $A_{3\text{-}4\text{-}2}$ antibacterial peptide from earthworm is a Gly-rich acid fortypeptide.

1.4.2.2 Spectrum of Fortypeptide

Spectrum of earthworm ABP in mixture and pure types is showed in Table 1.3. In Table 1.3, it shows that the diameter of inhibitory ring of *Erwinia carotovora* is the biggest, and inhibitory ring of *Xanthomonas campestris* as well as *E. coli* are the second, and that of *Staphylococcus aureus*'s is the smallest. But A has strong inhibitory effect on *S. aureus*. With the enhancing purity from A, A_3, to $A_{3\text{-}4\text{-}2}$, the bacteriostatic activity on various bacteria is depressed except on *X. campestris*. This showed that earthworm ABP have wide antibacterial chart, there are differences among various bacterial strains in their antibacterial efficiency. The substance A has obvious inhibitory effect on *Agrobacterium tumefacien*, which can cause the formation of plant tumor tissue, but A_3, and $A_{3\text{-}4\text{-}2}$ have no effect on this bacterium.

The antibacterial activity of $A_{3\text{-}4\text{-}2}$ was obliviously reduced and bacteriolysis was almost lost after heat treatment. The results showed that there are differences between A_3 and $A_{3\text{-}4\text{-}2}$ about their physicochemical properties and antibacterial activity, although both of them were derived from the last moving antibacterial substance PAGE gel. It may be other materials simultaneously taking part in the antibacterial function apart from ABP. It is very important to get full knowledge of earthworm's antibacterial mechanism and for these further studies on the structure and function of antibacterial components in total of substance A is essential.

1.4.2.3 Evaluation of Different Inducements for Antimicrobial Peptides

The PAGE inhibitory bands of antibionts from earthworm extract fluid in different *E. coli* treatments showed that inducing treatment by live *E. coli* injection could

produce maximum antibacterial substance and then it followed by dead *E. coli* injection and by live *E. coli* suspension. The low effect was found with dead *E. coli* suspension.

In physical treatments, the figure of PAGE inhibitory bands showed that all physical treatments had good effect on producing antibacterial substance, and the best methods were causing mechanical injury. The stronger the stimulation to earthworms the higher the antibacterial activities of the material. But the stimulation to earthworms must be in the limited level such that it can tolerate the shock.

In different chemical treatments, the best method was by Cd inducement. Then it was followed by DLMT, L-Cys, and $CuSO_4$. The least effect was found with EDTA. The inducement factor was dosage dependent. But earthworm was sensitive to high concentration of $CuSO_4$, and at 95% $CuSO_4$ earthworm could not survive.

The proportions and activity of component have relation with inducing sources, and have positive proportion with inducing intensity, which can provide a method for us to induce a certain component through designed induction way. For example, we can induce A1 with heavy metal induction. It was tested by the experiment that the best effect on inducing earthworm is to be at its tolerance level. It is interesting that DTMT is a good inducing agent, which not only can unveil the immune response of earthworm to pesticide DTMT but also put up a new question for studying the eco-toxicology of pesticide mechanism. Heavy metal Cd often causes soil pollution and can induce ABP. Cd can induce earthworm producing MT. Earthworms have the function of resisting and enriching metal at the same time, and the function can be gradually improved by adding the interface between earthworm and metal. The reasons for earthworm's response are not clear.

1.4.2.4 Comparison of Antibacterial Components of Coelomic Fluid Extraction

There are significant differences in components of induced substances derived from different methods. The A3 was mainly acted by live *E. coli* injection and DLMT, the A2 by physical treatments and the A1 by EDTA. Heavy metal can affect the quantity of the A3, A1, and A2. It may be that certain specific antibacterial substances are induced by different adopted methods.

Antibacterial substances can be induced by almost all inducing treatments, but there is a significant difference in their mode of induction. The best methods are live *E. coli* injection, mechanical injury, and heavy metal treatment. In the limited scope that earthworm can tolerate, the stronger the stimulation to earthworms the higher the antibacterial activities of the compound.

The study has demonstrated that earthworm can defend against invading exotic microorganisms by producing ABP. Earthworm's immune response is nonspecific unlike other invertebrates; earthworm is more easily hurt and invaded by bacteria than other higher animals. During the process of evolution, to survive in the adverse environment, it was essential for earthworms to produce immune active substances by nonspecific immune methods. Three components of antibacterial substances can

be induced by different treatments. Whether these components are synthesized by different genes or by a precursor composed of one or several genes are yet to be assessed.

1.5 Antibacterial Vermipeptide Family Found

The research of earthworm active protein was early reported in 1995 (Ukena et al. 1995). Since then, more earthworm active proteins and peptides were found frequently as the continual renovation with the microanalysis technology of protein and the foundation of biology activation determine method. Alexandra and Marguerite (1997) had found that the coelomic fluid of the *E. andrei* exhibited strong hemolytic activity against the erythrocytes of various mammalian species and bacteria, which was mediated by two proteins called fetidins, which are of apparent molecular masses 40 and 45 kDa (Alexandra and Marguerite 1997). Cho et al. (1998) found a novel antimicrobial peptide from *Lumbricus rubellus*, which was purified to homogeneity by a heparin-affinity column and C18 reverse-phase HPLC and named lumbricin I. In vitro, lumbricin I showed antimicrobial activity against a broad spectrum of microorganisms without hemolytic activity. Lumbricin I was a proline-rich antimicrobial peptide of 62 amino acids (15% proline in molar ratio; molecular mass, 7,231 Da), whose complete sequence was determined by a combination of peptide sequence and cDNA analysis (Cho et al. 1998). And two novel ABP named F-1 and F-2, which molecular weight were 535.27 and 519.27 Da, respectively, were isolated and purified from tissue of *E. fetida* by the following steps: ammonium sulfate precipitation, ultrafiltration, cation-exchange chromatography, and reverse FPLC in 2003. Both F-1 and F-2 performed a high-efficient antibacterial activity. On the contrary, a novel antibacterial short peptide named ECP_5-1 with the molecular mass of 510.8 Da was purified from the coelomic fluid of *E. fetida* by five steps, which included ammonium sulfate precipitation, ultrafiltration, DE52 ion exchange, Sephadex G-10 column chromatography, and C-18 reversed phase HPLC techniques (Liu et al. 2004). The primary structure of ECP_5-1 was Ala-Cys-Ser-Ala-Gly, which was determined by TOF MS-MS. Most of the above peptides were isolated and purified from the coelomic fluid and alimentary canal of the earthworms. Antibacterial peptide, named ESP-1, was isolated and purified from the earthworm's skin secretions (Wang 2005). This was the epidermal secretion and was exposed to the external environment directly and could form the foundation for the mechanism of earthworm immunity and furthermore for the chemotherapy of inflammation disease and (Table 1.4) antimicrobial peptides, which have been found in earthworms usually having amino acid compositions over 40 residues. A novel peptide family isolated from *Eisenia foetida*, as described in Table 1.2, was composed of only 5–7 residues that have the same or similar primary structural homology, AA sequence Ala-Met-Val-Ser-Gly (Wang et al. 2007). As short peptides have the obvious advantage of easy chemical synthesis and the new structural modification, it should be easy to synthesize artificial peptide

Table 1.4 Vermipeptides found in earthworms

Peptide name	Amino acid sequence	Molecular weight	Reference
Eisenia foetida tetradecapeptide	CFKDGAADRISHGF amide	1476.6	Ukena et al. (1995)
Pheretima vittata tetradecapeptide	GFRDGSADRISHGF amide	1521.0	
Fetidin I	/	40 kDa	Alexandra and Marguerite (1997)
Fetidin II	/	45 kDa	
Fetidin	MSSRAGIAEGYEQIEYDVVAVWKEGYVYENRGSTS VEQKIKITKGMRNLNSETKTLTASHSIGSTISTGDIFEI ATVDVSYSYSHEESQVSMTETEVYESKEIEHTITIPPT SKFTRVQLNADVGGADIEYMYLIDEVTPIGGTLSIPQ VIKSRAKILVGREIYLGETEIRIKHADRKEYMTVVSR KSWPAATLGHSKLYKFVLYEDMYGFRIKTLNTMYS GYEYAYSSDQGGIYFDQGSDNPKQRWAINKSLPL-RHPGDVVTFMVKYFTRSGLCYYDGATDVYCLD KREDKWILEVVKP	40 kDa	Lassegues et al. (1997)
Lumbricin I	22nd residue as Phe-Ser-Lys-Tyr-Glu-Arg-Gln-Lys-Asp-Lys-Arg-Pro-Tyr-Ser-Glu-Arg-Lys-Asn-Gln-Tyr-Thr-Gly	7,231	Cho et al. (1998)
Lumbricin I	MSLCISDYLYLTLTFSKYERQKDKRPYSERKNQYTGPQFL YPPERIPPQKVIKWNEEGLPIYEIPGEGGHAEPAA		Ju et al. (1998)
The GGNG peptides (*Perinereis vancaurica)*	/	734.72	Matsushima et al. (2002)
EP1	/ (40 amino acid)	4,832	Sun (1997)
EP2	*Ac-Ala-Met-Val-Ser-Ser*	535.27	Zhang (2002)
EP3	*Ac-Ala-Met-Val-Gly-Thr*	519.27	Zhang (2002)
EP4	/	About 2,0000	Zhang (2002)
EP5	*Ala-Cys-Ser-Ala-Gly*	510.80	Liu et al. (2004)
ESP-1	/ (50 amino acid)	5814.32	Wang (2005)

analogs with a known AA sequence. But chemically synthesized peptides did not show any functions against microbes (Xuelian et al. 2007).

As usual, it is almost impossible to form a second structure in a peptide chain with 5–7 AA residues. Perhaps there would be two possibilities in the functions of short peptides: direct antibacterial function and indirect function as a signal peptide. We found there is a cysteine residue in peptide EP5. Antibacterial mechanism of EP5 may be directed to enter into cells to influence duplication and synthesis. Another mechanism was to form multimers by the connection of cysteins with disulfide bonds, and also to make a hole in the cell membrane so as to induce the movement of the contents out of the cell. The antitumor activity of EP3 to MGC803 cells and the morphological changes of the cell observed may be supported in the latter mechanism.

1.6 Conclusion

Besides a general experimental protocol of ammonium sulfate precipitation, ultrafiltration, ion exchange, Sephadex G-X column chromatography, and C-18 HPLC techniques, it made simple and economical to get pure vermipeptides from coelomic fluid by using the character of good heat stability of antibacterial vermipeptides. More than 97% impure, mixed proteins were deposited from coelomic fluid by heat treatment, and then through different ultrafiltration steps, the goal peptide with molecular weight less than 1 or 10 kDa can be easily prepared.

The antibacterial characteristics of vermipeptides presented multifunctions in antibacterial spectrum and speciality. They performed antimicrobial activities not only against Gram-positive and Gram-negative bacteria but also against cancer cells and virus. It may be a mechanism for earthworm to adapt to varying environment, producing wide antimicrobiological chart of antibacterial peptide as a nonspecific immune response.

An AVPF was isolated from *E. foetida* containing 5–7 amino acids residues with the same or similar sequence of Ala-Met-Val-Ser-Gly. According to their structure and antibacterial characteristics, they will have wide use in medicine and agriculture as a new type antibiotic.

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Chapter 2
Optimizing Earthworm Sampling in Ecosystems

Jan Valckx, Gerard Govers, Martin Hermy, and Bart Muys

2.1 Introduction

To quantify the role of earthworms in ecosystems, a precise and accurate estimation of their diversity, abundance and biomass is needed. To date, a diverse set of earthworm sampling methods is available, and a diverse amount of chemical expellants have been used to greater or lesser success. Earthworm ecologists have so far often relied on expert judgement or past experience when it comes to spatial sampling designs and determination of sample size. However, based on the ever increasing data available in the literature, we start to understand the spatial organisation of earthworm populations. Moreover, straightforward techniques exist to assess earthworm species richness and the corresponding sampling effort needed to capture it, but so far these were not used in earthworm ecology.

In this chapter, we contribute to the optimization of earthworm sampling in terms of (1) how to sample, (2) where to sample and (3) how many samples to take.

First, we assess optimal concentrations of chemical expellants (allyl isothiocyanate (AITC) and mustard) recently recommended for earthworm sampling. The efficiency of these vermifuges is then evaluated against formalin application using a combined earthworm sampling method (extraction followed by hand sorting). Practical considerations are discussed.

Like many living organisms, earthworm populations are neither uniformly nor randomly distributed, but exhibit an aggregated distribution in patches. The range of spatial autocorrelation in these patches is an important variable to consider in spatial sampling designs. Based on a literature overview, guidelines for spatial sampling design are presented.

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Finally, species rarefaction curves are used to determine the optimal sample size to accurately represent earthworm diversity.

2.2 How to Sample? Optimizing Earthworm Sampling Methods

An array of earthworm sampling methods is available, basically belonging to two types: (a) passive (termed physical by Bouché (1969)), where the earthworms are physically sorted from soil, litter and other habitats; and (b) behavioural (termed ethological by Bouché (1969)), where the earthworms are captured after they are moved out from cover (Lee 1985), or a combination of both.

Isolating and hand sorting a soil sample of known volume from the bulk soil has been for a long time the reference sampling method. But this method is labour intensive and time consuming, especially in wet and heavy soils (Bouché and Aliaga 1986; Satchell 1971), and it is impractical in the case of rocky soils, in the presence of a dense root mat or when the study site should remain undisturbed (Bouché and Gardner 1984; Gunn 1992). Anecic species living in permanent vertical burrows (e.g., *Lumbricus terrestris* L.) can reside in or escape to deeper soil layers, making the method inefficient for such species (Bouché and Gardner 1984; Bouché and Aliaga 1986; Chan and Munro 2001). Moreover, cocoons and small juveniles are always underestimated by hand sorting (Bouché and Gardner 1984). Some of these limitations are overcome by washing and sieving the soil samples (Bouché and Beugnot 1972), but this again is a very time (and water) consuming activity.

As an alternative method, earthworms are commonly extracted from their habitat by using chemical expellants. Amongst others, formalin has become the standard vermifuge of the last decades after Raw (1959) demonstrated its superiority over hand sorting. However, extraction efficiency may strongly vary, which may be attributed to deleterious effects of the expellants used, earthworms not being adequately exposed to the chemicals (e.g., due to burrow orientation), or earthworms being unable to react to the irritant substances (e.g., due to low temperature, dormancy) (Daniel et al. 1992). Therefore, efficiency declines from epigeic, to anecic, to endogeic reflecting species behaviour and burrow orientation (Bouché and Gardner 1984).

Despite that its use is recommended in ISO standard ISO/DIS 23644-1 (Rombke et al. 2006), formalin is toxic not only to the earthworms but also to the treated soil system, and its carcinogenic nature poses serious health risks to people working with it (Gunn 1992; Eichinger et al. 2007). Recently, suspensions of prepared mustard (containing variable additives such as vinegar, citric acid, etc.) (Chan and Munro 2001; East and Knight 1998; Gunn 1992) and of dry mustard powder (Chan and Munro 2001; Lawrence and Bowers 2002) were tested as alternative expellants. Chan and Munro (2001) demonstrated that mustard is more efficient than formalin. Gunn (1992) found that it has a comparable efficiency as potassium permanganate and is better than formalin and household detergents. It has consistent

efficiency compared to hand sorting across soil and habitat types (Lawrence and Bowers 2002). Furthermore, mustard does not kill earthworms and shows no phytotoxic effects, as do potassium permanganate and formalin. Unlike formalin, mustard is not a carcinogen (Gunn 1992). However, some disadvantages come along with the use of mustard. Both East and Knight (1998) and Gunn (1992) had difficulties with keeping prepared mustard in suspension at high concentrations, which could influence efficiency. Dry mustard powder suspensions suffer from the drawback that the content of allyl isothiocyanate (further AITC), the active ingredient in mustard seeds believed to irritate the earthworms, is variable and not exactly known. Dry mustard powders are often ground seed mixes of different species (*Brassica nigra* Koch, *Brassica juncea* (L.) Czern. and sometimes *Sinapis alba* L.) and varieties. They all contain precursors of isothiocyanate, and the type and concentration of isothiocyanate produced can vary (Fahey et al. 2001; Zasada and Ferris 2004), also between years and regions of seed production (DeClerq and Daun 2003; Zaborski 2003). Given the known composition and reproducibility of pure AITC solutions and the resulting confidence in the comparability of results, Zaborski (2003) compared pure AITC solutions of different concentrations with formalin and hand sorting and found that 100 mg l^{-1} AITC was as efficient as 200 mg l^{-1} formalin, in particular for the capture of anecic earthworms.

Already in 1969, Bouché acknowledged the complementarities of both passive and behavioural sampling when he suggested his 'etho-physical' method consisting of formalin application followed by soil sampling. The ethological phase of the sampling allows for expelling deep burrowing species, attributing to more accurate biomass estimations, while a soil sample restricted to (e.g.,) 20-cm depth allows for recovery of smaller individuals living near to the surface and of anecics expelled by the ethological method but remaining hidden just below the soil surface, leading to more accurate numbers and biomass. In this chapter, we use Bouché's combined method to compare the efficiency of formalin, mustard and AITC as chemical expellants in earthworm sampling.

We first assess the optimal concentrations of AITC and mustard powder. Working with the optimal concentrations from the first experiment, we assess the earthworm sampling efficiency of formalin, mustard and AITC, using the combined method. Efficiency of methods is compared in terms of earthworm species composition, numbers, biomass, ecological groups (epigeic, anecic, endogeic) and development stages (adults + subadults vs. juveniles).

2.2.1 Material and Methods

2.2.1.1 Preparation of Expellants

Four concentrations (low – medium low – medium high – high) of Indasia™ mustard powder suspensions (0.75, 1.5, 3 and 4.5 g l^{-1}) and AITC solutions (50, 100, 150 and 200 mg l^{-1}) in water were prepared (Table 2.1). Mustard powder

Table 2.1 AITC and mustard concentrations used in the concentration optimization experiment

Expellant	Unit	Concentration			
		Low	Medium-low	Medium-high	High
AITC	mg l^{-1}	50	100	150	200
Mustard	g l^{-1}	0.75	1.5	3	4.5
	mg AITC l^{-1}	45–81[a]	90–162[a]	180–324[a]	270–486[a]

[a]Values from Yu et al. (2003)

Table 2.2 AITC, formalin and mustard concentrations per application in the efficiency assessment

Expellant	Unit	Concentration per application			
		First	Second	Third	Fourth
AITC[a]	mg l^{-1}	75	75	150	150
Formalin[b]	ml l^{-1}	2.5	2.5	5	5
Mustard[a]	g l^{-1}	3	3	6	6

[a]Concentrations based on the concentration optimization experiment
[b]Concentrations from Bouché (1969)

concentrations were based on AITC content in the mustard powder to obtain comparable doses for both expellants (Yu et al. 2003). Mustard powder suspensions were prepared 1 h before application by adding the appropriate amount of powder to 20 l of water and stirring heavily. Just before application, the suspensions were stirred again. Since AITC is not readily soluble in water, pure AITC (95% purity grade, 1.017 g cm^{-3}) was first diluted with isopropanol (technical purity grade, 0.785 g cm^{-3}) to provide a 5 g l^{-1} stock solution (Zaborski 2003). In the field, appropriate volumes of stock solution were then diluted with water to arrive at application volumes of 20 l and the final concentrations mentioned above.

The same procedures of preparing AITC solutions and mustard suspensions were used in the sampling efficiency experiment, using the recommended AITC and mustard powder concentrations from the concentration optimization experiment. Formalin solutions were prepared by diluting the appropriate volume of formalin (technical formaldehyde, ±35%, 1.088 g cm^{-3}) with 20 l of water (Table 2.2).

2.2.1.2 Earthworm Sampling

Expellant concentrations were tested in May 2003 in a regularly mown lawn on a sandy loam soil near Leuven, central Belgium. Earthworms were sampled by each expellant concentration in five replicate plots (0.707 × 0.707 m^2). Expellants were sprinkled with a watering can over the plot area and an adjacent area of 0.1 m outside the plot. Two successive applications of 10 l each with a period of 15 min between applications to collect emerging earthworms were used.

Sampling efficiency of mustard, AITC and formalin was assessed in November 2003 in a harvested wheat field in Court-Saint-Etienne situated in the loam belt of central Belgium. Within a radius of 3 m from eight sampling locations randomly selected in the field, a plot (0.707×0.707 m^2) was randomly located for each expellant. Total expellant volumes of 40 l were applied to the plots with a watering can in four successive applications of 10 l each. During the first two applications, low expellant concentrations were used, which were doubled in the two following applications (Table 2.2), as recommended by Bouché and Aliaga (1986). Between each application there was an interval of 10 min to collect earthworms emerging at the soil surface, corresponding to a total sampling effort of 40 min. Immediately after expulsion, a square soil sample (0.316×0.316 m^2) was dug out to a depth of 0.25 m in the centre of all plots at four randomly selected sampling locations. Soil cores were crumbled and hand sorted during about 1–1.5 h each under favourable light conditions in the laboratory to collect earthworms. All collected earthworms were conserved per plot and per sample fraction (extraction vs. hand sorting) in formalin (5%) immediately after capture. Earthworms were identified following the key in Sims and Gerard (1999) and counted and weighed (with gut content) in the laboratory.

Kruskall Wallis tests (Siegel and Castellan 1988) were used to find differences between methods at a significance level of 0.05.

2.2.2 Salient Observations

2.2.2.1 Concentration Optimization

AITC concentration did not significantly affect the extraction of earthworm numbers or biomass (Fig. 2.1a, b). However, medium-low AITC concentration tended to extract the highest earthworm numbers (Fig. 2.1a), after which numbers began to fall. All but the highest mustard concentrations were less effective in extracting earthworms than AITC, although increasing mustard concentrations consistently yielded higher numbers and biomass (Fig. 2.1a, b).

Five earthworm species were recovered at the lowest AITC concentration (*Lumbricus rubellus* Hoffmeister, *Octolasion tyrtaeum lacteum* Savigny, *Aporrectodea caliginosa* Savigny, *Aporrectodea rosea* Savigny and *Dendrobaena octaedra* Savigny), but only the two largest species were extracted at the highest concentration (*L. rubellus* and *O. tyrtaeum*) (Fig. 2.1c). Increasing AITC concentrations thus resulted in a consistent loss of ability to extract earthworm species in order of increasing average body size. At medium-low concentration *D. octaedra* is no longer recovered (30–40 mm, 123 mg, only one individual recovered), at medium-high concentration *A. rosea* is no longer detected (25–85 mm, 200–216 mg) and at the highest concentration *A. caliginosa* was no longer extracted (40–180 mm, 168–206 mg) (body size ranges from Sims and Gerard (1999)). The total species

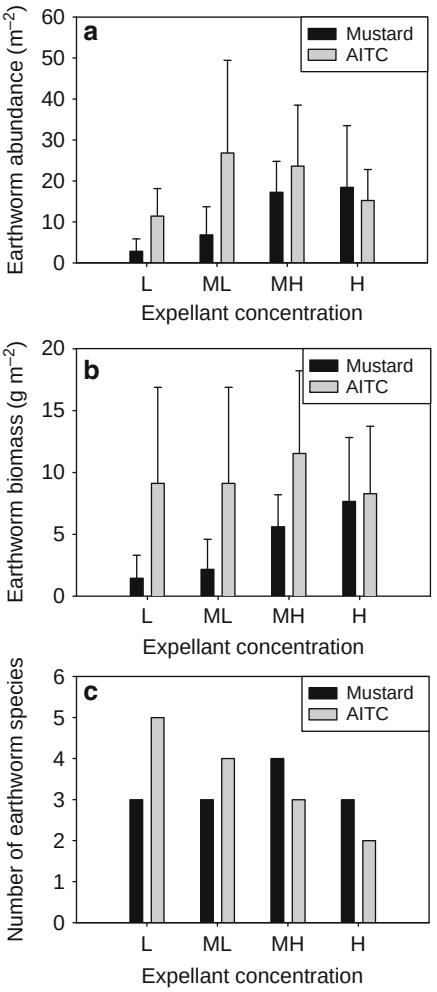

Fig. 2.1 Recovered earthworm numbers ($m^{-2} \pm$ standard error) (**a**) biomass (g $m^{-2} \pm$ standard error) (**b**) and species number (**c**) at low (L), medium-low (ML), medium-high (MH) and high (H) concentrations of mustard and AITC expellants

spectrum extracted by mustard was similar to AITC extraction, but species recovery by mustard was more constant across all concentrations compared to AITC.

2.2.2.2 Efficiency Assessment

Considering chemical extraction only, efficiency was not significantly different between expellants both in terms of numbers (Fig. 2.2a; 121 m^{-2} vs. 109 and 108 m^{-2} for AITC, formalin and mustard, respectively; $p = 0.535$) and biomass (Fig. 2.2b; 73.45 g m^{-2} vs. 70.79 and 58.23 g m^{-2} for mustard, AITC and formalin, respectively; $p = 0.360$). The hand sorted soil samples tended to yield higher earthworm numbers and biomass after mustard extraction than after formalin

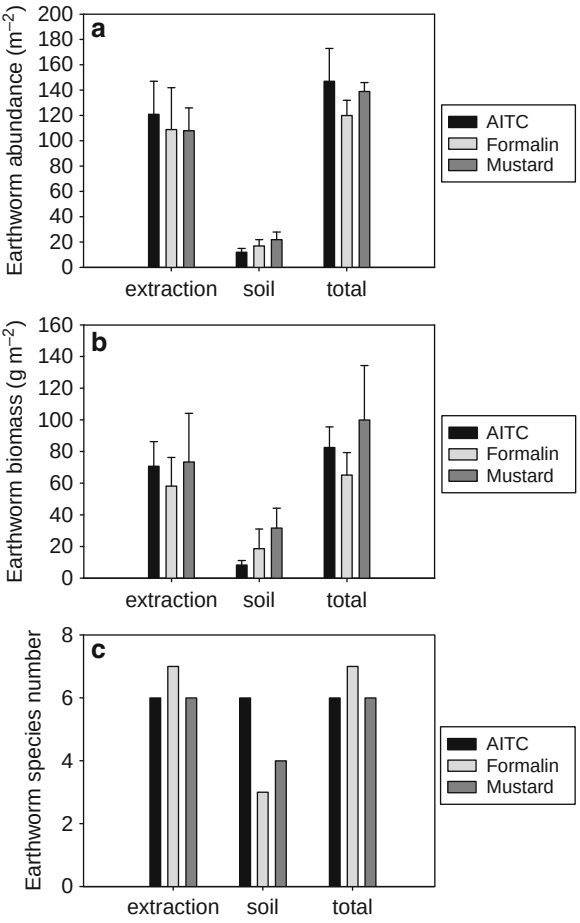

Fig. 2.2 Recovered earthworm numbers (m^{-2} ± standard error) (**a**) biomass (g m^{-2} ± standard error) (**b**) and species number (**c**) using different expellants (AITC, formalin, mustard) in a combined extraction – hand sorting sampling method. Results are given per sample fraction and in total

extraction, and contained significantly higher numbers (22 m^{-2} vs. 17 and 12 m^{-2}, $p = 0.037$) and biomass (31.72 g m^{-2} vs. 18.73 g m^{-2} and 8.38 g m^{-2}; $p = 0.015$) than after AITC extraction.

Total sampling efficiency (extraction + hand sorting together) was not significantly different between the three methods in terms of earthworm numbers, biomass and species number (Fig. 2.2). Although not significant, the AITC and mustard method showed slightly higher efficiency in sampling earthworm numbers than formalin (Fig. 2.2a, 147 m^{-2} and 139 vs. 120 m^{-2} for mustard and formalin, respectively; $p = 0.124$). In terms of biomass, mustard sampling tended to be more efficient than AITC or formalin (Fig. 2.2b; 99.99 g m^{-2} vs. 82.68 and 65.17 g m^{-2} for AITC and formalin, respectively; $p = 0.174$).

Six species were commonly recovered by all methods: *Allolobophora chlorotica* Savigny, *A. rosea*, *Aporrectodea longa* Ude, *A. caliginosa*, *L. terrestris* L. and *Octolasion cyaneum* Savigny (Fig. 2.2c). Using the formalin method, also one individual of the epigeic *Lumbricus castaneus* Savigny (0.143 g) was collected. In further calculations, it was omitted. Each expellant was able to extract all six (AITC, mustard) or seven (formalin) of the total number of species found. The soil cores after formalin extraction contained only the endogeic *A. chlorotica*, *A. rosea* and *A. caliginosa*, and the soil cores after mustard extraction additionally contained the anecic *A. longa*.

Although (sub)adults made up only a small portion of the total earthworm numbers (14, 9 and 14% for AITC, formalin and mustard, respectively), they constituted an important part of the earthworm biomass (54, 46 and 54% for AITC, formalin and mustard, respectively). There were no significant differences between the methods (both fractions together) in terms of numbers and biomass of both (sub)adults and juveniles ($p = 0.173$ and $p = 0.593$ for numbers, respectively; $p = 0.219$ and $p = 0.167$ for biomass, respectively). But AITC and mustard marginally extracted more (sub)adult numbers than formalin ($p = 0.060$) and hand sorting after mustard extraction yielded the highest juvenile biomass while the lowest was found after AITC extraction ($p = 0.030$).

Numbers and biomass of anecic species were equally found by the three methods ($p = 0.551$ and $p = 0.309$, respectively). Also comparable numbers of endogeic species (*A. chlorotica*, *A. caliginosa*, *A. rosea* and *O. cyaneum*) were recovered by the three methods ($p = 0.230$), but biomass recovery was marginally higher with mustard than with the other methods ($p = 0.077$). Extraction of anecics did not differ between methods ($p = 0.751$) but the formalin and AITC hand sorting fractions contained more anecic earthworm numbers than mustard ($p = 0.023$). AITC tended to extract more endogeics, both in terms of numbers and biomass, followed by mustard and formalin ($p = 0.076$ and $p = 0.093$, respectively). Hand sorting after mustard extraction recovered more numbers and biomass of endogeics than formalin and AITC ($p = 0.29$ and $p = 0.030$, respectively).

2.2.3 Interpretation

2.2.3.1 Concentration Optimization

In spite of the intentional use of similar AITC concentrations both in mustard and pure AITC treatments, the latter were generally spoken more efficient in sampling earthworms than mustard. Although mustard concentrations were stirred well and sufficiently long during preparation, it is probable that less than the maximum amount of AITC was formed from the glucosinates when mustard was added to the water (Fahey et al. 2001). Furthermore, AITC content of mustard powder is variable. The ranges we found in the literature (Yu et al. 2003) can be somewhat different from the actual content of the powder we used. It is thus possible that the

mustard treatments had an effectively lower AITC concentration than the pure AITC treatments.

The optimum of 150 mg l^{-1} pure AITC for sampling earthworm biomass and the lower optimum of 100 mg l^{-1} for earthworm numbers may indicate a trade-off between the recovery of smaller and more numerous individuals (juveniles and epigeics) on the one hand and heavier and less numerous individuals on the other (adults/subadults and anecics).

The inability to capture smaller earthworm individuals at increasing AITC concentrations could indicate that smaller earthworm species and juveniles cannot tolerate concentrations above 100 mg AITC l^{-1} and therefore do not reach the soil surface. Therefore, following Bouché and Aliaga (1986) in doubling expellant concentrations in subsequent applications, we recommend a concentration of 75 mg l^{-1} AITC in the first two applications, followed by two applications of 150 mg l^{-1} AITC. These concentrations compare very well both with the optimum of 100 mg AITC l^{-1} and the range of acceptable concentrations (60–200 mg l^{-1} AITC) found by Zaborski (2003).

The highest mustard concentrations (3 and 4.5 g l^{-1}) resulted in near-optimum extraction of earthworm numbers, but in a suboptimum recovery of earthworm biomass. This trade-off between numbers and biomass corresponds to the observations with AITC already discussed. Unlike AITC, however, earthworm species and species numbers were stable over all concentrations tested. Chan and Munro (2001) also noted this trade-off effect and found that concentrations between 1.6 and 4.7 g mustard powder per litre were optimal. They further suggested that these concentrations should produce equivalent results to that of English mustard suspension used by Gunn (1992) (15 ml l^{-1}) and better results than formalin (0.55%). They also found that their highest mustard powder concentration tested (~9.4 g l^{-1}) resulted in a significant lower number of collected juveniles compared to the use of lower concentrations. Therefore, we suggest, by analogy to the AITC method and as recommended by Bouché and Aliaga (1986), to use two applications of low (3 g l^{-1}) mustard concentration, and doubling the concentrations (6 g l^{-1}) in the following two applications. A concentration of 3 g l^{-1} lies well within the safe range as found by Chan and Munro (2001) and this study. The doubled concentration of 6 g l^{-1} is suggested by our data for more accurate biomass recovery, and it stays well below the less favourable concentration of 9.4 g l^{-1} identified by Chan and Munro (2001). However, it is advisable to further test a range of higher concentrations than tested here and compare them with standard methods such as hand sorting or with formalin or AITC extraction. From the previous we stress that sufficient mixing of the mustard powder in water is essential.

2.2.3.2 Efficiency Assessment

Overall, the three compared methods (extraction + hand sorting together) did not differ in sampling efficiency. But AITC and mustard extracted earthworms slightly better than formalin. Indeed, AITC and mustard tended to be more successful in

extracting endogeics and (sub)adults than formalin, and also most other tests gave the same trends in extraction efficiency. Interestingly, the greatest differences between methods were found in the soil fraction. On the one hand, the soil samples after formalin extraction contained more numbers of anecics (and not endogeics as could be expected from formalin's lower extraction efficiency) than after mustard extraction (with intermediate values for AITC). On the other hand, more endogeics and juveniles were collected in soil samples after mustard extraction than after AITC extraction (with intermediate values for formalin). If we assume that earthworms found in the soil fraction did not react to the vermifuges, we have to conclude that mustard fails in catching some endogeics and to a lesser extent some juveniles compared to the other expellants. We also would expect inverse results for the chemical extraction and hand sorting fractions but these were not observed. From this complex overall picture, it is not evident to push one of the methods forward as the best.

Characteristics of the expellants other than extraction efficiency may then play a decisive role in choosing between one and the other. Formalin proved to be lethal to earthworms (and other soil organisms and plants) (Gunn 1992; Eichinger et al. 2007), which is problematic if living worms have to be collected. Formalin is also known to be carcinogenic to humans, and in some countries its use is forbidden due to national health and safety regulations (Schmidt 2001). Together with its suggested lower extraction efficiency compared to AITC and mustard, these qualities make formalin a less preferred expellant.

Also some drawbacks with the use of AITC must be noted. First, AITC should be used with great care in the field since it is very toxic to aquatic organisms, and may cause long-term adverse effects in the aquatic environment. Second, the preparation and practical use of AITC solutions is hindered by the toxic and irritating damps of AITC, more disturbing than the ones of formalin. As a consequence, hand sorting after pure AITC application is not recommended for health and safety reasons, and it is not possible without proper protection. Third, earthworms emerging at the surface after AITC application are markedly groggy, even the bigger individuals. This could mean that some individuals, especially smaller ones, can get so drowsy that they do not even reach the surface while fleeing for the irritating AITC. Therefore, it is important to combine AITC extraction with succeeding hand sorting of a soil core to collect the left behind earthworms, which conflicts with our earlier discussion of the irritant nature of AITC. Another important demerit is that in many cases earthworms collected by AITC emerge with a loosened epidermis. This negative effect is most probably caused by the isopropanol in which AITC is diluted and seriously hampers identification after collection.

Mustard powder is a harmless and cheap product with comparable sampling efficiency as AITC. A disadvantage is that the concentration of AITC and its variability in mustard powder suspensions is not exactly known, although the extraction result was quite robust over a range of concentrations (Fig. 2.1). For reliable results, we, nevertheless, suggest to use mustard powder from the same manufacturer and from the same lot if possible and to order quantities sufficient for the whole sampling campaign (Gunn 1992).

2.3 Where to Sample? Optimizing Spatial Sampling Design

Like many living organisms, earthworm populations are spatially neither uniformly nor randomly distributed, but usually occur in spatial clusters. Both spatial heterogeneity and autocorrelation should be accounted for in the sampling design of any ecological field study, as many statistical tests rely on the assumption of independence of observations.

2.3.1 Spatial Autocorrelation and Sampling Design

Observed values of a variable (e.g., earthworm density) are said to be spatially autocorrelated when pairs of observations with a certain distance apart are more similar (positive autocorrelation) or less similar (negative autocorrelation) than expected for randomly associated pairs of observations. This higher (or lower) similarity among mutually closer observation sites is very common in nature. Autocorrelation in fact refers to the lack of independence among the error components of observations due to geographic proximity (Legendre and Legendre 1998). Because of this, the use of autocorrelated data in tests of statistical significance that rely on independent observations (e.g., *t*-test, correlation analysis, analysis of variance, linear regression; clustering and ordination methods do not use tests of statistical significance and may thus be safely used in the case of autocorrelated data) is problematic.

Unless the aim of the study is to analyze the spatial structure in the data per se (by correlograms, variograms, see Rossi et al. 1995 for a comprehensive introduction to geostatistical analysis of ecological data), spatial dependency among observations may be removed to validly use classical statistical tests. However, this is not recommended as discarding observations, until spatial independency is achieved, results in (costly) information loss (Fortin and Dale 2009). Similarly, the use of de-trended data cannot be advised if autocorrelation is inherent to the process under study.

Alternatively, various corrected tests that rely on modified estimates of the variance and on corrected estimates of the effective sample size and the number of degrees of freedom have been developed and their use should be advocated (e.g., Dutilleul 1993). When corrected statistical methods are not available, permutational tests may be used, given that the appropriate procedure of random permutation of observations to determine significance can be specified.

A more proactive way of preventing dependency among observations lies in the careful design of spatial sampling schemes, and will be discussed here. Classical statistical text books tell that without prior knowledge of the phenomenon under study, ecologists should rely on random or systematic sampling designs to avoid dependency among observations. But the random or systematic spatial allocation of observation sites per se does not rule out that observations may be spatially dependent to some degree: this will be the case if the average distance between observation sites is smaller than the zone of spatial influence of the underlying

ecological process. The zone of spatial influence is commonly determined by the range of the variogram model describing the spatial autocorrelation in the data (Rossi et al. 1995).

In recent years, studies addressing the spatial distribution of earthworm populations have yielded insights in the scales over which earthworm populations are spatially related (Table 2.3). Across a range of ecosystems, earthworm species are spatially distributed in clusters ranging from as small as 7 m in diameter (Rossi 2003b) to more than 100 m (Hernández et al. 2007). However, the majority of spatial ranges of autocorrelation lies within 20–50 m. Adult individuals of *L. terrestris* consistently live in small clusters (~20 m) compared to other species, presumably related to its surface mating behaviour. Another general trend is that juveniles tend to inhabit larger patches than conspecific adults.

Samples should ideally be taken at distances further apart than the variogram ranges as given in Table 2.3 for the given ecosystems and species. In practice, this means sampling locations should be at least 20–50 m apart to collect nonautocorrelated independent samples for most species and circumstances. Ideally, the site-specific spatial structure of the earthworm populations under study should be assessed beforehand in a pilot study or this information should be retrieved from previous surveys (Legendre et al. 2002). A risk that arises when spacing the sampling locations, according to the spatial structure, is that not enough independent samples can be collected from (strata within) the observation site or the experimental unit. As always, a good balance between theory and practice must be found, and other techniques (see earlier) can assist in the analysis of spatially dependent data.

2.3.2 Sample Unit Size and Shape

Observed spatial (and temporal) variability is a function of the 'window size' that one uses to look at the world (Levin 1992): as window size (i.e., support size (the volume (sample unit surface area $\times$ depth) collected per sample unit)) is increased, variability will decay. The inversely proportional relationship between window size and variability depends on how spatial autocorrelation decreases with distance within the range of spatial autocorrelation. Additionally, larger study objects need larger support sizes to adequately capture variability. In practice, different support sizes apply to nematodes, mesofauna and earthworms (Stein and Ettema 2003).

Clearly, lower sampling variance by well-chosen sample unit size and shape, in combination with well-chosen (nonautocorrelated) sample unit locations (see Sect. 2.3.1) lead to smaller confidence intervals, and thus more rapid detection of significant treatment differences for similar sampling efforts.

Little is known about the ideal support size in earthworm sampling. For example, many researchers adhere to a square sample unit area of 0.25×0.25 m^2 of variable depth, depending on the species of interest, life stage and season without further

Table 2.3 Overview of the types of fitted models and ranges of spatial autocorrelation from studies describing the spatial distribution of earthworms in a range of ecosystems

Country	Land use	Earthworm taxon	Life stage	Variogram parameters						Reference
				Model	Range	Model	Range	Model	Range	
		Individuals (m^{-2})								
Germany	Arable land	*L. terrestris*	–	Spherical	36					Poier and Richter 1992
			Adult	Spherical	21					
			Juvenile	Spherical	37					
		A. caliginosa	–	Spherical	53					
			Adult	Spherical	28					
			Juvenile	Spherical	53					
		A. rosea	–	Spherical	70					
			Adult	Spherical	50					
			Juvenile	Spherical	–					
Belgium	Arable land	*L. terrestris*	–	Spherical	30					Valckx et al. 2009
			Adult	Spherical	14					
			Juvenile	Spherical	34					
		A. longa	–	Spherical	64					
			Adult	Spherical	40					
			Juvenile	Spherical	63					
		A. caliginosa	–	Spherical	29					
			Adult	Spherical	33					
			Juvenile	Spherical	30					
		A. rosea	–	Spherical	45					
			Adult	Spherical	40					
			Juvenile	Spherical	44					
Canada	Arable land	Lumbricidae		Linear	16					Whalen and Costa 2003
	Hay field	Lumbricidae		Linear	18					
	Deciduous forest	Lumbricidae		Linear	21					
Spain	Pasture			2001		2002		2003		Hernández et al. 2007
		A. rosea		Gaussian	134	Spherical	18	Spherical	26	
		H. elisae		Exponential	41	Spherical	50	Gaussian	103	
		A. caliginosa		sph Erical	60	Spherical	30	Spherical	26	
Ivory coast	Savanna	Eudrilidae		Spherical	27					Rossi 2003a
		Millsonia anomala		Spherical	26					
				1995		1996				Rossi 2003b
		Filiform Eudrilidae		Spherical	8	Spherical	8			

(*continued*)

Table 2.3 (continued)

Country	Land use	Earthworm taxon	Life stage	Variogram parameters						Reference
				Model	Range	Model	Range	Model	Range	
		Individuals (m^{-2})								
				–	–	Spherical	17			
		Hyperiodrilus africanus		Spherical	7	–	–			
Colombia	Savanna			1993		1994		1995		Jiménez et al. 2001
		Andiodrilus sp.	Adult		–		–	Spherical	45	
			Juvenile		–		–		–	
		Aymara sp.	Adult		–		–	Spherical	41	
			Juvenile		–		–	Linear		
		Glossodrilus sp.	Adult	Spherical	37		–		–	
			Juvenile	Spherical	30	Spherical	36		–	
		Ocnerodrilidae sp.	Adult		–	Spherical	31		–	
			Juvenile		–		–		–	
	Pasture	*Andiodrilus* sp.	Adult		–	Spherical	29	Spherical	55	
			Juvenile		–	Spherical	27	Spherical	30	
		Aymara sp.	Adult		–		–		–	
			Juvenile		–		–		–	
		Glossodrilus sp.	Adult	Linear	–		–		–	
			Juvenile		–	Spherical	57	Spherical	42	
		Ocnerodrilidae sp.	Adult		–		–	Spherical	31	
			Juvenile		–		–		–	
		Biomass (m^{-2})								
Germany	Arable land	*L. terrestris*	–	Spherical	22					Poier and Richter 1992
			Adult	Spherical	21					
			Juvenile	Spherical	56					
		A. caliginosa	–	Spherical	35					
			Adult	Spherical	27					
			Juvenile	Spherical	47					
		A. rosea	–	Spherical	38					
			Adult	Spherical	53					
			Juvenile	Spherical	–					
France	Grassland	*L. terrestris*	Adult	Spherical	28					Cannavacciuolo et al. 1998
			Juvenile	Gaussian	18					
		A. caliginosa	Adult	Spherical	30					
			Juvenile	Linear	–					

justification. One of the few studies addressing sample unit size and shape in earthworm sampling (Dickey and Kladivko 1989) shows that the most efficient, acceptable sample unit size–shape was 10 cm along the row (the shortest increment in the experiment) by 45 cm across the row in a *Zea mays* row crop (75 cm inter-row distance) for an earthworm community composed of *Aporrectodea tuberculata* and *L. rubellus*. They also found as much as a threefold difference in the sampling efficiency (by hand sorting) within the range of tested sample unit size–shape combinations. The least efficient size–shape was also the largest (30 cm along the row by 75 cm across the row). The authors state that rectangular quadrants have a substantial advantage over square ones of equivalent sample unit area. Especially for patchily distributed populations, an elongated sample unit shape with its longest axis perpendicular to the expected or known density gradient results in a decreased variance (i.e., across corn rows in this case). The relative importance of sample unit size and shape for other ecosystems, sites, seasons, species and life stages may well differ from the study by Dickey and Kladivko (1989) but hitherto knowledge is lacking. Rossi and Nuutinen (2004), in their study of the spatial distribution of *L. terrestris* L. midden density in a Finnish forest, also found an considerable increase in total variance with decreasing sample unit, while neither the mean midden density nor the global distribution pattern were markedly affected by sample unit size (0.125, 0.25 or 1 m^2). Given that earthworm sampling is a time and labour consuming activity, in practice an acceptable level of sampling variance must be decided upon so that a corresponding compromise between the sample unit size and the number of samples can be found.

2.4 How Many Samples? Optimizing Sample Size

Commonly, earthworm communities are species-poor in a given area or ecosystem; in arable land they usually comprise only 4–6 species (Edwards and Bohlen 1996). Lavelle (1983) relates this to the simultaneous occurrence of species from different ecological categories, the plasticity of earthworm species, and temporal and spatial (both horizontal and vertical) niche separation. Nevertheless, to compare the effects of land use, land management and environmental factors on earthworm communities, it is important that a sufficient number of samples are taken in order to be a 'true' representation of local earthworm diversity.

Therefore, sample-based species rarefaction curves with 95% confidence intervals were computed by the free software Estimates (Colwell 2009), using the analytical formulas of Colwell et al. (2004). Sample-based rarefaction curves plot the expected number of species in a small collection of n samples drawn at random from the large pool of N samples, against a measure of cumulative sampling effort (e.g., the cumulative number of samples) (Gotelli and Colwell 2001). Thus, rarefaction curves represent the statistical expectation of the corresponding species accumulation curves, which themselves record the total number of species revealed as additional sample units are added in one particular random order to the pool of all previously

collected samples (Gotelli and Colwell 2001). Typically, both types of curves rise relatively rapidly at first, then much more slowly as increasingly rare taxa are added. In principle, an asymptote will eventually be reached for samples coming from a homogeneous study area (representing within-habitat α-diversity) (Gotelli and Colwell 2001). The point where the curve levels off is usually accepted as the optimal balance between sampling effort and an accurate estimation of local species diversity.

Species abundance data (number of individuals m^{-2}) from a study of the spatial distribution of earthworm density in an arable field in central Belgium (Valckx et al. 2009) served as input data. Data from two sampling methods (earthworms extracted by mustard powder (0.5 m^2) vs. earthworms hand sorted from a soil monolith (0.1 m^2 to a depth of 20 cm) after mustard extraction) from 30 sampling locations were analyzed both separately and combined to determine which method was more efficient in capturing earthworm diversity. The expected number of species was calculated against both the cumulative number of samples and the cumulative sampled area (m^2). The latter was done to account for differences in sample plot area used in both sampling methods.

Here, we do not discuss the determination of sample size to protect against both type I and type II errors, or to estimate the variables of interest with sufficient precision to detect (ecologically) meaningful differences between treatments. For this, we refer to classic statistical textbooks (e.g., Neter et al. 1996) where the relevant techniques (e.g., the power approach) are explained in much detail.

Figure 2.3 shows that all species rarefaction curves reached an asymptote well before total accumulated sampling effort, whether expressed as number of samples or sampled area. This suggests that species inventory was complete in the study area, that is, 'true' species richness was observed and not a single species remained undetected or rare species were present. Indeed, the occurrence of rare species (unique and duplicates, i.e., species occurring in exactly one or two samples, respectively) may severely hamper to reach asymptotic species richness (Mao and Colwell 2005).

Circa ten samples of 0.5 m^2 extracted by mustard were needed to capture the total observed species richness of six species (*L. terrestris* L., *A. longa* UDE, *A. caliginosa* SAVIGNY, *A. chlorotica* SAVIGNY, *A. rosea* SAVIGNY and *O. cyaneum* SAVIGNY) (Fig. 2.3a). For the same purpose, more than twice as much hand-sorted soil samples (±25) were needed (Fig. 2.3c). However, rescaling the number of samples to the actually sampled area (m^2) in the *x*-axis revealed that on a per m^2 basis, hand sorting of soil samples is more efficient than mustard extraction to observe the asymptotic local diversity (Fig. 2.3d vs. b): only a total area of ±2.4 m^2 need to be sampled by hand sorting, while a total sampling area of ±5 m^2 is needed for earthworm extraction. This result reflects the lower efficiency of the extraction sampling method compared to the hand sorting method to catch endogeic species richness of the earthworm assemblage dominated by endogeic species in this particular arable land context (4 out of 6 observed species were endogeic). Earthworm communities dominated by epigeic and/or anecic species may well be more efficiently sampled on a per m^2 basis by the mustard extraction method. Using the combined sampling data (extraction + hand sorting) (Fig. 2.3e, f), it is clear that depending on the species composition and how sampling effort is expressed, the

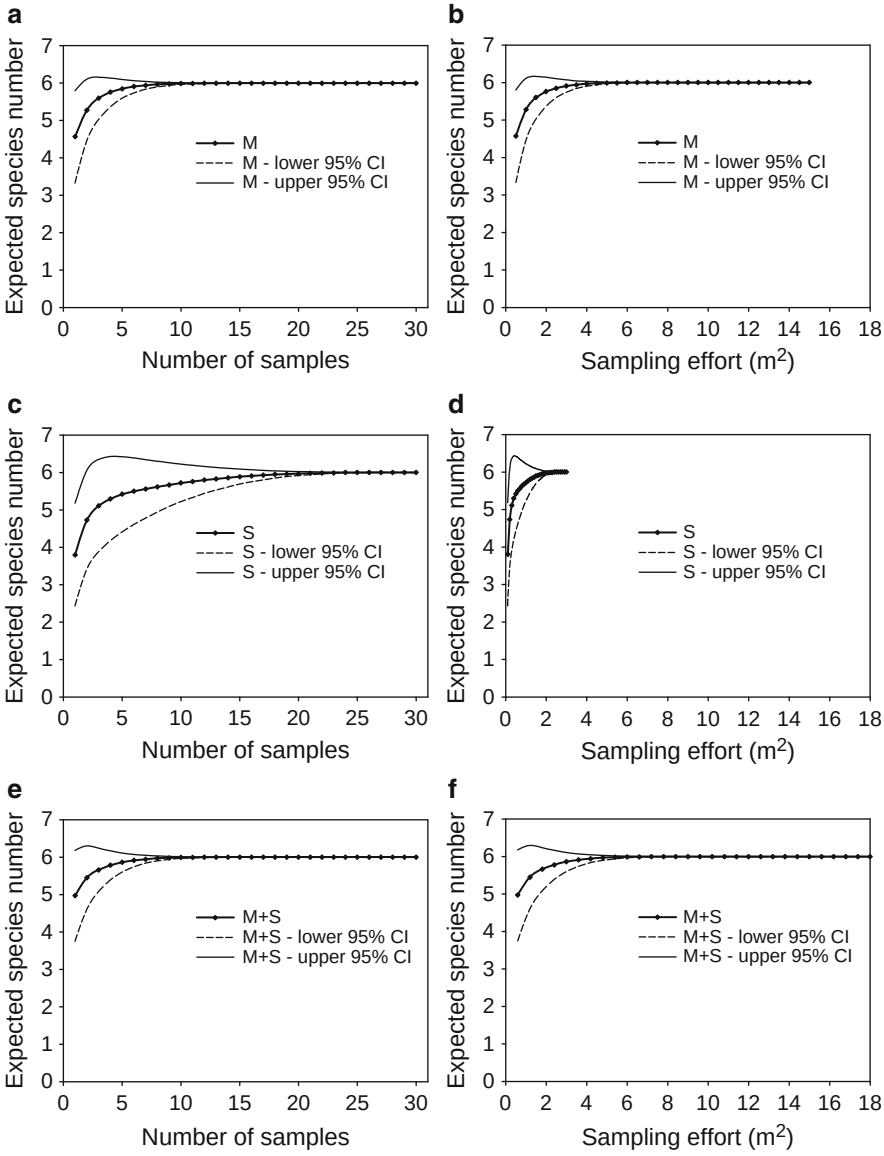

Fig. 2.3 Sample-based species rarefaction curves (*thick lines*) with 95% confidence intervals (*thin lines*) of earthworms collected by mustard extraction (M; 0.5 m^2 plot) (**a** and **b**), by hand sorting a soil monolith after mustard extraction (S; 0.1 m^2 plot) (**c** and **d**), and by a combination of both methods (M + S) (**e** and **f**). *Graphs* on the *left* show the expected species number as a function of sample size; in the *graphs* on the *right* sample size is rescaled to sampling effort expressed as total area sampled (m^2)

combined sampling method may be more or less efficient than its constituent sampling methods.

2.5 Conclusion

Because of the toxicity to the soil ecosystem and the irritant nature to the operators of formalin and AITC, these expellants are less recommended for earthworm sampling compared to mustard. Mustard extraction consisting of two initial applications of low expellant concentration (3 g mustard l^{-1}) followed by two applications of doubled concentrations (6 g l^{-1}) is at least equally efficient as the other vermifuges, and has the advantage to be harmless to the environment.

Sample unit locations should ideally be randomly selected at distances larger than the range of spatial autocorrelation, and sample unit size and shape should reflect the variability to be expected. Much of this important information to assure the collection of independent data is lacking when research projects start, hence the importance of pilot studies. More research is needed about ideal support size.

Species rarefaction curves are a useful and promising technique to assess asymptotic earthworm species richness and the corresponding sampling effort needed to capture it. Despite its wide use in ecology, hitherto it was not applied in the context of earthworm research. Earthworm communities are usually species-poor, which has the advantage that a relatively small number of samples usually suffice to capture total species richness compared to other taxa. However, species rareness may complicate conclusions about the ideal sampling effort because it prevents rarefaction curves to reach an asymptote. It is recommended to explore this technique's potential with data from other ecosystems, with different species compositions and rareness. Also the effects of spatial patchiness on estimation of asymptotic species richness needs further research as it can be assumed that higher spatial aggregation means that a higher sampling effort is needed to capture total earthworm richness compared to randomly distributed organisms.

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Chapter 3
Earthworms and Soil Structure

Yasemin Kavdir and Remzi İlay

3.1 Introduction

Soil structure effects on pore size distribution, aeration, plant nutrient uptake rate, root development, hydraulic conductivity, infiltration, water holding capacity of soils and erosion. Many factors effect on soil structure development and one of them is biota including earthworms. Earthworms consume and excrete plant and soil residues, incorporate these into aggregates and contribute formation and stabilization of soil aggregates. As a result, physical conditions of soil are improved to support plant growth. Earthworms act on soil structure by creating burrows and casts and by breaking down organic matter (OM). Influences of earthworm on soil aggregate formation and soil structure will be discussed in this chapter.

3.2 Soil Aggregates and Structure

Aggregates are formed through the combination of clay, silt, and sand with organic and inorganic substances. There are two main factors responsible for aggregation, which are flocculation and cementation. The aggregates are separated from the adjacent aggregates by surfaces of weakness. Aggregate stability is very successful to be used as an indicator of soil structure (Six et al. 2000). Aggregation is mediated by soil organic carbon (SOC), biota, cation bridges, clay, and carbonates. The SOC acts as a binding agent during the formation of aggregates. Biota and their organic products contribute to the development of soil structure (Table 3.1). Cations form bridges between mineral and organo-mineral particles. Clay particles have negative

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DOI 10.1007/978-3-642-14636-7_3,

Table 3.1 Overview of the impact of earthworms on aggregate stability (AS) and tensile strength (TS)

Place	Soil type and crop	Species	Increased	Reduced	Reference
Lower Saxony, Germany	Loamy	*L. terrestris*	TS	AS	Schrader and Zhang (1997)
Lower Saxony	Loamy	*A. caliginosa*	TS	AS	Schrader and Zhang (1997)
Lower Saxony	Clay	*L. terrestris*	–	TS + AS	Schrader and Zhang (1997)
Lower Saxony	Clay	*A. caliginosa*	–	TS + AS	Schrader and Zhang (1997)
Soil and Fertilizer Research Institute (SFRI, Hanoi, Vietnam)	Loam	*M. posthuma*	–	AS	Bottinelli et al. (2010)
Braunschweig, Germany	Compacted (with barley mulch)	*L. terrestris, O. cyaneum*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Uncompacted (with barley mulch)	*L. terrestris, O. cyaneum*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Compacted (with lupin mulch)	*L. terrestris, O. cyaneum*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Uncompacted (with lupin mulch)	*L. terrestris, O. cyaneum*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Compacted (with sugar-beet mulch)	*L. terrestris*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Uncompacted (with sugar-beet mulch)	*L. terrestris, O. cyaneum*	AS	–	Buck et al. (2000)
Braunschweig, Germany	Compacted (with sugar-beet mulch)	*O. cyaneum*	–	AS	Buck et al. (2000)
El Molar, Madrid	Sandy	*H. elisae*	TS + AS	–	Garvin et al. (2001)
Lower Saxony, Germany	Loam	*L. terrestris*	AS	TS	Flegel et al. (1998)
Lower Saxony, Germany	Loam	*L. rubellus*	AS	TS	Flegel et al. (1998)
Lower Saxony, Germany	Loam	*D. octaedra*	AS	TS	Flegel et al. (1998)
Lamto (Cote d'Ivoire)	Sandy- Alfisol-Savanna	*M. anomala, C. zielae, S. porifera*	–	AS	Blanchart et al. (1997)
Lamto (Cote d'Ivoire)	Ferralsol	*M. anomala*	–	AS	Blanchart (1992)

charges and flocculates to form aggregates in the presence of cations such as Ca^{++}, Mg^{++}, Fe^{++}, Al^{+++}, and OM.

Aggregation is a very complex procedure that includes environment, soil management factors, plant influences, and soil properties such as mineral composition, texture, SOC concentration, pedogenic processes, microbial activities, exchangeable ions, and moisture availability (Kay 1998). Roots and hyphae enmesh and

release organic compounds that act as glue to hold particles together. Consecutive wetting and drying cycles help to stabilize the aggregates.

Aggregates occur in a variety of manners and sizes and are often grouped by size: macroaggregates (>250 μm) and microaggregates (<250 μm) and can be further divided by size (Tisdall and Oades 1982).

Soil structure refers to the size, shape, and arrangement of solids and voids, continuity of pores and voids, their capacity to hold and transmit water and organic and inorganic substances, and ability to support root growth and development (Lal 1991). Soil structure is one of the most important properties affecting crop production because it determines the depth that roots can penetrate, the amount of water that can be stored in the soil, and the movement of air, water, and soil fauna (Hermavan and Cameron 1993). Soil structure influence on root distribution and water and nutrients uptake by plants (Rampazzo et al. 1998; Pardo et al. 2000). Favorable soil structure and high aggregate stability are important to improve soil fertility, to increase agronomic productivity, increase porosity, water holding capacity, and to reduce erosion. Erosion, desertification, rapid recycling of nutrients, crusting, reduced water and air availability to roots and susceptibility to compaction originate from soil structure degradation in intensive arable lands. Subsurface structure tends to be composed of larger structural units than the surface structure. Subsoil structures also tend to have the binding agents on aggregate surfaces rather than mixed throughout the aggregate. Phase changes such as shrinking–swelling, freezing–thawing result in volume changes in the soil and produces soil aggregates. Shrinking and swelling, freezing and thawing, tillage, soil biota, and roots development effect on formation and stabilization of soil structure. Plant roots and associated hyphae can be seen to enmesh soil particles, while root mucilage, hyphae, bacteria, earthworms involve in stabilizing the soil aggregates. Soil fauna on soil structure are achieved mainly by earthworms, termites, and ants. It is often difficult to separate the multiple effects of organisms on aggregation.

3.3 Earthworms and Soil Structure

The history of earthworm effects on soil physical properties started in 1837 and 1881 by Darwin, who reported the importance of earthworms on formation of granular soil structure. Earthworms move through the soil by pushing soil aside and by ingesting and egesting (cast) soil. Earthworms consume and then excrete plant and soil residues, incorporate these into aggregates, and contribute to formation and stabilization of soil aggregates. Earthworms improve physical structure of the soil, increase drainage and aeration, enhance soil fertility, recycle nutrients, reduce run-off, supply better conditions for plant root growth. Drilosphere is a burrow linings, which is directly and indirectly modified by earthworms (Bouché 1975).This hot spot contains plant nutrients, high amount of

polysaccharides, and has higher enzyme activity, which increases soil aggregate stability. The concept of drilosphere was later expanded to include earthworm itself, its gut, soil that is in contact with the earthworm, casts, middens, and burrows (Lavelle 1988).

Earthworms directly influence soil structure by creating burrows and casts (Fig. 3.1). Casting enhances soil aggregation, while burrowing regulates soil physical properties. Earthworm's activity reduces soil bulk density by creating larger pores. These macropores permit water (Chan and Heenan 1993) and gasses flow in the soil. Earthworms translocate surface OM to deeper soils (Shuster et al. 2001) and produce organo-mineral casts (Zhang and Hendrix 1995; Decaens et al. 1999; Wilcox et al. 2002).

When earthworm passes through the soil, it faces two opposite mechanical effects. First, soil particles are broken and second the ingested soil is compacted during passage through the gut prior to excretion. Barré et al. (2009) tested these two hypotheses and concluded that earthworms were shown to bring initially loose and compacted soil to an intermediate mechanical state that is more favorable for structural stability and root growth.

Soil aggregate size distribution is significantly affected by earthworms in the 0–2 cm layer (Snyder et al. 2009; Fig. 3.2). Earthworm's presence shifted soil aggregate size to the >2,000 μm fraction from smaller fraction by reducing the amount of soil in the 2,000–250 and 250–53 μm fractions. Blanchart et al. (1999) reported that increment in the proportion of large aggregates in soil was due to cast production by earthworms.

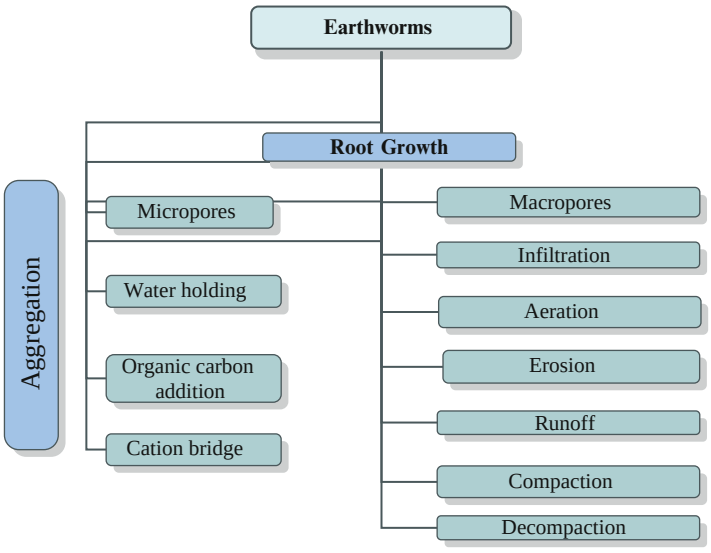

Fig. 3.1 Effects of earthworms on soil structure

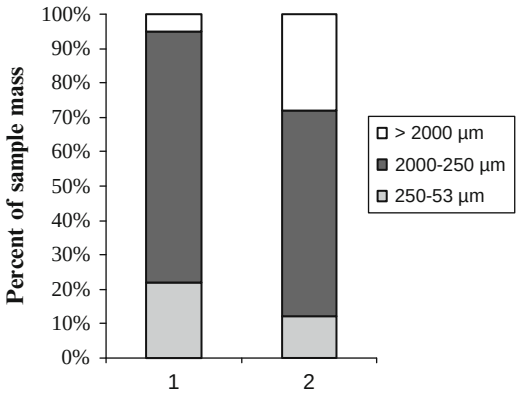

Fig. 3.2 Aggregate size distribution in 0–2 cm layer. (1: none, 2: earthworm) (Snyder et al. 2009)

3.3.1 Earthworm Species Effects on Burrowing and Casting

Earthworms are classified into three ecological groups according to the localisation in soil, feeding behavior, and burrowing (Lamandé et al. 2003; Lee 1959; Bouché 1977): (1) Epigeic (1–2.5 mm in diameter, live and feed above the soil surface, forest litter, rarely burrow and ingest, and have very little effect on soil structure), (2) anecic (4–8 mm in diameter, feed at the ground surface and decaying organic residues, live in semipermanent burrows, more or less vertical and opened to the soil surface), and (3) endogeic (2–4.5 mm in diameter, mostly live on 10–15 cm surface, ingest soil, dig extensive systems of burrows to search foods and they immediately refill with their casts, the burrows are mostly subhorizontal oriented).

Burrows increases water infiltration, water storage, and aeration (Beven and Germann 1982; Devliegher and Verstraete 1997), increases solute and water movement to deeper soil horizons (Kung et al. 2000; Beven and Germann 1982), modify root penetration (Whalley and Dexter 1994).

Jegou et al. (2000) compared the burrowing activity of two earthworm species, which were *Lumbricus terrestris* and *Aporrectodea giardi*. The weight of the "casts," "burrow wall," and "burrow periphery" compartments was much higher for *A. giardi* compared with *L. terrestris*. Results showed that the burrow system of *A. giardi* was three times more important than that of *L. terrestris*. Also *L. terrestris* seemed to use the same burrows, while the extensive volume found in the *A. giardi* column suggests that the burrows are made as earthworm moves through the soil.

Recent studies showed that there was a significant influence of earthworm activity and residue application on stable aggregate formation (Bossuyt et al. 2006). More larger aggregates were formed (>2 mm) when residues are incorporated in soils then that were placed on the surface with earthworms. Earthworm species were also important for larger aggregate formation. Soil aggregates were 4.3 times greater than the control (no earthworm) when *Aporrectodea caliginosa* were present in residue incorporated soils. In the presence of *Lumbricus rubellus*, soil aggregates were three times greater than the control. When residues were placed on the soil surface, less large aggregates were formed by earthworms.

Similarly proportion of stable microaggregates in macroaggregates was higher in earthworm contained soils when residues were incorporated into the soils. This suggests that earthworms break up small microaggregates and incorporate those and forms in new formed macroaggregates. *A. caliginosa* had greater effect on water stable aggregate formation than *L. rubellus*, which lives on the surface soils and has less effect on aggregate stabilization than *A. caliginosa*. Organic carbon content was higher in casts of *L. terrestris* than *A. caliginosa* (Schrader and Zhang 1997).

Blanchart et al. (1997) demonstrated that different species of earthworm had contrasting effects on the structure of the upper 15 cm of the soils. For instance, *Millsonia anomala* (17 cm in length and 0.8 cm dia) ingest aggregates <2.0 mm and excrete aggregates larger than 5.0 mm. This species is responsible for the formation of macroaggregates (casts) and large macropores. Conversely, *eudrilid* (3–7 cm in length, 0.5–l mm dia) earthworm casts were between 0.63 and 5.0 mm in diameter, and restrict the formation of large macropores. As a result, the small worms destruct the casts of large worms such as *M. anomala* and shorten the lifespan of large aggregates.

3.3.2 The Effect of Burrows on Soil Structure

Earthworms vertically and horizontally burrow soil during feeding and moving. Burrow size and continuity depend on soil properties as well as on earthworm species, and size of burrows can be >10 mm in diameter (Lee 1985). The anecic species such as *L. terrestris* L. tends to build large and vertical burrows in the soil when compared with endogeic species (Bastardie et al. 2005). Endogeic species such as *Octolasion tyrtaem* Savigny consumes soil as they form horizontal burrows at the depths of 20 cm. Earthworm species such as *L. rubellus* Hoffmeister belongs to the epigeic group, and can burrow through the upper 20 cm of soil when conditions are unfavorable at the soil surface.

Francis and Fraser (1998) reported that burrows of *A. caliginosa* were more continuous and connected to the soil surface than burrows of *Octolasion Cyaneum* in the topsoil. In contrast, *O. cyaneum* created the most continuous burrows through the subsoil of New Zealand agricultural soils. Annual production of burrow length of *L. terrestris* was estimated as 82.3 km ha^{-1} (Langmaack et al. 1999).

Earthworms with bigger burrowing activity may also enhance soil drying (Ernst et al. 2009). They reported that anecic and endogeic earthworm species may enhance the soil drying process, relative to epigeic earthworm species.

Burrows influence soil-water transport in the soil. Chan and Heenan (1993) reported a significant correlation between macropores >1.0 mm that contribute to water flow and earthworm populations. Sixty-five percent of the total macropores were transmitting macropores under no-till/stubble retained system compared with 1% under conventional tillage/stubble-burnt systems. The anecic earthworms are

particularly effective in the creation of vertical macropore channels, which can modify aeration and infiltration.

Because of their reduced porosity, the burrow walls may influence water and air circulation from the burrows to the soil matrix. Moreover, Edwards et al. (1992) showed that, owing to its high content of OM, the drilosphere of burrows built by *L. terrestris* may have a higher affinity for herbicides sorption than does the bulk soil material.

Soil properties as well as management practices (tillage, cropping, chemicals, and fertilizers) also affect earthworm abundance and burrowing. Review of Chan (2001) summarized that earthworm population of no-till systems were greater than conventionally tilled systems. Tillage intensity, depth, and soil types were important factors effecting earthworm abundances in soil. Minimum tillage increases earthworm population compared with plowing, discing, or chiseling (Wuest 2001; Lachnicht et al. 1997). In grasslands, there were higher burrowing rate than in vineyard soil (Eijsackers et al. 2005) because of a higher bulk density of the vineyard soil. Compaction is an important factor to control earthworm numbers in the soil (Chan and Barchia 2007). When soil bulk density increased from 1.32 to 1.52 Mg m^{-3}, earthworm abundance decreased from 52 to 22 m^{-2} (Sochtig and Larink 1992). However, burrowing activity is not directly related to soil strength but rather on soil water content (Dexter 1978).

3.3.3 The Effect of Casts on Soil Structure

Casts are generally enriched in OM and cations, and their density is often higher than the surrounding soil bulk density. Microaggregates are formed while passing through the earthworm's gut, organic and inorganic matrices are egested as cast, and water loss from the cast strengthened the bonds among organic and inorganic particles (Shipitalo and Protz 1989). As a result earthworm cast formation can increase soil aggregation, structure, and aggregate tensile strength (McKenzie and Dexter 1987). In general, cast stability is higher than bulk soil but sometimes it can be less than surrounding soil (Shipitalo and Protz 1988).

Casts may be present on the soil surface or within soil. Surface casts have different shapes, sizes, structures and compositions, and therefore their role on soil structure may be different. Globular casts are made of coalescent round or flattened units, and granular casts are made of an accumulation of small fine-textured pellets (Lee 1985).

The effects of earthworms on soil detachment and erosion might be positive or negative depending on the age of casts. Aged casts are usually more stable than the surrounding soil, while fresh casts are highly susceptible to dispersion (Blanchart et al. 1999). The shape, size, and composition of casts depend on earthworm species. In general, small species produce smaller casts, with a finer structure than the large species (Lee 1985). The size of casts depends on earthworm size and ranges from a few millimeter to a few centimeter in diameter. Casts have

different properties compared with surrounding soil. All these properties have important consequences on aggregate stability and erosion. Water runoff simulation clearly showed an enhancement of water infiltration with earthworm casting activity. Although the above-ground casting activity decreased surface runoff, they were not involved in soil detachment (Jouquet et al. 2008). Bulk densities of casts are usually greater than that of the surrounding soil. Jouquet et al. (2008) reported that macro aggregates occurred from casts had a higher soil density (1,600 kg m^{-3} when compared with 970 kg m^{-3} for the surrounding soil). Jouquet et al. (2008) studied the effects of above-ground earthworm casts on water runoff and soil erosion in steep-slope ecosystems in Northern Vietnam. The water runoff simulation clearly showed that surface runoff decreased with earthworm surface casting activity. They distinguished regular soil aggregates from earthworm cast as free biogenic aggregates (rounded shape macroaggregates, which belong to old casts and physicogenic aggregates) (angular to subangular blocky macroaggregates) (Fig. 3.3).

The values of surface cast production in tropical regions range from almost 0 to 200 Mg ha^{-1} per year (Madge 1969; Lavelle 1978; Reddy 1983; Lavelle et al. 1998 and Norgrove and Hauser 1999), while the values of total cast production may reach up to 1,250 Mg ha^{-1} per year (Blanchart et al. 1999). Casts produced on the soil surface increases soil roughness and affects water and infiltration into soil in temperate ecosystems (Binet and Le Bayon 1999; Le Bayon and Binet 2001). The stability of earthworm casts has been found to vary between earthworm species.

Marashi and Scullion (2003) reported that aggregation was closely related with numbers of *Aporrectodea longa* for all sampling depths while number of *L. terrestris* and *A. caliginosa* were closely correlated with aggregation, respectively, at the 0- to 5- and 5- to 10-cm depths. Numbers of *L. rubellus* and *Allolobophora chlorotica* were not associated with improved aggregation.

Zhang and Schrader (1993) ranked earthworm species in terms of their ability to promote interparticle binding in the order of *L. terrestris* > *A. longa* > *A. caliginosa*.

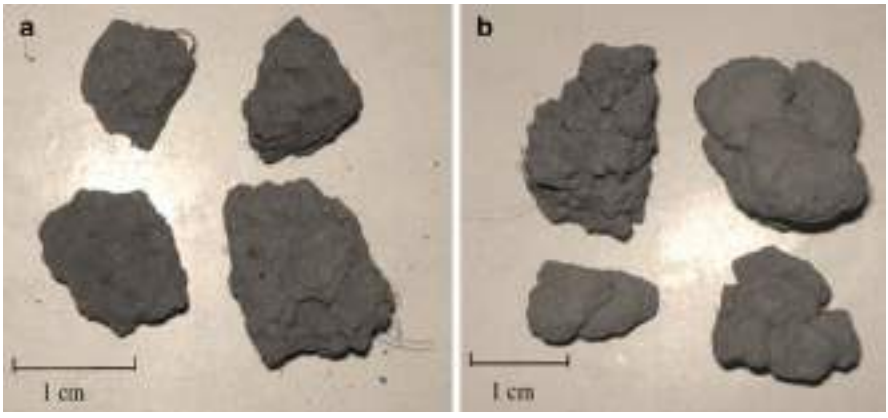

Fig. 3.3 Macroaggregates examples: (**a**) ROUND: rounded shape aggregate belonging to old cast (i.e., biogenic aggregates); (**b**) ANG: (sub)angular blocky aggregate (i.e., physicogenic aggregate) (Photos obtained from Jouquet et al. (2008) with his permission)

Schrader and Zhang (1997) conducted research to determine the effect of the gut passage through earthworms on the aggregate stability of soils varying in texture, carbonate and OM content. The soil material was loam soil (Gleyic Luvisol) and a clay soil (Calcaric-Vertic Cambisol). The anecic detritivorous *L. terrestris* and the endogeic geophagous *A. caliginosa* have been used in the experiment. The contribution of earthworm on the cast stability depended on the parent soil. The tensile strength of casts was positively correlated with the clay and carbonate content of the parent soil. They concluded that the organic carbon content of the earthworm casts is higher than the parent soils. They measured cast and bulk soil's organic carbon contents, which were greater in casts of all earthworm species than in the control soils. Casts of *L. terrestris* in loam soil contained more than twice the organic carbon found in the control soil aggregates. Organic carbon content was 0.75% for control soil and 1.70% for cast of *L. terrestris* in Bt horizon. Packing voids within casts control soil mesoporosity, in which water and solutes are transported and retained (Lamandé et al. 2003).

3.4 Conclusion

Earthworms are important biological factors in soil ecosystems. On the one hand, they have mainly positive effects on soil structure with reducing compacted soil's bulk density, increasing tensile strength and aggregate stability, opening macropores in soil, improving water holding properties of soil. On the other hand, these modifications are significantly related with soil type, cropping systems, and earthworm species. There are comprehensive studies of burrowing effects on soil porosity and water conductivity. Several studies showed that earthworm activity and casting could contribute to the stabilization of organic carbon in stable soil aggregates (Blanchart et al. 1999; Pulleman et al. 2005). Size of earthworm is also important for larger and stable soil aggregate formation. In general, large earthworms are responsible for the formation of the macroaggregate, while smaller earthworms can destroy large aggregates and excrete small aggregates. Nevertheless, more research is needed to estimate earthworms' importance on soil aggregate formation and stabilization.

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Chapter 4
Comparative Anatomy of the Calciferous Gland of Lumbricid Earthworms

María Jesús Iglesias Briones and Trevor George Piearce

4.1 Introduction

The calciferous glands of earthworms (Fig. 4.1a) are commonly referred to as "calciferous glands" "kalkdrüsen", "chylustaschen", or "glandes de Morren", and they reach the highest degree of complexity in the family Lumbricidae. Since their discovery in 1820, several authors have tried to describe the structure and activity of these organs with special emphasis on *Lumbricus terrestris* (e.g., Leo 1820; Morren 1829; Darwin 1881; Harrington 1899), but detailed anatomical and histological descriptions have also been provided for *Eisenia fetida*, *Eiseniella tetraedra*, *Octalasion lacteum*, *Octodrilus complanatus*, *Dendrobaena octaedra*, *Dendrodrilus rubidus*, *Helodrilus oculatus*, *Allolobophoridella eiseni*, *Allolobophora chlorotica*, *Aporrectodea caliginosa*, and *Aporrectodea rosea* (Beddard 1894; de Ribaucourt 1901; Stephenson 1917, 1930; Stephenson and Prashad 1919; Smith 1924; Myot 1957; Semal-van Gansen et al. 1960; Cădariu 1963, 1965; Bertolini et al. 1967). It should be noticed here that in these early descriptions they are referred as "pairs of glands", extending from segment X to segment XIV. For example, Lankester (1864) called them "oesophageal glands" and said that there are three pairs located in segments XII (actually somite XI), XIII, and XIV. Harrington (1899) described the two posterior rounded swellings in segments XI and XII as "two posterior glands (CG^2 and CG^3)." Similarly, de Ribaucourt (1901) indicated that in *L. terrestris* there are four pairs of glandular specializations: the three rounded swellings in segments X (anterior gland), XI, and XII (two middle glands) and the posterior gland in segment XIV. However, further research has

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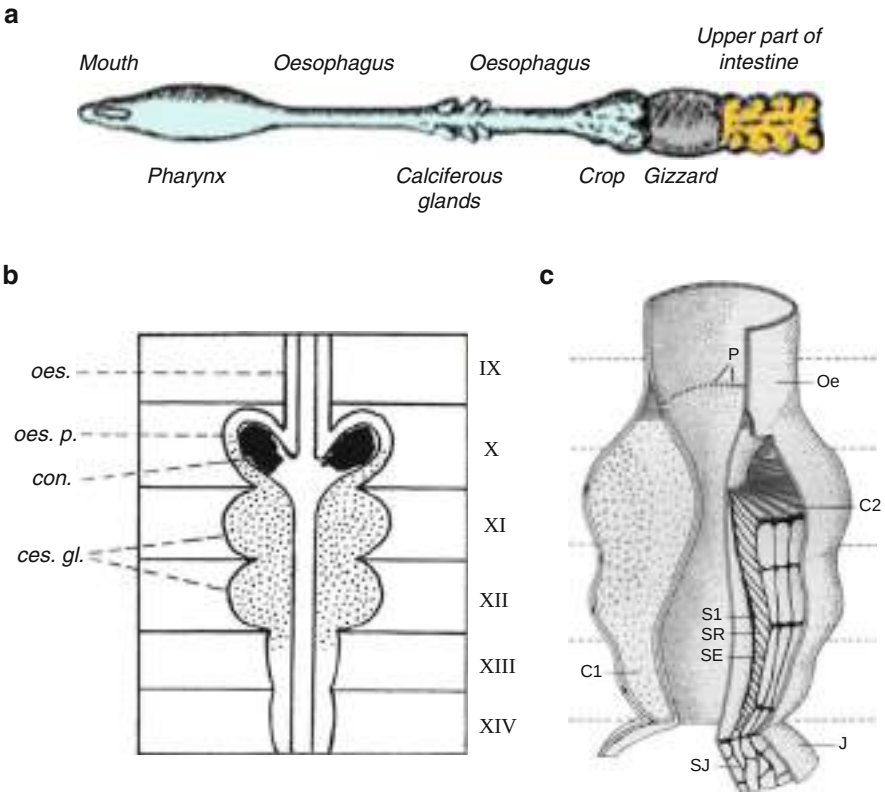

Fig. 4.1 (**a**) Alimentary canal of *Lumbricus* from Lankester (1864); (**b**) diagrammatic horizontal section through the calciferous gland of *Lumbricus terrestris* showing the communication of the pouches with the esophagus (from Robertson (1936)); (**c**) anatomy of the calciferous gland of *Eisenia fetida* showing the distribution of the pores (P) in front of segment 10/11 (from Semal-van Gansen (1959, 1962))

shown that they are indeed one single organ with a paired structure in which most of the glandular activity takes place in the secretory epithelium of segments XI and XII (the "true gland cavities" according to Harrington (1899)), where a concentrated suspension of amorphous calcium carbonate ("milky fluid") in the form of spherulitic deposits is produced (Gago-Duport et al. 2008).

The communication between the gland and the esophagus constitutes another important aspect, which has been debated over the years. Claparède (1869) concluded that the calcareous secretion broke through the epithelial layer anywhere, whereas later observations demonstrated that the secretion passes forward to the esophageal pouches in segment X (Stephenson 1930; Myot 1957). In addition, Harrington (1899) described a second opening at the middle of segment XIV; however, neither Combault (1907) nor Myot (1957) ever found this communication in any of the specimens of *L. terrestris* and *E. fetida* investigated.

In view of this, it is reasonable to assume that the secretion is always released to the esophagus through the foramens present in the pouches (the so-called diverticula of Perrier) (Fig. 4.1b) or through pores in the anterior part of segment XI (Fig. 4.1c) in those species lacking these structures to be finally released into the gut lumen and eventually to the soil.

Despite all this information available, the gland structure of many lumbricid earthworms has not been reported in detail and, for this reason, the interpretation of its macromorphology creates a lot of confusion, making it difficult to use in taxonomical studies (Bouché 1972). Therefore, in this study, we extend the number of species whose gland anatomy has been described and provide new insights into the earthworm gland anatomy at the macroscopic level. The principal morphotypes are established and possible taxonomical implications considered.

4.2 Materials and Methods

Thirty earthworm species belonging to 13 genera (*Allolobophora*, *Aporrectodea*, *Allolobophoridella, Dendrobaena*, *Dendrodrilus*, *Eisenia*, *Eiseniella*, *Lumbricus*, *Murchieona*, *Octalasion*, *Prosellodrilus*, *Satchellius*, and *Scherotheca*) were collected from different locations in Europe (Table 4.1). Species names were assigned following the most recent reviews of lumbricid nomenclature (Blakemore 2007; Briones et al. 2009).

Earthworms were hand-sorted in the field, taken back to the laboratory, and then dissected in distilled water under a SZX12 OLYMPUS microscope to determine the macroscopic morphology of the gland with digital photographic records also made.

4.3 Calciferous Gland Types Based on Anatomy

The morphological study of the different genera showed that the anatomy of the gland falls into three main general morpho-types: (1) pouches and esophageal enlargements present, (2) pouches present but no enlargements, and (3) pouches absent but enlargements present. However, due to the degree of variability of some of these diagnostic characters, distinct subtypes can also be recognized, which helps toward a better understanding of the morphology of this organ in the different species.

4.3.1 Well Developed Esophageal Pouches in Segment X and Lateral Enlargements in Segments XI and XII (Subtype I: Anterior and Posterior Lateral Enlargements of Similar Size)

This type of calciferous gland is typical of the *Lumbricus* species. It extends from segment X to XIV with the calciferous subspherical sacs opening into the

Table 4.1 Taxa examined for this study

Species	Site	Collector/identifier
Allolobophora chlorotica	York, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
	Dublin, Ireland	Dr. O. Schmidt/Dr. O. Schmidt
A. nocturna	Avignon, France	Dr. Y. Capowiez/Dr. Y. Capowiez
A. molleri	Parga, Lugo, Spain	Prof. M.J.I. Briones/Prof. M.J.I. Briones
A. oliveirae	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
Allolobophoridella eiseni	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Ap. caliginosa	Lyons, Ireland	Dr. O. Schmidt/Dr. O. Schmidt
Ap. icterica	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Ap. longa	Lancaster, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
Ap. rosea	York, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
Ap. trapezoides	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
Dendrobaena attemsi	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
D. hortensis	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
D. madeirensis	Pontevedra, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
D. octaedra	York, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
D. veneta	Preston, UK	Dr. K.R. Butt/Dr. K.R. Butt
Dendrodrilus rubidus	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Eisenia fetida	Kents Bank, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
E. andrei	Kents Bank, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
Eiseniella tetraedra	Glen of the Downs, Dublin, Ireland	Dr. O. Schmidt & Prof. M.J.I. Briones/ Dr. O. Schmidt & Prof. M.J.I. Briones
Lumbricus terrestris	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
L. friendi	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
L. rubellus	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
L. castaneus	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Murchieona muldali	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Octolasion cyaneum	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
O. lacteum	Coruña, Spain	Ms. A.C. Harrison/Prof. M.J.I. Briones
O. tyrtaeum	Bayfordbury, UK	Prof. M.J.I. Briones/Prof. M.J.I. Briones
Prosellodrilus praticola	Olite, Navarra, Spain	Dr. P. Bescansa/Prof. M.J.I. Briones
Satchellius mammalis	Lancaster, UK	Dr. T.G. Piearce/Dr. T.G. Piearce
Scherotheca gigas aquitania	Olite, Navarra, Spain	Dr. P. Bescansa/Prof. M.J.I. Briones

esophagus ventrally in segment X and just in front of septum 10/11 and consisting of conspicuous paired swellings in XI and XII (Harrington 1899; de Ribaucourt 1901; Stephenson and Prashad 1919; Stephenson 1930; Smith 1924; Myot 1957; Bouché 1972; Sims and Gerard 1999) (Fig. 4.2a).

The four species investigated here (*L. terrestris*, *L. friendi*, *L. rubellus*, and *L. castaneus*) show well-developed glands with the greatest secretory activity of

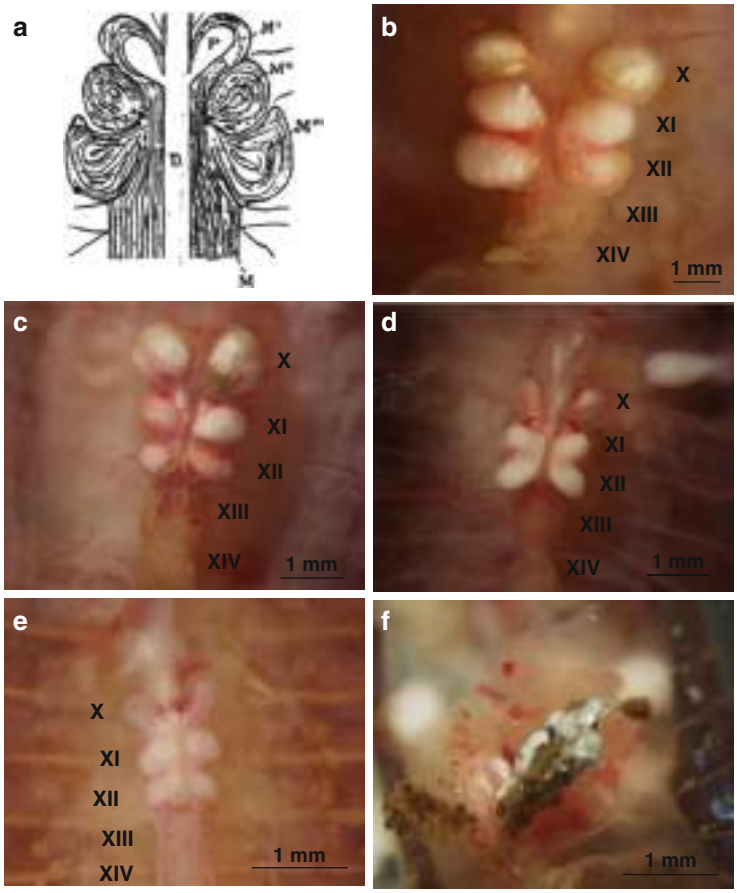

Fig. 4.2 (**a**) Scheme of the calciferous gland of *Lumbricus herculeus* (syn. *L. terrestris*) from de Ribaucourt (1901); (**b**) calciferous gland of *L. terrestris*; (**c**) calciferous gland of *L. friendi*; (**d**) calciferous gland of *L. rubellus*; (**e**) calciferous gland of *L. castaneus*; (**f**) milky fluid wrapping the esophageal content of *L. rubellus*

any of the species investigated (Figs. 4.2b–e). The two glandular portions in segments XI and XII are obviously enlarged and the "milky fluid" entirely fills the internal spaces. The pouches in segment X are elongated and often contain aggregates of calcite crystals of 0.5–2.5 mm size (Canti and Piearce 2003; Gago-Duport et al. 2008), which are then released to the soil. In contrast, the remaining section of the gland is less broadened and shows no obvious difference from the rest of the esophagus.

The high complexity and activity of the glands is associated with the occurrence of these species in soils that are often highly organic, with abundant decaying plant material on the soil surface, and also under dung pats, logs, and stones. These are mostly relatively active, mobile, acid-tolerant species with a rich diet. The calcium

carbonate secretion was often seen wrapping the ingested material in the most acid-tolerant species, such as *L. rubellus* (Fig. 4.2f).

4.3.2 *Well-Developed Esophageal Pouches in Segment X and Lateral Enlargements in Segments XI and XII (Subtype II: Glandular Portion in Segment XII Smaller in Size)*

The earliest description of the calciferous gland of *Octalasion* can be found in de Ribaucourt (1901) for the species *Octalasion profugum* (*Octalosion profugum*; syn. *O. lacteum*, Csuzdi and Zicsi (2003)), which briefly indicates that the glandular activity takes place in the posterior segment of the gland (XIV) and there is no mention of swellings or the presence of pouches in segment X. However, the illustration accompanying this description (Fig. 4.3a) shows some lateral enlargements in the approximate position of segment XI.

Later descriptions have demonstrated the presence of pouches in *Octalasion* spp. (Smith 1924; Cădariu 1963; Bouché 1972), which open vertically to the equator of segment X (Sims and Gerard 1999), but none of them have indicated the presence of lateral enlargements.

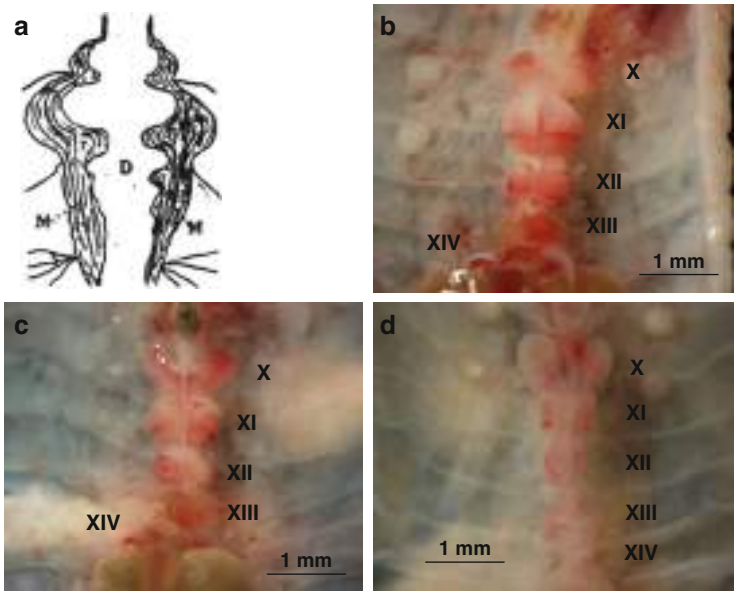

Fig. 4.3 (**a**) Scheme of the calciferous gland of *Octolasion profugum* (syn. *O. lacteum*) from de Ribaucourt (1901); (**b**) calciferous gland of *O. cyaneum*; (**c**) calciferous gland of *O. lacteum*; (**d**) calciferous gland of *O. tyrtaeum*

The three species included in this study, *O. cyaneum*, *O. lacteum* and *O. tyrtaeum* (Figs. 4.3b–d), show the same anatomical arrangement of the gland with conspicuous pouches in segment X and two rounded and swollen portions in segments XI and XII, the anterior enlargement being bigger in size. No white fluid was observed through the transparent body and gland walls, which suggests that, perhaps, the organ is less active in this genus. Indeed previous work (Cădariu 1963) has shown that the Golgi apparatus is less developed in *Octalasion* than in *Lumbricus*, which is an unequivocal sign of less secretory activity. Furthermore, the pouches are conspicuous but were often empty or contained very little material. Interestingly, laboratory cultures in calcium enriched soils (Canti and Piearce 2003) have allowed the collection of a sufficient number of granules to conclude that their average size is less than 0.3 mm. Our investigations showed that granules vary in size, and one big solid concretion or several granules can be found in a given pouch.

All three species investigated here have in common a broad pH tolerance, from low values such as 4.3 to alkaline ranges above 8 (Sims and Gerard 1999). However, Bouché (1972) regards *O. lacteum* as nonacid tolerant, *O. lacteum gracile* (syn. *O. tyrtaeum*) as relatively acid tolerant, and *O. cyaneum* as highly acid tolerant.

4.3.3 *Small Esophageal Pouches in Segment X and Lateral Enlargements (Subtype I: Glandular Portion in Segment XI Bigger in Size)*

The species included here have pouches in segment X, although they are less conspicuous than in *Lumbricus* and *Octalasion* and only segment XI is obviously dilated (Fig. 4.4). In the case of the dissected specimens of *Allolobophoridella eiseni* (Fig. 4.4a), a great production of milky fluid was observed, suggesting that the gland is very active in this species. This could be related to its habitat: acidic environments, namely decaying leaves and logs, peaty and podzolic soils. It should

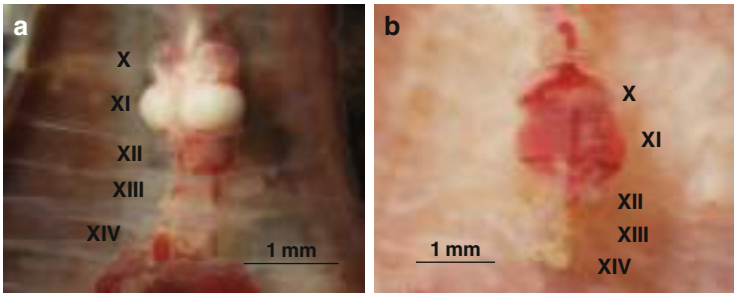

Fig. 4.4 (**a**) Calciferous gland of *Allolobophoridella eiseni*; (**b**) calciferous gland of *Dendrobaena madeirensis*

also be mentioned here that *Ad. eiseni* has been traditionally considered to be a *Lumbricus* species in the literature (Sims and Gerard 1999) and an *Eisenia* species in the French literature (Bouché 1972). However, recent molecular work (Briones et al. 2009) has shown that it should be removed from this genus. Our results clearly demonstrate that the anatomy of its calciferous gland is very different from that of the rest of the *Lumbricus* species but, unlike *Eisenia* species, *Ad. eiseni* has pouches. Therefore, these results add more support to its exclusion from these two genera and its correct position in the genus *Allolobophoridella* created by Mršić (1990).

Interestingly, the endemic species of the north-western area of the Iberian Peninsula (Alvarez 1971), *Dendrobaena madeirensis*, shows a similar arrangement in its calciferous gland (Fig. 4.4b) but in this case less secretory activity has been detected. Very little information on the internal anatomy of this species is available, just the number of seminal vesicles and spermathecae; therefore, this is the first time the calciferous gland of this species is described. This species was originally described as *Allolobophora madeirensis* by Michaelsen (1891); however, 2 years later Rosa (1893) included it in the subgenus *Dendrobaena* (i.e., *A. (Dendrobaena) madeirensis*) and it was raised to generic level by Alvarez (1971). Although it has been retained in *Allolobophora* by other authors (de Ribaucourt 1896; Easton 1983), it is now considered to be a valid taxon (Blakemore 2007). However, the anatomy of its gland suggests that this species might need to be reassigned to another genus.

4.3.4 *Small Esophageal Pouches in X and Lateral Enlargements (Subtype II: Variable Number of Dilated Segments)*

Three species belonging to three different genera are included here: *Dendrodrilus rubidus*, *Satchellius mammalis* and *Prosellodrilus praticola*. The earliest description of the calciferous gland of these species is provided by de Ribaucourt (1901) for *Dendrobaena putris* (syn. *Dd. rubidus*). The gland is similar to that of *Eiseniella tetraedra*, although the posterior region is more elongated and less differentiated, and the anterior region and the calciferous sacs are well developed (Fig. 4.5a).

Our dissection work suggests that the calciferous gland of these three species is very similar (Figs. 4.5b–d). In particular, they all have in common the smaller size of their calciferous sacs, when compared with *Lumbricus* and *Octalasion* species. Indeed, Bouché (1972) indicates that the calciferous sacs in *S. mammalis* are quite reduced, whereas Sims and Gerard (1999) do not make any reference to the size of the pouches; however, these authors found the opening of its gland into the esophagus to be posterior and ventral like in *Lumbricus*. In the case of *Dd. rubidus*, the gland is described as having paired diverticula in segment X (Bouché 1972) which open posteriorly into the esophagus ventrally in front of septum 10/11 (Sims and Gerard 1999). According to Bouché (1972), the calciferous gland of *Pr. praticola* lacks pouches and it extends from segment XI (dilated) to XIV.

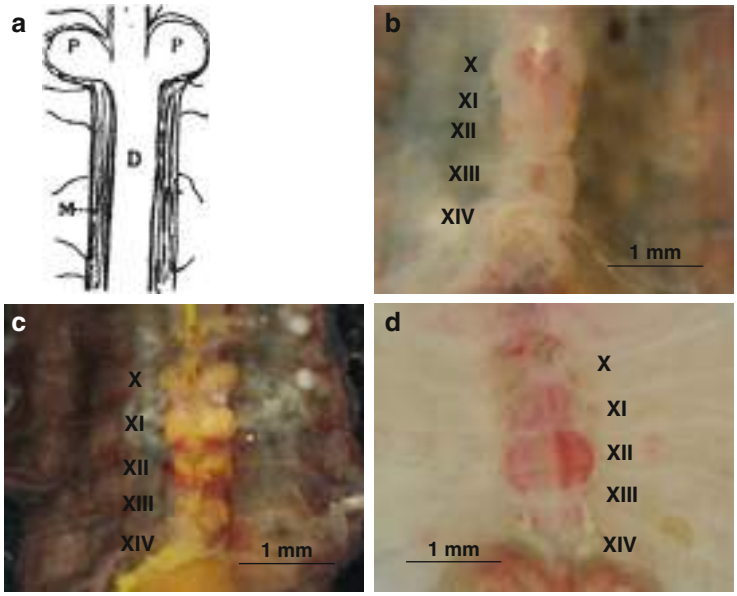

Fig. 4.5 (**a**) Scheme of the calciferous gland of *Dendrobaena putris* (syn. *Dd. rubidus*) from de Ribaucourt (1901); (**b**) calciferous gland of *Dd. rubidus;* (**c**) calciferous gland of *S. mammalis*; (**d**) calciferous gland of *Pr. praticola*

Our dissection work shows that pouches are present in these three species and that all the glandular segments (XI–XIV) are somewhat dilated. But in the case of *Dd. rubidus*, none of them are more swollen than the others (Fig. 4.5b); in *S. mammalis*, they gradually decrease in size toward the posterior region such as in the *Octalasion* species (Fig. 4.5c) and in *Pr. praticola*, segment XII is distinctively dilated (Fig. 4.5d).

The presence of pouches in these three species suggests that they may be well capable of producing solid concretions but their morphological characteristics are still unknown. Both *Dd. rubidus* and *S. mammalis* are regarded as relatively acid tolerant, whereas *Pr. praticola* is neutrophile, according to Bouché (1972).

4.3.5 Well-Developed Esophageal Pouches in X But No Lateral Enlargements

The only obvious characteristic of this type of calciferous gland is the presence of well-developed pouches in segment X. The earliest descriptions are given by de Ribaucourt (1901) who described *Allolobophora turgida* (syn. *Aporrectodea caliginosa*), *Allolobophora (Notogama) rosea* (syn. *Ap. rosea*), *Allolobophora*

chlorotica, and *Allurus tetraedrus* (syn. *Eiseniella tetraedra*) as all lacking evaginations in the esophagus but having two prominent calciferous sacs in segment X (Fig. 4.6a). Later, Smith (1924) described *Helodrilus roseus* (syn. *Ap. rosea*), *Helodrilus tetraedrus* (syn. *Ei. tetraedra*), *H. chloroticus* (syn. *A. chlorotica*), and *H. caliginosus trapezoides* (syn. *Ap. trapezoides*) as having pouches but no other lateral enlargements. Similar descriptions are given by Stephenson (1930) for *A. chlorotica* and *A. caliginosa trapezoides* (syn. *Ap. trapezoides*) and in Bouché (1972) for *A. chlorotica*, *A. icterica* (syn. *Ap. icterica*), *Nicodrilus longus* (syn. *Ap. longa*), *N. caliginosus caliginosus* (syn. *Ap. caliginosa*), *Nicodrilus caliginosus alternisetosus* (syn. *Ap. tuberculata* = *Ap. caliginosa*), *N. caliginosus meridionalis* (syn. *Ap. trapezoides*), *N. nocturnus* (syn. *A. nocturna*), *A. muldali* (syn. *Murchieona muldali*), and *Scherotheca* spp. It is interesting to notice that he, sometimes, has observed an enlarged segment XIII in specimens of *A. rosea* (syn. *Ap. rosea*).

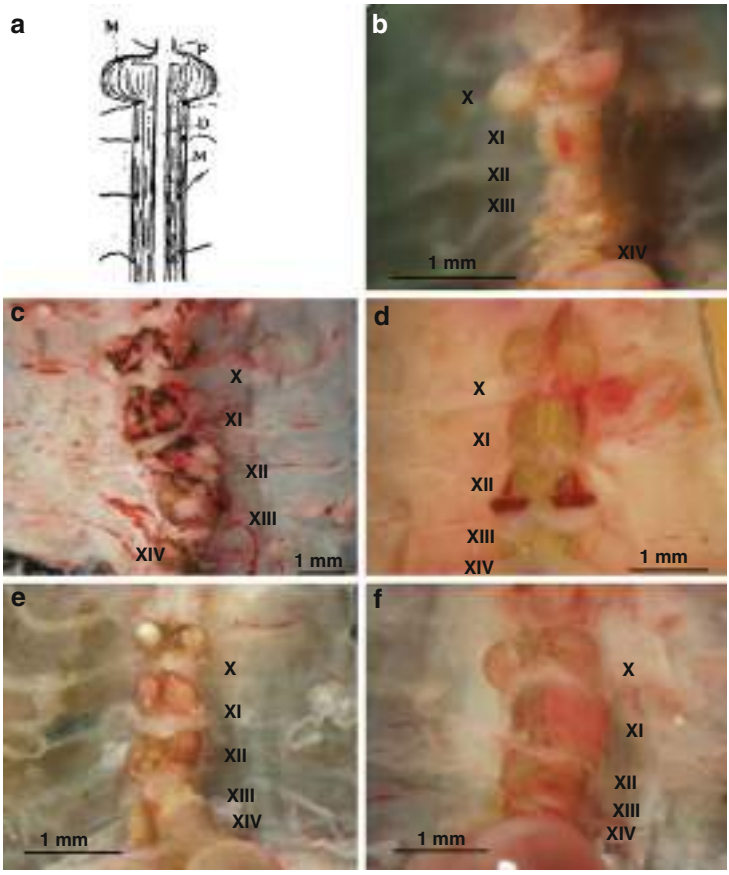

Fig. 4.6 (continued)

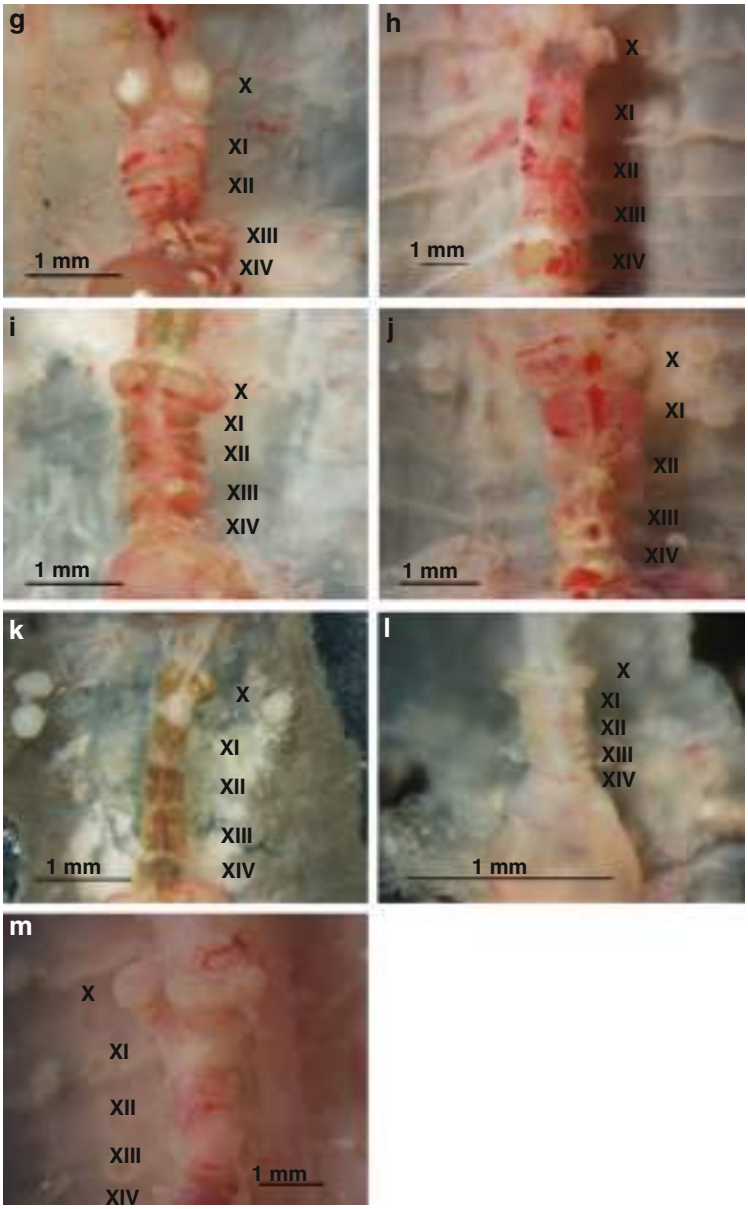

Fig. 4.6 (**a**) Scheme of the calciferous gland of *Notogama rosea* (syn. *Ap. rosea*) from de Ribaucourt (1901); (**b**) calciferous gland of *A. chlorotica*; (**c**) calciferous gland of *A. molleri*; (**d**) calciferous gland of *A. nocturna*; (**e**) calciferous gland of *A. oliveirae*; (**f**) calciferous gland of *Ap. caliginosa*; (**g**) calciferous gland of *Ap. icterica*; (**h**) calciferous gland of *Ap. longa*; (**i**) calciferous gland of *Ap. rosea*; (**j**) calciferous gland of *Ap. trapezoides*; (**k**) calciferous gland of *Ei. tetraedra*; (**l**) calciferous gland of *Mu. muldali*; (**m**) calciferous gland of *Sc. gigas*

The opening of these pouches into the esophagus and the extent of the lamellae are slightly different in each of the genera and thus, in *Allolobophora* and *Eiseniella*, the pouch opening is located ventrally in front of septum 10/11 and the lamellae extend along the lateral walls, whereas in *Aporrectodea*, the pouches open at the equator of segment X and the lamellae continue onto the posterior walls (Sims and Gerard 1999). The genus *Murchieona* deserves special consideration as the genus was established by Murchie (1956) to accommodate those species presenting a unique structure of the calciferous sacs, which has been described as finger-like in shape and with two compartments, the anterior one opening dorsally directly into the esophagus and the posterior one into the intralamellar tunnels of the calciferous gland.

At the macroscopic level, the calciferous glands of the 12 species belonging to the five genera (*Allolobophora*, *Aporrectodea*, *Eiseniella*, *Murchieona*, and *Scherotheca*) investigated here confirm these previous results (Figs. 4.6b–m): the glandular segments XI and XII are not swollen and their delimitation from the rest of the esophagus is not appreciable. No obvious signs of secretory activity were detected, although on some occasions a few crystals were collected from the pouches suggesting that the organs are sometimes active. Indeed, production of concretions in soil has been recorded for *A. chlorotica*, *Ap. caliginosa*, *Ap. longa*, and *Ap. icterica*, but in significantly lower quantities than in other genera with more active glands such as *Lumbricus* spp. (Canti and Piearce 2003); according to these findings the anecic *Ap. longa* and the epigeic *A. chlorotica* produced the highest number of granules during the 10 months incubation period.

4.3.6 *Esophageal Pouches in X Absent But Lateral Enlargements Present in Segments XI and XII (Subtype I: Segment X Not Enlarged)*

The most obvious characteristic of the calciferous gland of the three species included here is the maximum development of the glandular segments XI and XII, which are well enlarged as in the *Lumbricus* species. However, unlike them, the esophageal pouches are absent, although early descriptions by de Ribaucourt (1901) showed the presence of a small pouch in segment X for *Dendrobaena octaedra* (Fig. 4.7a). The anatomy of the calciferous gland in *D. octaedra* and *D. attemsi* (Figs. 4.7b, c) indicates that it is a highly active organ as the two enlargements in segments XI and XII contain great amounts of milky fluid. Because of the small size of these two worms, these two white structures can also be seen through the transparent body wall in the intact worm. On some occasions, the secretion fills the gut and extends onto segment X resulting in the enlargement of this part of the esophagus (Fig. 4.7c) and perhaps, this is what led to the belief that there was indeed a pouch in this segment.

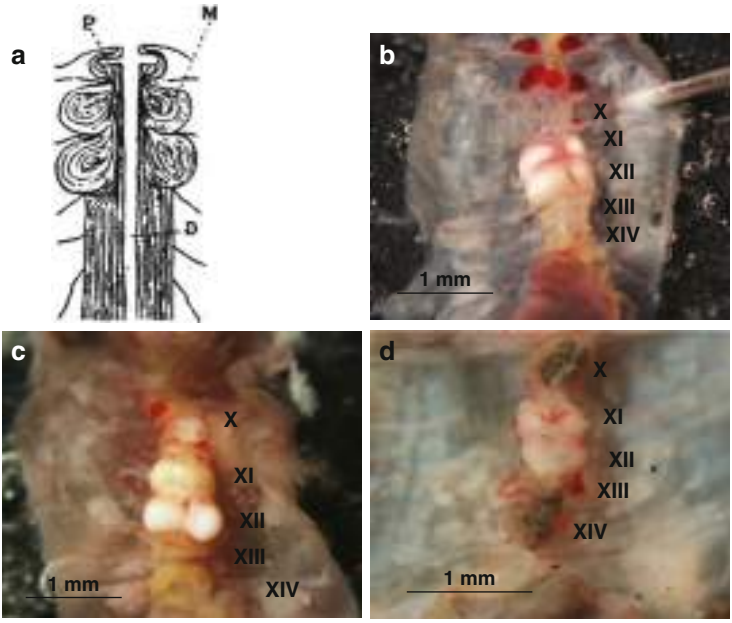

Fig. 4.7 (**a**) Scheme of the calciferous gland of *Dendrobaena octaedra* from de Ribaucourt (1901); (**b**) calciferous gland of *D. attemsi;* (**c**) calciferous gland of *D. octaedra*; (**d**) calciferous gland of *D. hortensis*

In the other *Dendrobaena* species inhabiting compost heaps, *D. hortensis*, these two glandular segments contain a much lower amount of calcareous secretion and that is why they are also less distended (Fig. 4.7d). Interestingly, Smith (1924) indicated that the gland in *Helodrilus venetus hortensis* starts in segment XI and differs from that of the typical form, *Helodrilus venetus* (syn. *Dendrobaena veneta*; see also below) where the gland begins in somite X.

4.3.7 Esophageal Pouches in X Absent But Lateral Enlargements Present in Segments XI and XII (Subtype I: Segment X Dilated)

Like the above genera, the calciferous gland of the species included here does not have calciferous sacs in segment X, although a small diverticulum has been described (de Ribaucourt 1901; Fig. 4.8a). This means that the only communication of the gland with the esophagus is through the secretory portions of the gland, possibly at segment XI (Semal-van Gansen 1962; Sims and Gerard 1999; see also Fig. 4.1c).

Two lumbricid genera present this anatomical arrangement in their calciferous gland, *Dendrobaena* and *Eisenia*. A great variability in the secretory activity of the

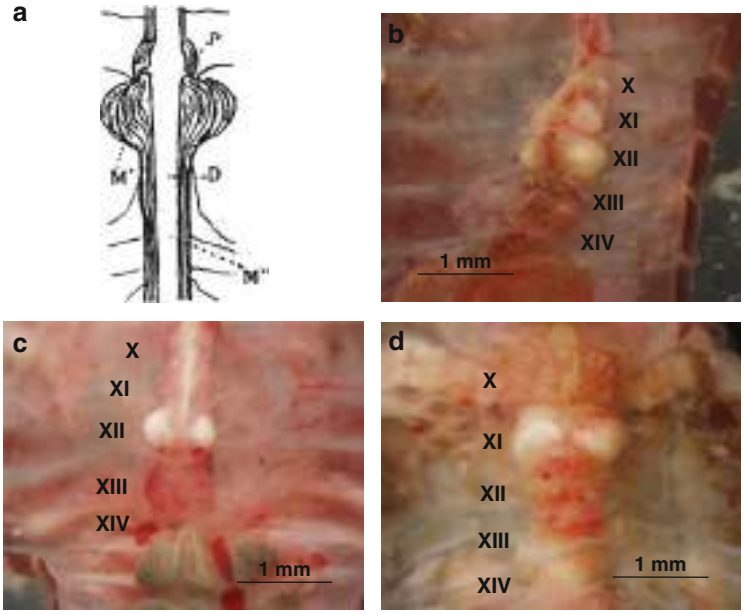

Fig. 4.8 (**a**) Scheme of the calciferous gland of *Notogama foetida* (syn. *E. fetida*) from de Ribaucourt (1901); (**b**) calciferous gland of *E. andrei;* (**c**) calciferous gland of *E. fetida*; (**d**) calciferous gland of *D. veneta*

calciferous gland of the dissected species included here was observed. This is associated with variations in the size of the lateral enlargements (in segments XI or XII) containing secretion. Thus, in the two compost worms, *Eisenia andrei* and *E. fetida*, the greatest amount of calcareous secretion was observed in segment XII (Figs. 4.8b, c), whereas in the case of *D. veneta*, only the anterior glandular portion in segment XI contained milky fluid (Fig. 4.7d). This is in agreement with earlier observations by Bouché (1972). In relation to this, a distinct functional regionality has been suggested for these two glandular portions, at least for *E. fetida* (Myot 1957; Cădariu 1965); with segment XI being the "conductive region" with a ciliated prismatic epithelium, whereas in the "secretary region", which extends from segment XII to XIV, it is clearly syncitial with a well-developed Golgi complex.

Early observations revealed the seasonal activity of the glands. Morren (1829) wrote that the glands disappeared in the winter, and other authors pointed out that these organs only contain calcium carbonate at certain seasons (Marshall and Hurst 1887). More detailed ultrastructural studies have revealed that these variations in the production and location of the calcareous secretion are the result of different secretion cycles among different individuals (Harrington 1899) and secretory-destructive cycles within the same individual, which involves histological alterations of the glandular epithelium that have been described for *E. fetida* (Cădariu 1965).

4.4 Conclusions

The morphology of the calciferous gland in the members of the family Lumbricidae shows a great variability both in anatomical aspects as well as secretory activity. This could be related to the habitat and the feeding activities of the different species for example, those living in acidic environments and consuming fresh organic matter tend to have more complex glands producing great amounts of calcareous fluid. Furthermore, changes in the environmental conditions and the associated resting phases (e.g., diapause) could have a direct effect on the activity of these organs.

Importantly, the anatomical variability of this organ makes it difficult to use it as a diagnostic character in taxonomical studies but provides valuable information for reassigning species names. For example, the results of this study have added more supporting evidence for removing *Lumbricus eiseni* from the *Lumbricus* genus. Interestingly, the presence of pouches in the calciferous gland of the endemic species *Dendrobaena madeirensis* raises some doubts about its inclusion in the genus *Dendrobaena*. Previous molecular work (Briones et al. 2009) did not allow a good elucidation of its phylogenetic position, and further research is needed to clarify its taxonomic status. Similarly, the anatomy of the calciferous gland of *D. veneta*, another conflictive species from the taxonomical point of view (Briones et al. 2009), shows more similarities with the *Eisenia* species than with the *Dendrobaena* ones and highlights the need of more studies to solve this nomenclature problem.

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Chapter 5
Reproduction of Earthworms: Sexual Selection and Parthenogenesis

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5.1 Introduction

Earthworms are generally considered to be cross-fertilization hermaphrodites (i.e., using reciprocal insemination, transferring, and receiving sperm in the same copulation). Although not all earthworms use this reproductive strategy, the best known species, *Lumbricus terrestris*, is a cross-fertilization hermaphrodite and this strategy seems to be the most widespread in earthworms. Nevertheless, cases of self-fertilization have been reported in earthworms; Domínguez et al. (2003) discussed that *Eisenia andrei* individuals bend themselves, allowing their spermathecal pores to contact the ventral zone of their clitellum. The sperm is then transported from the male pores to the spermathecae. This finding explains why 33% of isolated individuals in this study produced viable cocoons.

However, hermaphroditism is not the only reproductive mechanism and more parthenogenetic earthworms are being discovered all the time, most of which are polyploid. Parthenogenetic reproduction is very frequent in the family Lumbricidae, with more than 30 parthenogenetic species occurring in North America (Reynolds 1974). Parthenogenesis has also been reported in families such as Megascolecids, but has not been observed in other families, including Glossoscolecids.

"Asexual" reproduction by means of bipartition, stolonisation, budding, or similar processes has not been observed in earthworms and their ability to regenerate is limited. There are several reproductive models: discontinuous, semicontinuous, or continuous. In *Hormogaster elisae*, male and female gametogenesis are synchronized, beginning in autumn and ending in the summer. Male funnels are full of spermatozoa and the spermathecae contain spermatozoa throughout the year, but

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two peaks of reproduction have been observed, with the largest peak occurring in the spring and the second peak occurring in autumn (Garvín et al. 2003).

An excellent description of the earthworm reproductive system can be found in general zoology volumes and monographs such as Jamieson (2006), so it will be only succinctly described in the present chapter. Earthworms are usually hermaphrodites in which the testes and ovaries are accompanied by a series of organs with a male or female function. The female components typically include the ovaries (generally one pair in the 13th segment), ovisacs (in the 14th segment), oviducts, female pores (in the 14th segment), and spermathecae (of variable position and number). Male components typically include the testes and male funnels (in most cases, there are two pairs in the 10th and 11th segments and singularly a single pair in the 11th segment), seminal vesicles (of variable number, with 2–4 occurring in segments 9–12), deferent ducts, and male pores surrounded by atrial glands that are more or less developed. Other organs, such as testicular sacs (*Lumbricus* and *Octolasium*), accessory glands (prostates), or the thecal glands associated with the spermathecae, may also be present.

Some of the external reproductive organs, such as the clitellum, tubercula pubertatis, and sexual papillae, are developed at sexual maturity. The sexual papillae include modified genital chaetae and chaetal glands, which could be used to inject substances into the partner (see Sect. 8.2.2).

The union during copulation, which could last between 69 and 200 min in *L. terrestris*, is secured by tubercula and quetae. Copulation can occur at the surface in epigeic and anecic earthworms, which increases the depredation risk, and also occurs in deeper layers of the soil in the case of endogeic species. The more primitive type of copulation seems to be a simple juxtaposition of the male pores of one individual and the spermathecal pores of the other, with the direct transfer of spermatozoa. The presence of a penis has been observed in some cases, which in reality seems to be just an elevated papilla, as in the case of some *Pheretima* species.

In most of the species in the Lumbricidae family and in other families, the clitellum moves backwards and seminal groves are developed from the male pores to the tubercula pubertatis. Spermatozoa flow through the seminal groves to get into the partner's spermathecae pores. Details of sperm transfer are not well known with the exception of a few species such as *Pheretima sp*., in which, according to Tembe and Dubash (1961), the sperm appears to be transferred sequentially, passing first to the anterior spermathecae and later to the posterior ones.

Bouché (1975) indicated that spermatophores have been observed in more than 20 species of lumbricids. Spermatophores are small capsules that adhere to the body wall and can be iridescent and full of spermatozoa. Their function is not clear. It has been suggested that the spermatophores may play a role in sperm transfer (Edwards and Bohlen 1996), thus avoiding sperm digestion in the spermathecae and fertilizing the ova during cocoon formation Michiels (1998). Nevertheless, Monroy et al. (2003) showed that spermatophores have no effect on the reproductive success of *Eisenia fetida* and were not able to demonstrate the specific function of these capsules.

Complex precopulatory behaviors have been described in partner selection in some species, including *L. terrestris*, in which individuals perform visits to their neighbors' burrows (Nuutinen and Butt 1997; Michiels et al. 2001, see Sect. 8.2.1). Development is direct in earthworms. Fertilization occurs within cocoons and one or more juveniles are produced for each cocoon.

The presence of parthenogenesis in earthworms was first observed many years ago, thanks to the contributions of authors such as Omodeo (1951), Casellato (1987), Jaenicke and Selander (1979) and Victorov (1997), among others.

Reynolds (1974) pointed out that in North America 35 species are anphimictic, 11 probably sexual, 4 facultative parthenogenetic, 1 possibly parthenogenetic, and 30 parthenogenetic. Casellato (1987) cited 25 parthenogenetic species or subspecies (12 of which had even ploidy numbers and 13 of which showed odd ploidy) and Victorov (1997) pointed out that in Russia, the number of polyploids almost equals the number of diploids, with a ratio of 46 polyploids: 52 diploids. He observed that polyploids (in cases of sympatry) tend to occupy the margins of the distribution areas. According to Edwards and Bohlen (1996), the association between parthenogenesis and high polyploidy in earthworms produces an unexpected level of heterozygosity, an advantageous condition that provides resistance to environmental stress.

5.2 Sexual Selection in Cross-Fertilization Earthworms

In simultaneous hermaphrodites, a trade-off between male and female sexual functions is expected because the two sexes share limited resources from the same individual. In addition, the strategy that maximizes fitness is different for the male and female functions. This has been explained previously by Bateman (1948), who showed that the higher the number of partners, the higher the fitness for the male function because it produces small sperm cells. Nevertheless, female function maximizes its fitness by seeking high quality mates because it produces large eggs and this function has to invest in cocoon production. As a consequence, there is a conflict between the sexes. Indeed, Porto et al. (2008) found a negative relationship between the present investment in male function and the future fertility of the female function in their research on *E. andrei*. Sexual selection is expected to occur because of female function as long as a sufficient number of mates are available.

5.2.1 Precopulatory Sexual Selection

Copulation is very costly and involves sperm and mucus production and long periods of time. Consequently, precopulatory selection is expected in environments where the density of earthworms is high.

One of the factors that could influence precopulatory sexual selection is the female fecundity of the partner, which may be related to body size. Large earthworms have not been found to produce more cocoons (Tato et al. 2006; Butt and Nuutinen 1998) but they do tend to produce heavier cocoons and larger offspring (Michiels et al. 2001). Size-assortative mating was indeed observed in the field for the epigeic *E. fetida* (Monroy et al. 2005) and for the endogeic *H. elisae* (Novo et al. in press), as well as in laboratory experiments for the anecic *L. terrestris* (Michiels et al. 2001). Earthworms selected similar-sized partners. Because every earthworm seeks a bigger partner, equilibrium is finally reached, resulting in partners with a similar weight, thus balancing the expectations of both mates on female and male functions. In the particular case of epigeic and anecic worms, which can copulate at the surface, this general tendency could be reinforced by a trade-off; worms can either select a bigger, more fecund partner or a smaller partner, which would decrease the risk of predation.

In ongoing laboratory experiments with *H. elisae*, we have observed that there is no such size selection in virgin individuals, although the bigger virgin individuals always managed to copulate so they seem to be more desirable.

Aside from size, reciprocation is sought from a potential partner. In simultaneous hermaphrodites, the primary purpose of mating is to fertilize the eggs of their partners, rather than to fertilize their own eggs. Therefore, the conflict of two earthworms copulating would be the amount of sperm that each of them is allowed to give (Michiels 1998).

Finally, the quality of the place where cocoons are deposited after copulation and the suitability of the burrow for offspring development (i.e., the moisture or litter content) could be important factors for precopulatory assessment. Ortiz-Ceballos and Fragoso (2006) studied parental care in *Pontoscolex corethrurus* and *Balanteodrilus pearsei*. They found that both species build up a chamber that they periodically clean and surround with fresh casts where a single cocoon is deposited. Grigoropoulou et al. (2008) observed that *L. terrestris* deposits the cocoons inside burrows, which may offer a protective location from the physical environment or may represent parental investment as they were also found to be coated with earthworm casts. These casts could be a means of maintaining the moisture content or protecting cocoons from predators.

The mechanism through which earthworms choose a mate, assess size, test reciprocity, or assess the burrow quality of their potential partners remains unknown, although there are some data on these factors. Chemical cues have been suggested in earthworms as a mechanism of finding and attracting the mate (Olive and Clark 1978; Edwards and Bohlen 1996).

Grove and Cowley (1926) suggested the existence of a courtship in *E. fetida* as they observed short and repeated touches between partners before mating. This type of contact, executed with the prostomium, was also observed by Nuutinen and Butt (1997) in *L. terrestris* and could last 90 min. The prostomium has been described as a sensory lobe with many chemoreceptors or sensory cells (Wallwork 1983).

During contact, the clitellum and associated structures could be indicators of female functionality and glandular margins of the male pores could be indicators

of male functionality. These structures could provide a means of evaluating the partner and assuring reciprocation. Reciprocation can also be assured by increasing the copulation time, which would prevent the partner from copulating with other earthworms. In addition, Nuutinen and Butt (1997) observed that *L. terrestris* visited the potential mate's burrow by inserting its anterior segments, but retaining the posterior segments in their own burrows, as a mechanism to evaluate the quality.

In case of the size assessment, it is also suggested that assortative mating could be due to a physical incompatibility of the copula among individuals of different sizes (Michiels et al. 2001), although this incompatibility would only result from large differences in size.

These selective forces depend on other factors, such as the density of earthworms or the distance of potential mates. Indeed, the low dispersal ability of these animals provides a restriction in the number of available mates. Earthworms have low migration rates, with observed natural dispersal rates of only 1.4–9 m year^{-1} (Ligthart and Peek 1997; Hale et al. 2005) and are therefore expected to mate with partners living in their vicinity. In addition, in the case of the earthworms who copulate at the surface, a smaller distance to the partner would also minimize the risk of predation. There is evidence for this selective limitation produced by distance. Nuutinen and Butt (1997) investigated burrow visit patterns in *L. terrestris* and found that the nearer the burrow opening was, the more visits the worms made to assess the potential partner quality. In addition, Sahm et al. (2009) showed mate choice in the same species for its closest partner and Novo et al. (in press) found that *H. elisae* do not move long distances to find mating partners. Nevertheless, this low dispersal could cause inbreeding, which is generally accepted to be unadaptative and would reduce the fitness of the offspring. Partner selection has not been found to be dependent on relatedness (i.e., kin recognition), and Novo et al. (in press) did not find a correlation between mating probabilities and the level of heterozygosity in *H. elisae*. Regarding this, differential investment in offspring is thought to occur (Velando et al. 2006, see Sect. 8.2.2).

Finally, parasite concentrations may influence mate choice, since they can have a negative effect on earthworm growth as shown by Field and Michiels (2005) for the association between *Monocystis* and *L. terrestris*. In addition, earthworm skin color could be positively correlated with parasite concentration (Field et al. 2003), which could be a sign used to evaluate partners. Nevertheless, Sahm et al. (2009) failed in an attempt to show a relationship between parasite concentration and mate choice, and more studies are needed to assess this correlation.

5.2.2 Postcopulatory Sexual Selection

In spite of the precopulatory sexual selection, multiple mating is common in earthworms (Monroy et al. 2003; Sahm et al. 2009; Novo et al. in press) and all the allosperm received is stored and sometimes mixed (Novo et al. in press) in the

spermathecae. Therefore, postcopulatory sexual selection such as sperm competition (Parker 1970) or cryptic female choice (Thornhill 1983) could be expected.

The sperm remains viable in the spermathecae until fertilization. Butt and Nuutinen (1998) observed that *L. terrestris* was capable of successfully maintaining the received sperm up to 6 months. Meyer and Bowman (1994) reported that *E. fetida* continued cocoon production for up to 12 months after the earthworms were isolated from their partner, although these authors did not measure viability. Garvín et al. (2003) reported spermathecae full of spermatozoa during diapause in *H. elisae*. This would be advantageous for species with poor dispersal capacities or for species that occur in low densities that can copulate at any time of the year.

The maintenance of sperm for such a long time implies the existence of some kind of preservation mechanism. There is evidence suggesting that the spermathecal epithelium actively contributes to the successful maintenance of sperm by providing a favorable luminal environment (Grove 1925; Varuta and More 1972) or by producing nourishing substances (Vyas and Dev 1972; Jamieson 1992; Novo et al. (unpublished data))

A possible mechanism for postcopulatory sexual selection developed by the recipient is sperm digestion. Richards and Fleming (1982) observed spermatozoal phagocytosis by the spermathecae of the facultative parthenogenetic *Dendrobaena subrubicunda* and other lumbricids. This is likely related to the removal of aging or aberrant sperm during the months when cocoon production was minimal. Novo et al. (unpublished data) found sperm degeneration in the central area of spermathecae from *H. elisae* (Fig. 5.1a, b). These authors also observed sperm intrusions into the epithelium of spermathecae with high sperm contents, although these intrusions seemed to occur in areas where the sperm sought more nutrients rather than phagocytosis processes (Fig. 5.1c). Future ultrastructure studies will shed light on these mechanisms.

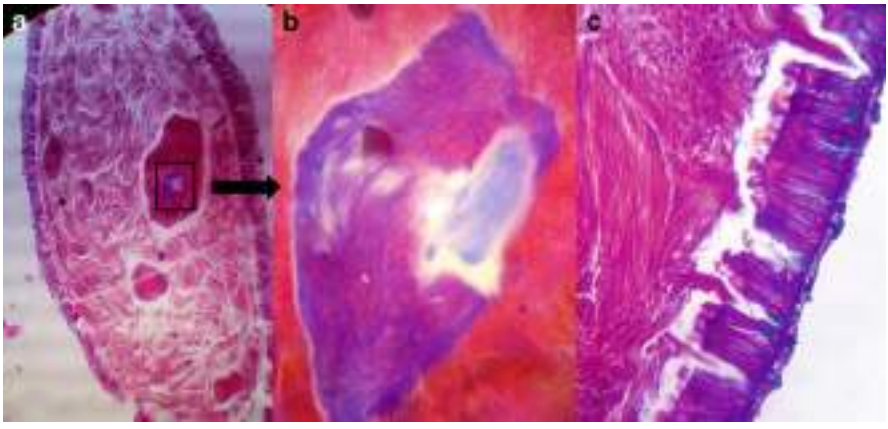

Fig. 5.1 Histological preparations of the spermathecae from *H. elisae*. Sperm degeneration (**a** and **b** in detail). Sperm intrusions in the epithelium of the spermathecae (**c**)

Another strategy for cryptic female choice could be the differential storage of the received allosperm within the spermathecae. The recipient can control the storage of sperm by increasing the complexity of these organs. Different species of earthworms have different numbers of spermathecae (Sims and Gerard 1999), although Novo et al. (in press) demonstrated using microsatellite markers that the four spermathecae from *H. elisae* contained sperm from the same individuals. Grove and Cowley (1926) observed that the transmission of sperm in *E. fetida* typically occurs on both sides of the individual, whereas in *L. terrestris* some individuals were found to have spermatophores on only one side of their body (Butt and Nuutinen 1998).

Moreover, some earthworms present different sperm loads within a single spermathecae. This has been observed in some hormogastrids (Qiu and Bouché 1998), and in *Megascolides australis*, in which spermatozeugmata (i.e., sperm in orientated bundles) were reported by Van Praagh (1995). In addition, the spermathecae may include one or more diverticula that arise from the duct (Butt and Nuutinen 1998).

Finally, the amount of sperm stored in each spermatheca could be controlled, and this occurs for *L. terrestris*, which predominantly store sperm in the two posterior spermathecae when there is no injection of allohormones (Koene et al. 2005, see later). Garvín et al. (2003) also observed that the second pair of spermathecae seems to be the main recipient of spermatozoa in *H. elisae*. However, Velando et al. (2008) showed that *E. andrei* distributes the sperm equally among the four spermathecae.

Cryptic female choice may also be achieved through differential investment in offspring. Velando et al. (2006) found that *E. andrei* adjusted the breeding effort to the degree of mate relatedness, showing that inbreeding and outbreeding cause a strong reduction of cocoon production, especially in genetic lines with high reproductive rates.

Sexual selection drives the evolution of strategies that increase the chances of fertilization for the donated sperm as a means of increasing paternity. Some of these strategies have been observed in earthworms. Velando et al. (2008) reported a behavior that could promote sperm competition in *E. andrei*, which can have a high degree of control over their own ejaculate volume after evaluating their partners. This species donated three times as much sperm as they did normally when mating with a nonvirgin mate. Moreover, such increases were greater when the worms mated with larger partners. Mariño et al. (2006) also showed a sperm trade in *E. andrei*, which adjusted the amount of sperm they release to the volume they receive from their mating partner during copulation. In addition, the total sperm volume they found in the spermathecae was correlated to the recipient's body mass, indicating that this adjustment is in accordance with the quality of the partner.

Koene et al. (2002) proposed that during mating, *L. terrestris* use their copulatory setae to pierce their partner's skin to inject an allohormone produced by the setal glands which manipulates the reproductive physiology of the partner and damages the body wall. The injection of this substance provokes a higher uptake of sperm, a more equal sperm distribution over the four spermathecae, and an increase the amount of time occurring before the next mating. The damage caused

by the injection itself could incur a considerable cost that inhibits another mating (Koene et al. 2005).

5.3 Parthenogenesis

5.3.1 Definition

Parthenogenesis is a very wide collective concept. Historically, classical authors addressed this concept on several occasions; although not defining the concept or providing an experimental approach, authors posed hints regarding the existence of this kind of reproduction. Although Bonnet provided experimental proof for this kind of reproduction in aphids in 1762, it was not until 1849 that Richard Owen coined the term. He defined parthenogenesis as "procreation without the immediate influence of a male". As this general concept could include several typically asexual modes of reproduction such as fission or budding, several authors attempted to create new definitions for this term. A century later, Suomalainen (1950) defined it as "the development of the egg cell into a new individual without fertilization". Later, Beatty (1957) defined it first as "the production of an embryo from a female gamete without the concurrence of a male gamete, and with or without eventual development into an adult", but modified the definition in 1967 (Beatty 1967) by substituting "without any genetic contribution from a male gamete" for "concurrence of a male gamete". In this way, Beatty extended the definition to include special types of parthenogenesis such as gynogenesis. Nevertheless, all of these definitions give rise to some terminological difficulties.

5.3.2 Types of Parthenogenesis in Earthworms

Several classifications have been used to define the different types of parthenogenetic mechanisms. To understand earthworm classification of parthenogenesis, it is worth mentioning the classifications proposed by Thomsen (1927); Ankel (1927); Suomalainen (1950) and White (1973); these are mainly based on the mode of reproduction, sex determination, and cytology.

The system of classification proposed by Thomsen (1927) and Ankel (1927) points out two main points: the zygoid–azygoid status of an individual and the maintenance of the zygoid chromosome number. It includes two main categories: generative or haploid parthenogenesis (in which chromosome reduction takes place in the eggs, and consequently the parthenogenetic offspring have an azygoid – haploid-number of chromosomes), and somatic parthenogenesis, in which parthenogenetic offspring have a zygoid–diploid or polyploid-chromosome number.

The difference between the two categories basically depends on the absence (apomixis) or presence (automixis) of chromosome conjugation and reduction. Both concepts are synonymous with White's concepts of ameiotic and meiotic parthenogenesis, respectively.

When considering sex determination, it is especially useful to use the classification of parthenogenesis proposed by Suomalainen et al. (1987): arrhenotoky, thelytoky and deuterotoky, or amphitoky (unfertilized eggs producing only male descendants, only females, or descendants of both sexes, respectively).

Parthenogenetic earthworms are generally automictic and thelytokous. Following the cytological studies of Muldal (1952); Omodeo (1951, 1952, among others) and Casellato and Rodighiero (1972), there is a premeiotic doubling of the chromosome number at the last oogonial divisions resulting in endomitosis, followed by the formation of chiasmatic bivalents and regular meiosis with the extrusion of two polar bodies. The genetic consequences of this cytological mechanism are similar to those of apomixis (i.e., the formation of clonal animals), as synapsis is restricted to sister chromosomes that are exact molecular copies of one another. The immediate genetic consequence of this mechanism is that heterozygosity is maintained. Following White (1973), all bivalents are structurally homozygous and multivalents are never formed. Consequently, this kind of reproduction is perfectly compatible with different degrees of polyploidy, especially in odd-numbered levels (Fig. 5.2).

Only one exception to the parthenogenetic mechanism described above has been found. *Dendrobaena octaedra* shows apomictic parthenogenesis: the chromosome number is not doubled in the oogonia, the chromosome number of the oocytes is unreduced, and there is only one equational maturation division (Suomalainen et al. 1987). For this species, Omodeo (1953) and later Gates (1972; as explained later in this chapter) described different parthenogenetic forms with a huge degree of morphological variation, which makes it very difficult to establish the evolutionary relationships among them. Omodeo (1953) suggested that "it could be the result of a breakdown of developmental canalisation in the absence of stabilizing selection", while White (1973) indicated that "it seems more likely that it indicates the coexistence of numerous biotypes differing significantly from one another genetically, even if not in their visible cytology".

Parthenogenesis is one of the main sources of morphological variability within reproductive structures of earthworms. This variability is related to the reduction in the investment in male structures: seminal vesicles, testes, spermathecae, genital setae, and prostates are reduced or even lacking; there is no sperm production (i.e., lack of iridescence in male funnels and spermathecae); and spermatophores are lacking (in some cases they are produced but are invariably empty). In *Octolasion tyrtaeum* (Muldal 1952; Jaenicke and Selander 1979), male structures are not reduced and pseudogamy is shown: individuals copulate to exchange spermatophores that are invariably empty. Thus, although spermatozoids are not necessary, this species needs a mechanical or chemical stimulus to trigger reproduction. Polymorphic degradation of reproductive structures is often observed in parthenogenetic organisms. In some species, such as *Eiseniella tetraedra* even hypergynous

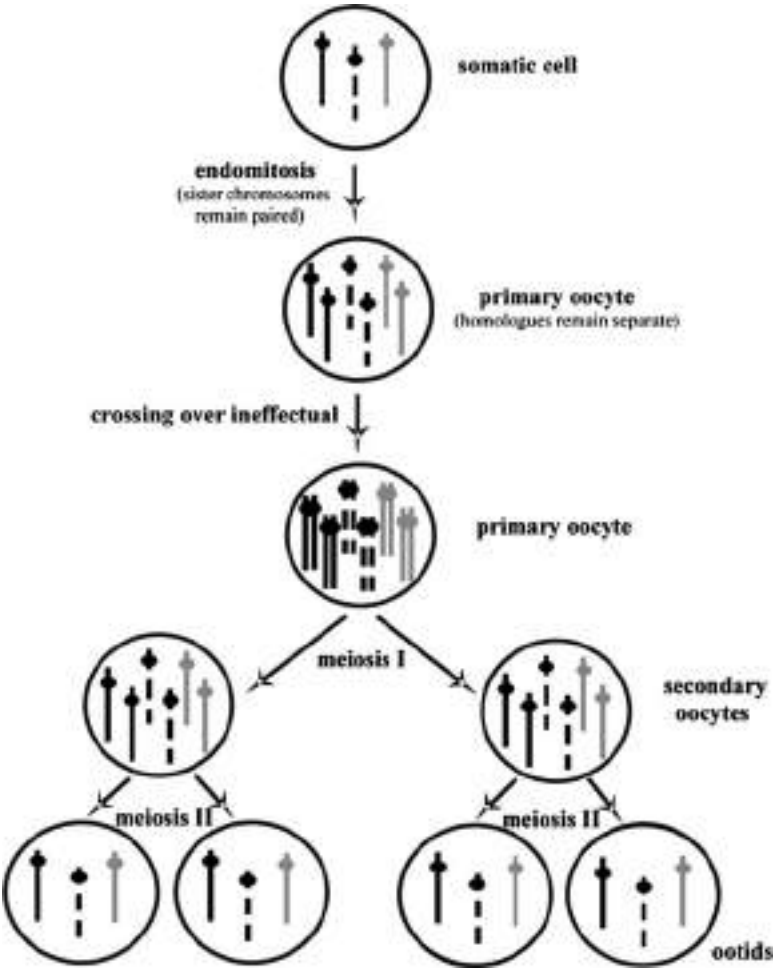

Fig. 5.2 Automictic parthenogenesis: the genetic consequences of premeiotic restitution

individuals (with an extra pair of ovaries) can be found (Jaenicke and Selander 1979). However, in other parthenogenetic earthworms such as *Aporrectodea trapezoides*, both primary and secondary male sexual characters, such as perithecal papillae, tubercula pubertatis, spermathecae, swollen male porophores, and seminal vesicles, are retained. Recent studies show that pseudogamy is not observed in this species (Fernández et al. 2010). As discussed later, this seems to suggest very different origins of parthenogenesis in the different species.

Parthenogenesis is not homogenously distributed in earthworms; it is only found in lumbricids and megascolecids. It is curious that it is not found (or not known to occur) in glossoscolecids or hormogastrids; this clearly shows that their life traits or evolutionary histories should be completely different and that somehow parthenogenesis and even polyploidy are not compatible or viable in this family.

5.3.3 Parthenogenesis and Polyploidy

Most part of the parthenogenetic earthworms are polyploids. Polyploidy ranges from tri- to dodecaploidy. From a cytogenetical point of view, automictic biotypes should be diploid (White 1973); nevertheless, in parthenogenetic lumbricids, polyploidy is the most common phenomenon. This is because, as explained later, the automictic mechanism in most lumbricids is premeiotic doubling, which leads to genetic consequences similar to an apomictic mechanism, leaving levels of heterozygosity unchanged from generation to generation (Suomalainen 1950). Because of premeiotic doubling, no multivalents are formed, so pairing only occurs between genetically identical sister chromosomes; this mechanism is compatible with odd-numbered polyploidy, as only bivalents are formed. This is the complicated chromosomal background that can give rise to different ploidy levels even within the same species. For example, in *Dendrobaena rubida*, diploid, triploid, tetraploid, hexaploid, and octoploid biotypes are known to occur, which clearly shows the extremely high liability of the genetic system. It has been proposed that automixis could be a step before apomixis (White 1973), which could mean that most lumbricids could be evolving toward an apomictic parthenogenesis. Polyploidy could be common in earthworms, as animals lacking the chromosomal determination of sex are particularly prone to this kind of reproduction, which is the main mechanism preventing the establishment of polyploidy in animals (White 1973). One of the main advantages of polyploidy in parthenogenetic species is the increase in genetic variability.

Since no study to date has elucidated the origin of parthenogenetic earthworms (as explained later in this chapter), it is not known if parthenogenetic earthworms may have arisen from hybridisation processes. These kinds of processes have been found to be very common mechanisms causing asexuality (only to the extent that parthenogenesis can be considered to be asexual reproduction) in animals and plants (Delmotte et al. 2003). Following this assumption, polyploidy (and particularly allopolyploidy) could provide important advantages, such as heterosis, to parthenogenetic species. This strong advantage could lead the parthenogenetic morphs to have more general purpose genotypes, allowing them to adapt to a wider range of environmental conditions than their sexual amphimictic ancestors (White 1973). There is much evidence that hybrid vigor could be responsible for the success of polyploids, but there is insufficient information to determine this with certainty.

5.3.4 Genetic and Ecological Consequences of Cloning

As stated by Hughes (1989), it is extremely difficult to define the advantages or disadvantages of parthenogenesis, as these depend on the situation; for some groups of animals, parthenogenesis is tremendously advantageous, while in others it is not.

Therefore, natural selection should control the pattern of occurrence in each group of animals.

Using molecular tools, very different degrees of genetic variability have been reported in different species. Both with allozyme electrophoresis and with mitochondrial gene sequencing, genetic variability was recorded as being high in *D. octaedra* (Haimi et al. 2007; Terhivuo and Saura 1996; Cameron et al. 2008) and *Aporrectodea rosea* (Terhivuo and Saura 1993; King et al. 2008), but low in *O. tyrtaeum* (Jaenicke et al. 1980; Heethoff et al. 2004) and *O. cyaneum* (Terhivuo and Saura 2003). In *A. trapezoides*, both mitochondrial and nuclear sequences resulted in an extremely high number of clones (Fernández et al. unpublished data.).

Judging from the number and distribution of parthenogenetic earthworms, one could expect that parthenogenesis is quite advantageous in this group. Parthenogenetic earthworms are widespread and very abundant, especially among peregrine species (Blakemore 1994) such as *A. rosea*, *A. trapezoides*, or *O. tyrtaeum*. Hughes (1989) pointed out the following advantages of parthenogenesis: both high levels of heterozygosity and exceptionally fit genomes, which are maintained and inherited by avoiding recombination and segregation; high reproductive rates, which could potentially be doubled by avoiding the production of males (i.e., no twofold cost in parthenogenetic reproduction); high colonizing abilities, since there is no need to mate; high values of reproductive potential, enabling clones to quickly replace losses; advanced polymorphism generated from selection at the level of the genome; and the delay or prevention of senescence as somatic replicas from undifferentiated somatic cells are generated. In reference to the last advantage, Hughes (1989) pointed out that several clones of oligochaetes did not show any signs of senescence after having been maintained for many generations.

5.3.5 *The Species Concept in Parthenogenetic Earthworms*

Parthenogenetic earthworms were wisely defined as "systematist's nightmares" by Blakemore (1999). The biological species criterion cannot be applied to parthenogenetic earthworms, as each individual meets the criterion of being completely reproductively isolated not only from the parental species, but also from every sister clone. Several authors have attempted to resolve this problem, but an agreement has never been reached. Mayr (1963) suggested that the best solution would be to use a morphological criterion. Following this author (1963), a parthenogenetic species would be the one that "results in the combination of a single species of those asexual individuals that display no greater morphological difference from each other than from conspecific individuals or populations in related bisexual species". He also proposed that clones can be combined into collective species when no essential morphological or biological differences have been observed. To complete this criterion, the author also argued that if a parthenogenetic line originated from an amphimictic species by an irreversible chromosomal event (such as polyploidy), it should be considered to be a separate and sibling species, although almost no

morphological differences could exist. This criterion has traditionally been used to define species in parthenogenetic lumbricids, though it can be difficult to apply as the degree of morphological variation is sometimes slight and the features defining parthenogenetic and even amphimictic species can overlap. This is a particularly big problem in complexes of very similar species containing both amphimictic and parthenogenetic species such as the "*Aporrectodea caliginosa* species" complex. In this context, other approaches, as discussed later, could be essential not only for properly defining parthenogenetic species, but also for determining the taxonomic status of each form in these species complexes.

Following Gates (1974), "the species is understood to include not only the interbreeding population, but also all recently evolved uniparental strains, clones, or morphs that clearly are affiliated with it". This statement is useful when intermediate forms are found, but still does not solve the problem of how to resolve the status of parthenogenetic species with unknown (or extinct) amphimictic parental species. Another option would be to use the phylogenetic concept of species based on molecular markers, which would provide information about the genetic divergence between morphs or species. However, these tools are not so well developed in earthworms that they could provide a good idea as to the exact amount of divergence that should be used to differentiate between species. In addition, there is evidence of different degrees of divergence among closely related species in the different earthworms groups. The best way to define a parthenogenetic species (and amphimictic species, particularly when dealing with complex of species) is to use an integrative concept of species, using ecological, behavioral, morphological, and molecular data. A species should not be given a name if its biology is not well understood, but then, it is completely necessary to name the species. Parthenogenetic species are very common among the earthworms, and thus a solution needs to be found. The ideal study would be one using all of the available approaches to examine the same individuals so as not to incorporate any source of error or introduce any possible mistakes when identifying species. Making comparisons with previously published data is dangerous because different authors might have incorrectly identified species when dealing with parthenogenetic morphs or species from a complex, in which intermediate forms are typically found. The best means of eliminating this uncertainty is to deposit the individuals used in the experiments into a collection.

Gates (1974) categorized parthenogenetic morphs of *D. octaedra* using the presence or absence of different reproductive male structures. Gates (1974) defined morphs lacking spermathecae, male terminalia, testes, testis sacs, or seminal vesicles or those lacking several of these structures (e.g., athecal anarsenosomphic, with or without testes). He also included two categories of intermediate morphs with an incomplete or asymmetrical deletion of the above organs: hermaphroditic parthenogenetic morphs were defined as those that had reproductive organs in a juvenile state, while hermaphroditic morphs used biparental reproduction and were also parthenogenetic. Unfortunately, few studies have demonstrated the existence of these forms in every parthenogenetic species; the knowledge about the extension and degree of parthenogenetic morphs in parthenogenetic species is quite limited.

This is a problem both for clarifying the taxonomy of earthworms using this type of reproduction, and for understanding the origin of parthenogenesis in these species.

Gates (1974) and Blakemore (1999) suggested that parthenogenetic morphs should be given a name only when the parental amphimictic species can be determined. We totally agree with this statement. Nevertheless, as Blakemore suggested, the origin of the name, regardless of whether it was based on morphs or parthenogenetic forms, has no effect on the availability of a taxonomic name (ICZN 1999, Article 17.3). Moreover, Gates (1972) suggested that provision of names for all intermediate morphs of such species complexes was *ridiculous*.

Another limitation, as stated by Suomalainen et al. (1987), is that there are still very few examples of taxonomic diversification beyond the species level in parthenogenetic earthworms.

5.3.6 The Origin of Parthenogenetic Forms

Amphimictic ancestors of parthenogenetic forms are well known in many different animal groups, but this is not the case for Lumbricids. Hybridization has been proposed several times (e.g., Suomalainen et al. 1987) as a common origin of parthenogenetic animal species such as fishes, lizards, and salamanders. Among invertebrates, there are many examples of parthenogenetic forms originating from Hybridization in the literature. This is the case, for example, for parthenogenetic forms in delphacid leaf-hoppers or stick insects belonging to the genus *Acanthoxyla* which were described as having two haploid genomes, one of which came from an amphimictic parental species (Buckley et al. 2008). Suomalainen et al. (1987) also gave some examples among invertebrates in which parthenogenesis seems to have arisen through a single mutational event, or through multiple events. In these cases, parthenogenesis was a polyphyletic condition within a single species as, for example, in the psychid moth *Solenobia triquetrella*.

Little is known about the origin of parthenogenetic earthworms. Molecular biology will be very useful in shedding light on this topic. Several tools can be useful in reaching this goal. Traditionally, some studies using allozymes have been used to check genetic variability in parthenogenetic and sexually reproducing species that are related, such as *A. trapezoides* and *A. caliginosa* (Cobolli Sbordoni et al. 1987). However, the information obtained using this technique was not sufficient to evaluate hypotheses regarding the origin of parthenogenetic forms. An appropriate first approach would be to compare phylogenies using both mitochondrial and nuclear genes. To determine whether parthenogenetic species originated from hybridisation, alleles could be cloned in nuclear genes to check for the presence of different haploid genomes in diploid and, especially, polyploid parthenogenetic earthworms.

As stated earlier, there is a strong variation among parthenogenetic earthworms regarding the type of parthenogenesis that is observed; most of the species are automictic, but at least one is apomictic. Similarly, some species are pseudogamic

while others are not; some lack spermathecae while others have an extra pair of ovaries. The fact that parthenogenetic mechanisms are very labile in earthworms provides strong evidence that parthenogens could have originated in a number of different ways. Molecular biology will allow us to better understand why parthenogenetic earthworms have been so successful.

5.4 Conclusion

Reproduction models in earthworms are much more variable than it could seem *a priori*. Although direct cross-fertilization hermaphroditism may be seen as the most usual model, it is common to find different ones as self-fertilization or parthenogenesis. Even within the most widespread strategy, it is possible to find variations, such as presence of spermatophores.

During the last years, a great research effort has been made to shed light on some aspects of sexual selection, such as mate assessment, copulatory behavior, and sperm competition. Nevertheless, very interesting processes as origin and maintenance of parthenogenesis in earthworms are mainly unknown. Deeper research on both aspects would allow us to better understand the reproductive biology of these animals.

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Chapter 6
The Earthworm Inoculation Unit Technique: Development and Use in Soil Improvement Over Two Decades

Kevin Richard Butt

6.1 Introduction

Researchers have appreciated the soil-forming capabilities of earthworms for decades (see below). To this end, the utilisation of earthworms to improve soil quality has been the subject of many investigations. A seminal paper, produced by Brun et al. (1987) on 'biostimulation', took a critical view of this process and tried to balance arguments both in favour of and against such practises. This chapter examines one direction taken by a group of researchers who sought to develop a technique to maximise the possibility of successful soil stimulation by the addition of earthworms. This technique is explored over a 20-year period and shows developments and applications along the way.

6.2 Earthworms as Ecological Engineers

There can be no doubt on the positive effects of earthworms on soils. Over the past 50 years, numerous authors have described the main areas in which they are involved (summarised for example by Lee (1985), Edwards and Bohlen (1996), Lavelle and Spain (2001)). Both laboratory-based and field trials have demonstrated effects of earthworms on soils that may be physical, chemical, biological or combinations of all three. For example, earthworm burrowing can increase soil aeration and drainage, whilst soil gut passage can improve soil crumb structure and lead to enhanced water holding capacity. The incorporation of organic material and its mixing with mineral soil can also lead to increased nutrient availability.

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Such attributes have led researchers to regard earthworms as ecosystem engineers – organisms that directly or indirectly control the availability of resources to other organisms by causing physical state changes in biotic or abiotic materials (Jones et al. 1994). Earthworms are considered as one of the most important ecosystem engineers in soil (Jiménez et al. 2001) as their activities can dramatically change the structure and properties of soil and affect distribution of resources to other soil animals and plants.

Long before a soil-engineering role had been applied to earthworms, utilisation of their soil-related activities had been investigated by a number of researchers on different continents. The examples that follow are well documented in the scientific literature but are considered essential for inclusion here, as they helped to shape the thinking which led to the processes described in this chapter. Four examples drawn from the UK (Rothamsted and Hillingdon), The Netherlands (former Ijssel Lake) and New Zealand (Uplands in Otago) are described, but others could easily have been included (e.g. as covered by Curry (1988), Scullion (1992), Baker et al. (2006)). Predictably, the majority of the work relating to earthworms and soils was investigated with respect to effects on agricultural systems, with direct returns measured in terms of increased productivity.

Observations at Rothamsted Experimental Station, Hertfordshire, in the 1970s showed that increased numbers of earthworms in a soil resulted in improved crop yields and a better quality of grassland. In a series of experiments, batches of mixed species of earthworms were inoculated directly into plots previously sterilised with a soil fumigant, on sites direct drilled for at least 5 years. The earthworms were introduced (broadcast) directly on to the soil surface and the area initially covered with plastic sheeting to encourage burrowing. Plots inoculated with deep burrowing *Lumbricus terrestris* and *Aporrectodea longa* significantly increased barley plant populations – weight and depth of roots and height and quantity of cereal leaves. These field tests were confirmed by laboratory experiments using barley plants grown in soil blocks dug from the same fields, but also showed that shallow working species (*Aporrectodea caliginosa*, *Allolobophora chlorotica* and *Aporrectodea rosea*) could be responsible for increased root growth close to the soil surface, particularly beneficial during early seedling growth (Edwards and Lofty 1980; Lofty Pers. Com.).

In an upland area of southern New Zealand, farmers recognised that grassland around introduced European fruit trees was more productive than surrounding areas. A closer examination revealed the presence of a population of lumbricid earthworms, which had been introduced with the soil around tree roots. Experiments showed that the introduced earthworms were responsible for the observed increase in productivity. In their absence, a thick, undisturbed mat of dead plant material had accumulated. The non-indigenous earthworms, mainly *A. caliginosa*, broke down the mat in the immediate area around the trees and the release of nutrients initially led to an increased grass production of over 70%. This levelled off at around 30% above former records following the first few years after earthworm introduction, as the stored nutrients became exhausted (Stockdill and Cossens 1966).

To further investigate this and deliberately inoculate earthworms into fields, a machine was developed capable of digging turfs from earthworms-rich soils, for relocation at earthworm-deficient sites (Fig. 6.1). Turfs, 20 cm square and 5–7.5 cm deep, were laid grass side down to prevent rooting taking place, but ensure good contact for earthworm transfer and provide a food supply for the earthworms of semi-rotting grass. This proved successful and it was established that complete colonisation of an area with few or no earthworms could be expected from turfs placed at 10 m intervals within 6–7 years (Stockdill 1982). The expected economic return on earthworm introduction projects of this type was calculated to be more than 300%. Baker et al. (2006) reported that similar economic results were obtained more recently through utilisation of the same technique in northern Tasmania.

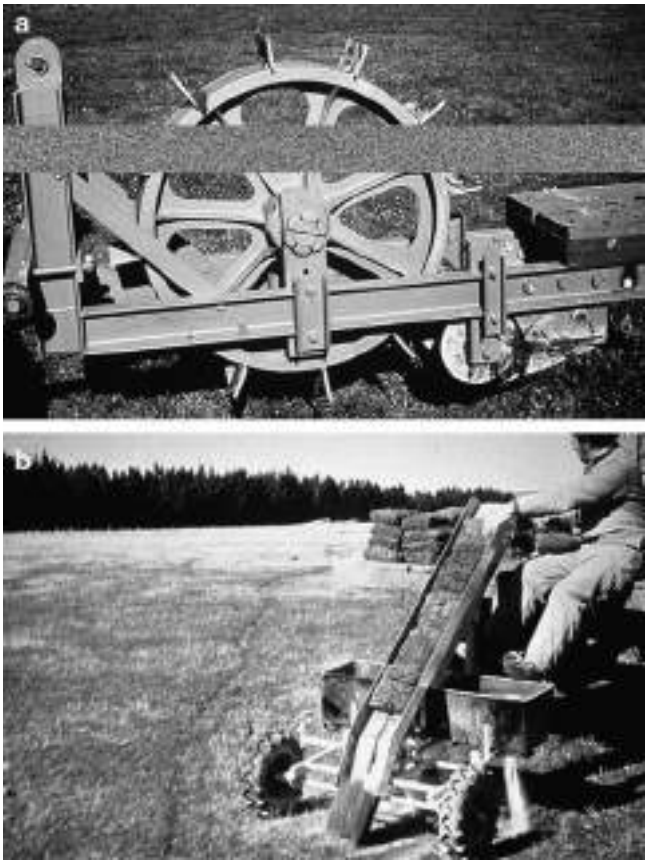

Fig. 6.1 (**a**) Turfcutting and (**b**) deposition machinery produced by the New Zealand Agricultural Engineering Institute for earthworm inoculation. Turfs were cut from earthworm-rich soils, transported to the receptor site and laid grass side down. Two men were capable of inoculating 25 ha (100 turfs ha^{-1}) in a day (Murray Stockdill)

Back in Europe, the value of earthworms in soil amelioration was simultaneously documented on another type of grassland. In the Netherlands, where land reclamation is part of the national heritage, the draining of areas formerly flooded by the sea led to the creation of polders which have subsequently been put into cultivation. In soils such as Ijssel Lake drained in 1957, which may take 50–100 years to mature, van Rhee (1969, 1971) introduced *A. caliginosa* and *L. terrestris*, under pasture and in orchards. The activity of these earthworms and that of *A. caliginosa*, in particular, was found to increase the rate of soil development. More tree roots grew in earthworm-inoculated soils than in those without earthworms, but van Rhee did not detect any influence of earthworm activity on grass or fruit yields. The populations of *L. terrestris* declined rapidly after inoculation, suggesting that soil conditions did not favour this species.

Unlike van Rhee, Hoogerkamp et al. (1983) investigating similar polder areas did record an increase of grass production caused by earthworm inoculation, but the results obtained were variable. Again *A. caliginosa* was thought to be the most important species in terms of organic matter incorporation. Rates of population increase and spread of this species were estimated by use of an infrared scanning technique that measured differences in heat exchange from areas where a surface organic mat was present, or had been removed by earthworms. When earthworms were well established, the surface mat disappeared, which resulted in greater heat exchange between the soil and air, and reduced daily fluctuations in temperatures at the soil surface. The mat was ingested and incorporated into the soil within 3 years of earthworm invasion and a dark A_1 horizon developed within 8–9 years. Improved soil conditions positively influenced root growth and distribution. This was reflected in better sward attachment and fewer turfs were detached by cattle grazing. Urine damage on earthworm-inoculated plots was less common, and grass yields were on average 10% higher, compared with un-inoculated plots. However, during wet weather, some damage by treading and soiling of grass was observed on earthworm-inoculated plots.

In southern England, the Rothamsted team also experimented with large-scale earthworm inoculation to assess the effects on soil development and grass production in less fertile settings. A former landfill site at Hillingdon, near London, was capped in 1983 with clay, dressed with a layer of sewage sludge and subsoil (top grow) and sown with perennial rye grass. A year later, some 4,000 earthworms (*A. caliginosa*, *A. longa* and *L. terrestris*) were collected nearby using formalin extraction (Raw 1959), washed and broadcast on to the soil surface across 1 ha of the Hillingdon site (Marfleet 1985). After 6 years, areas where earthworms had successfully established showed improved soil structure and all top grow had been incorporated (Butt et al. 1993). However, results were mixed, earthworm colonisation was patchy with some top grow still very obvious (Fig. 6.2). These earthworm species may not have been ideal candidates for the site, at the stage of development, due to the high organic matter content. Litter dwelling and shallow working species such as *Lumbricus rubellus* and *A. chlorotica* might have been more suitable (but perhaps less easily collected).

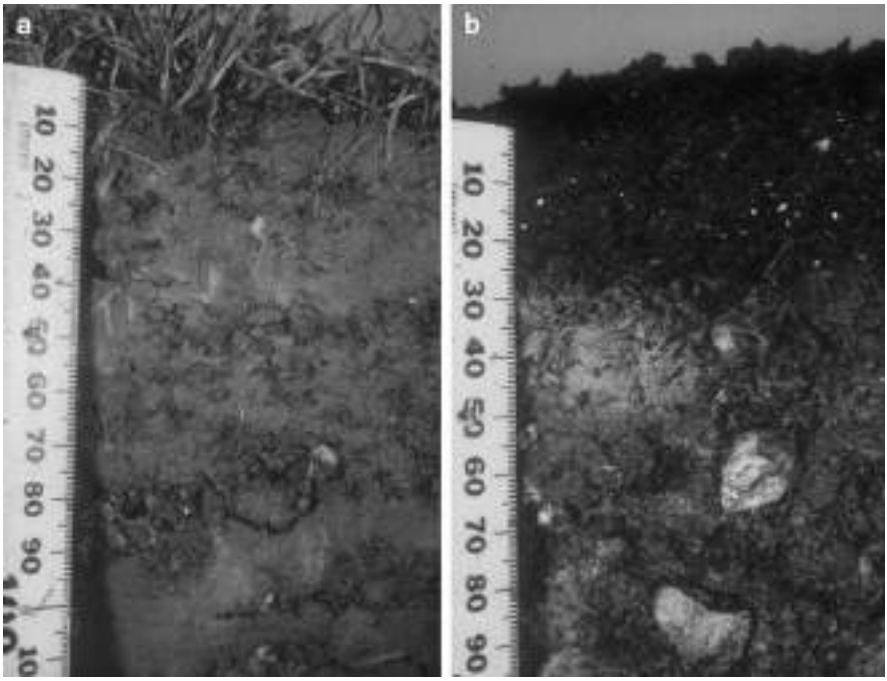

Fig. 6.2 Soil profiles from Hillingdon in (**a**) earthworm-rich and (**b**) earthworm-deficient areas. The layer of top grow (a combination of sewage sludge and subsoil) remained after a period of 7 years where earthworms were absent (Jim Frederickson)

6.3 Established Inoculation Techniques

The above examples illustrate that the potential for introducing earthworms to areas where they were absent or present in low numbers was a growing research area in the 1960s to mid-1980s across the world. It was possible to create soil conditions that would encourage earthworm population/community development e.g. through the addition of organic matter and improvement of soil physical factors e.g. through soil ripping. However, direct manipulation of earthworm numbers, by translocation from elsewhere, seemed to be a more instant way of increasing desired activities. Two major techniques were in operation. These, as illustrated, were either turf cutting and transfer or mass collection and broadcasting. Each technique had advantages and disadvantages in terms of the species obtained, the quality of the inoculum and the life stages present. These are summarised in Table 6.1. In all instances, the requirement was for the inoculum to survive and then reproduce to promote a growing, sustainable population. To achieve such results, best use was really needed from aspects of each existing technique. Linked to cultivation of soil dwelling earthworms, this was a potential way forward for earthworm inoculation.

Table 6.1 Relative merits of earthworm inoculation techniques (developed from Butt et al. (1997))

Technique	Advantages	Disadvantages
Turf cutting and relaying	Protective micro-environment	Densities usually low
	Cocoons transferred	Little control over species/numbers
		Mainly shallow working earthworms
		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 earthworms
		Earthworms may be injured during extraction
		Laborious and expensive
		Damage to collection site
Earthworm Inoculation Unit (EIU) method	Protective micro-environment	Laborious and potentially expensive (compared with above methods)
	Species selection possible	
	Cocoons transferred	
	High densities possible	
	Earthworms of known origin	

6.4 Development of the EIU Technique: From Tea Chests to Plastic Bags

During the mid-twentieth century, earthworm cultivation (excepting vermiculture of litter dwelling species) was viewed as a problematic science. Pioneering experiments on a small scale had been conducted, for example by Evans and Guild (1948), to determine basic requirements of soil dwelling species. However, equating burrowing requirements of larger species to culture conditions meant that unrealistic expectations were held. Ashby (1976) when describing the care of laboratory animals suggested that large quantities of soil (the size of a tea chest) might be required. Working with *L. terrestris*, Tomlin (1979) kept 30–40 individuals in 18-l vessels and suggested that mature individuals required a soil depth of at least 30 cm for successful culture. If this was not supplied, it was suggested that an individual 'will attempt to leave the soil and container. If it is unable to escape the shallow soil, it lies on the surface and dies.' However, less than 10 years later, this was disputed by Lofty (Pers. Com.) who suggested that *L. terrestris* would breed successfully in soil only 7–8 cm deep and did not necessarily need a permanent burrow. The amount of soil necessary for culture of earthworms was set to critically influence experimentation in development of an intensive production system.

With a focus on *Lumbricus terrestris*, work in the Biosystems Research Group at the Open University was undertaken to assess the possibility of mass producing this species in culture. This was developed in conjunction with Rothamsted, where previous inoculation work using field-collected earthworms had been undertaken (e.g. Marfleet 1985). Life cycle traits and environmental parameters were manipulated

to reduce the length of the life cycle as much as possible and also to try and minimise the volume of culture material (specifically the depth) required for successful culture. Results showed that manipulation of temperature (Butt 1991), food quality (Butt et al. 1992) and earthworm density (Butt et al. 1994) were key areas and allowed the life cycle of *L. terrestris* to be reduced to 6 months. Of equal importance, a requirement for a fully formed deep burrow was shown to be unnecessary. Reece Lofty was absolutely correct; this species would copulate and produce cocoons in a soil depth as shallow as 4 cm. Academically, these finding were of merit and demonstrated the plasticity in behaviour of this and later other species (Butt 1993a). Nevertheless, the work was envisaged as a precursor to utilisation of earthworms in soil amelioration schemes.

The next step was to determine the best way of using earthworms produced to maximise the potential survival, reproduction and hence development of sustainable populations within the chosen soils. Discussions between the author and Jim Frederickson at the Open University debated the merits of containerisation and unit size, always trying to formulate an approach which used the initial scientific findings but coupled these with practicalities associated with proposed soil inoculation. Use of units was seen as an advance on large-scale breeding bed production as used with 'compost worms' as it reduced the likelihood of mass death, allowed for sampling to monitor progress without major disruption at any stage and an ease of manipulation for the inoculation phase.

Ultimately, the Earthworm Inoculation Unit (EIU) technique evolved. This patented technique (GB2 240 456B) then went through a series of staged developments, with improvements after each field trial. The technique set out to cultivate earthworms in a small unit allowing development of all life stages, adult, cocoon and hatchling. Contents of the unit were then inoculated into the desired field site in a way that ensured maintenance of the micro-environment in which the population had developed to maximise survival. The work of Frederickson and Frederickson (1997), written after the event, encapsulated an element of the systems thinking (Checkland 1981) which went into EIU development. The science was deemed to be sound, but there was a need to ensure that the practicalities of using the technique could be integrated into standard working practises of soil rehabilitation, often with unskilled labour.

In an academic sphere, the concept of the EIU, although then in its infancy, was first discussed publically at the Fourth International Symposium on Earthworm Ecology at Avignon in 1990. However, the first field trials were not undertaken until the following year (see below) and first results from the technique were initially presented at a British Grassland Society meeting in Northern Ireland (Butt 1992).

6.5 First Trial: Calvert 1991

From the original concept, it was envisaged that polythene-bound units containing soil, earthworms and a source of food could be set up and maintained under controlled culture conditions, until the carrying capacity of the unit was reached.

The latter was determined by earlier laboratory-based finding (e.g. Butt et al. 1994). To this end, 4-l plastic bags with sealable tops were provisioned with soil (sterilised in batches to remove any potential predators, competitors or pathogens), moistened to an acceptable level (25–30% by mass). Field-collected *L. terrestris* were added (six per unit, approximately 25 g) ensuring that all were mature and apparently healthy. Surface-fed organic matter was in the form of waste from the paper-making industry mixed with yeast extract, a proven food for this species (Butt 1993b). The units were housed at 18 ± 2°C in an insulated polythene greenhouse on sand with heating cables below. Duration of cultivation was 6 months with repeated feeding throughout.

Prior to inoculation, a small number of the EIUs were destructively sampled to determine the size of population that had developed and was available for inoculation. Results were a little discouraging. Reproduction and cocoon production had occurred, and viable adults, cocoons and hatchlings were recovered. However, hatched cocoons revealed that many hatchlings may have escaped from the unsealed units and carrying capacity was not approached (Butt et al. 1997). Nevertheless, the inoculation phase took place at Calvert, Buckinghamshire, on a partially restored landfill cap. The hostile nature of the site (substrate bulk density manipulated to 1.4–2.0 g cm^{-3} and comprising of organic matter-deficient subsoil) was chosen deliberately. This was to stringently test the technique. EIUs were inoculated into the landfill cap by drilling holes with a tractor-driven soil auger, splitting and removing the plastic envelope and inserting the unit into the hole with as little disturbance to EIU contents as possible. The units were positioned in a grid pattern to permit assessment of earthworm spread. The inoculation exercise (Fig. 6.3) took 2 days but was undertaken relatively easily although transportation and handling of the 4-l units was physically demanding.

Results after a period of 10 months showed that survival following inoculation was minimal. Very few animals were located in and around the points of inoculation. This might have been the death-knell of this technique, but thinking had progressed following inoculation, and a number of factors had been discussed and incorporated into a second trial which was already underway. These included unit size and basic design, choice of earthworm species, type of food and its provision, length of the cultivation phase and more thought of the objectives with regard to earthworm community creation.

6.6 Down Sizing and Mixed Cultures

Unit volume was reduced from 4 to 2 l for subsequent trials. This was decided upon from two standpoints. The smaller units led to ease of handling at all stages and a mechanised digger was no longer required for inoculation. Digging a hole was possible with an appropriately (round) shaped posthole spade. Also, a desire to seek all life stages to enhance inoculum survival, rather than proceed to carrying capacity, was thought appropriate. The latter also led to a marked reduction in

Fig. 6.3 Inoculation of a 4-l Earthworm Inoculation Unit (EIU) into the landfill cap at Calvert in 1991 (Jim Frederickson)

cultivation period, from 26 to 12 weeks. This had the knock-on effect of reducing the amount of food required in the cultivation phase, thus allowing a single feeding event at the production stage. Therefore, reduction of unit volume solved a number of previous problems. The type of plastic envelope was also changed, with bags produced specifically for purpose with the bag length extended. This permitted tying the units with string (see Fig. 6.4) rather than relying on a seal. The single feeding event also meant that the organic material could be incorporated directly into the soil at the outset and not necessarily surface applied. This was critical as the choice of earthworm species was also amended.

In the first trial, use of *L. terrestris* had largely been determined by the site managers who wanted what was potentially deemed to be the most 'useful' earthworm species for the site. (This did not take into account burrowing ability or (K-selected) life strategy.) Lessons learned from Calvert suggested that another deep burrowing species, *A. longa*, might be more appropriate and that addition of a shallow working species such as *A. chlorotica* might also be advantageous. Much has subsequently been written about species interactions (reviewed by Uvarov (2009)), but at the time, this was quite novel (Butt 1998).

To this end, 2-l EIUs were set up using a purchased sterilised soil (Boughton loam, now used widely in earthworm ecotoxicology experiments e.g. Arnold et al. 2008) mixed in a (85-l) tip-up concrete mixer with dried and rewetted, separated cattle solids. The bags were then provisioned with mature specimens of field-collected *A. longa*, *A. chlorotica* or a combination of the two (Butt et al. 1997).

Fig. 6.4 Two-litre Earthworm Inoculation Units (EIUs) transported to an organically enriched landfill cap in southern England post-cultivation phase in 2003. The plastic envelopes, tied at the neck, are removed prior to soil inoculation (Kevin Butt)

Cultivation in an insulated polythene greenhouse was restricted to 12 weeks and destructive sampling of some units at this and subsequent time intervals showed this length of time to be the most productive (Butt et al. 1997). Once again, inoculation was into a (different, recently capped) section of the Calvert landfill site. Post-inoculation protection was provided by pegging netting above the position of each EIU. This was to deter avian predation which was also thought to have been one problem in the first trial. Inoculation units were used in conjunction with trees planted subsequently by the Forestry Commission. The experimental design allowed for treatments examining the species combinations in the EIUs (plus blanks with no earthworms) coupled with planting of Alder (*Alnus glutinosa*) and Sycamore (*Acer pseudoplatanus*).

Results from this work are reported fully by Butt et al. (1999a, 2004) and Moffat et al. (2008). The major findings were: (1) EIU inoculation led to population establishment of *A. longa*, whilst Broadcast inoculation of the same species did not; (2) presence of *A. longa* promoted growth of *A. chlorotica* populations; (3) tree presence (only alder survived) enhanced earthworm community development; (4) under the hostile soil conditions, earthworms had little effect on soil conditions even after a decade; (5) whilst other earthworm species (such as *L. rubellus*) naturally colonised the site, those inoculated did not, but did spread over the whole inoculation area.

In terms of plot disruption (by road building), problems associated with the management of the site have already been highlighted (Butt 2008). Nevertheless, a site visit in spring 2009 revealed that the whole inoculation experiment – earthworms, trees and agricultural cap – had been completely stripped away by excavators (in preparation for further waste disposal – and possibly to prevent a requirement for a thorough ecological assessment). This was a somewhat sad (but inevitable) end to a large-scale earthworm/tree inoculation experiment that had been monitored for 16 years. A further experiment, to examine the effects of bulk

organic matter addition on EIU performance (Fig. 6.4) set up at Calvert in 2003 (Butt 2008), was also found to be stripped by excavators in 2009, even though this site was nowhere near to the 1992 EIU location. Earthworm inoculation experiments at Calvert landfill site have therefore ceased to exist.

6.7 An Industrial Application: In Scotland and Poland

A project at Hallside, near Glasgow in Scotland was devised in the early 1990s by the Scottish Greenbelt Company (SGC) to regenerate a disused steelworks site for biomass production. This was in the form of willow (*Salix* sp.) and poplar (*Populus* sp.) short rotation coppice (SRC) (Craven 1995). It was envisaged as a 'win–win' situation as the substrate for tree growth was shale-rich colliery spoil from nearby waste heaps, which was mixed with sewage sludge. The spoil freed up land for housing and the sewage was disposed of and acted as an organic amendment to the newly formed soil. The SGC undertook provision of earthworms, to assist soil development, through use of the EIU technique.

Two thousand (3 l) EIUs were produced, but the substrate used was similar to that spread on site (unproductive colliery spoil and sewage sludge). The earthworm starter culture comprised 8,000 commercially purchased *L. terrestris* and after 2-month cultivation in a straw-insulated outhouse (to April 1996), EIUs were inoculated into site by a commercial labour force. However, as in the first Calvert trial, this species was not particularly suitable to the given soil conditions, was not helped by the indelicate inoculation and none of this species were recorded during monitoring of the inoculation area over the following 2 years (Bain et al. 1999). Laboratory trials replicating the conditions under which the EIUs were produced demonstrated that the substrate was not conducive to *L. terrestris* reproduction (very few cocoons were produced), and survival of the starter culture was also poor (approximately 20%) with some survivors losing reproductive condition (Bain et al. 1999).

The SGC should be applauded for undertaking a field trial with the EIU technique, but might have better followed advice and used locally sourced individuals as a starter culture and possibly opted for a mixture of earthworm species more suited to the given soil conditions (Butt 2008). More recent sampling at Hallside in 2005 showed that after almost 10 years, the site had a viable community of earthworm species which was composed of ten species from all three ecological categories and included *L. terrestris* (unpublished data). Perhaps, some of the animals from the EIU exercise survived and their descendants are still resident.

In the south-west of Poland, a site with soils despoiled by sulphur from an industrial process was destined to become another area to receive attention of the EIU technique in 2006. This followed provision of instructions for EIU production using field-collected earthworms and field-collected soil (Box 6.1). The EIUs were established at the University of Rzeszow and after soil sterilisation were provisioned with *L. terrestris* and *A. caliginosa*. Unfortunately, problems associated with temperature control meant that the majority of these starter cultures perished

during the cultivation phase. Ongoing work now seeks to replicate this exercise and, in the short term, continue through to soil inoculation.

Box. 6.1 Production of Earthworm Inoculation Units (EIUs) Using Field Soil

1. Collect soil and put approx. 2 l into each EIU plastic bag.
2. Tie neck of bag with string.
3. As soil is collected from field, freeze bag and contents to kill any earthworms present, plus potential competitors or predators.
4. Defrost EIUs and allow to stand for at least 3 days.
5. Collect appropriate species of earthworm (possibly *Lumbricus terrestris and Aporrectodea caliginosa*).
6. Select mature, healthy specimens for use and keep these in moist soil for a few days to ensure they are viable.
7. Before putting into EIUs, weigh a number of specimens (ideally all, but at least 20 of each species).
8. Make pin prick holes in the EIUs around the area where the string is tied ($n = 6$ or so per bag).
9. Untie EIUs and add earthworms (but see No 11 first): 4 Lt per 'Lt' EIU; 6 Ac per 'Ac' EIU; 2 Lt and 3 Ac peer 'Combined' EIU.
10. Allow earthworms to burrow down into soil. Add water spray as appropriate.
11. Feed EIUs with manure. This is best applied at the surface for Lt but mixed into the soil for Ac. Therefore, there is a need to go back to 'point 8' and empty out soil from the 'Ac' and 'Comb' units and mix in the food before earthworm addition. (Food should be dried and rewetted cow or horse manure.[a]) For the 'Comb' EIUs some food also applied at the surface.
12. Retie the EIU when soil earthworms and food are in position, as appropriate. These are now ready for the cultivation phase and should be maintained as close to 15–18°C as possible to encourage reproduction. (If kept in a cool place, then they could be put tightly together and insulated with straw or other appropriate material (if outside, care that mice do not nibble at the EIUs).)
13. These need to be kept for 3–5 months (overwinter), so will need to be re-fed at least once during that period (all can be surface fed at this time; there is no need to take out soil and re-feed in situ; just untie, add and re-tie (spraying with water as appropriate)).
14. At the end of cultivation phase, select a sample of each treatment e.g. 5 of 'Lt', 'Ac' and 'Comb'. These are to be destructively sampled to give baseline data on what will be inoculated into the field. All earthworms to be collected and weighed and condition recorded e.g. fully clitellate, in diapauses. Soil to be sorted for hatchlings and wet sieved to collect any cocoons of each species.

(continued)

15. At similar time to '14', the inoculation phase is to take place. EIUs are to be transported to inoculation site. Grid pattern established as appropriate and EIUs inserted in appropriate manner. Locations to be recorded accurately.[b]
16. Then wait and see what happens. Observations/sampling after e.g. 6 months and then sampling after 12 months will start to give an indication of colonisation.
17. During the 'waiting phase', a need to progress with further EIU production, possibly concentrating on a single species or combinations (perhaps of other species).

[a]Note a trial with a few earthworms to ensure that they are not killed by the food might be advisable before setting up the EIUs.

[b]A need to be aware of which earthworms species (if any) are present on site at this point. Therefore standard sampling by hand-sorting and vermifuge application required.

[c]Note – For every 60 EIUs: (20 per treatment) $20 \times (4 + 2) = 120$ Lt; $20 \times (6 + 3) = 180$ Ac

(This is a relatively large number of mature, healthy animals, so may take some time to collect.)

6.8 An Agricultural Trial: In the USA and in Finland

In a standard agricultural setting, the EIU technique has been tested on two occasions. The first instance was at the North Appalachian Experimental Watershed site in Coshocton, Ohio. Here, much research has been undertaken on rainfall infiltration and interest lay in trying to establish *L. terrestris* in a particular watershed. This earthworm species was of particular interest as it is capable of creating permanent vertical burrows, which are also considered as macropores by soil hydrologists (e.g. Edwards et al. 1990). This was put to the test in November 1992, with the earthworms in this trial collected 4 months earlier from a nearby watershed and kept in 4-l EIUs. At inoculation into the small (less than 1 ha) site (#123), a parallel inoculation using broadcasting of recently collected *L. terrestris* also took place. It was not certain, but thought that the absence of *L. terrestris* from this particular watershed was a function of water table height. The outcome of this trial was that no *L. terrestris* were later found in the inoculated areas. Reported by Butt et al. (1999b), this work showed that the EIU technique was not a panacea. Although it failed to bring about population establishment, so did more traditional broadcast inoculation. This seemed to indicate that the particular watershed did not support *L. terrestris* for good reason and that inoculation of any type would prove difficult. The problem here was not one related to mode of introduction, but rather one of long-term environmental conditions preventing population persistence.

In Jokioinen, south-west Finland researchers at MTT (Agrifood Research Finland) are responsible for the management of a particular 1.9 ha cultivated heavy clay field (Kotkanoja) which contains the earthworms *A. caliginosa* and *L. rubellus*. Even though *L. terrestris* was present in other fields with lower clay content, only 600 m distant, none had been recorded from Kotkanoja. To this end, workers undertook to inoculate the grassy field margins with *L. terrestris* in 8-l EIUs during October 1996, after a 5-month cultivation period. The starter culture had been collected by mustard extraction from a parkland area nearby (Nuutinen et al. 2006). Monitoring was undertaken after 2 years close to the inoculation points and more widely after a further 5 years in 2003. After 2 years, *L. terrestris* persisted at the field boundaries in almost half of the samples taken with median densities of ten individuals m^{-2}, but none was located within the field itself. Five years later, *L. terrestris* remained in the field boundary area (at higher densities) but was also detected at very low median density (one individual m^{-2}) up to 4 m from the nearest point of inoculation (Nuutinen et al. 2006).

Further monitoring in 2009 showed that the population of *L. terrestris* has continued to grow and spread into the field from the points of inoculation. Figure 6.5 illustrates that after 13 years, spread from points of inoculation has reached 50–60 m. Tillage trials in subsections of the field and effects of tile drain presence both appear to significantly affect *L. terrestris* establishment at this site. Linked to earthworm establishment, these factors are currently under investigation (Nuutinen and Butt in preparation). Here, use of the EIU technique has led to *L. terrestris* population establishment and development, but it will be a matter of time before monitored physical and chemical soil parameters give an indication as to the desired agricultural improvements of earthworm inoculation.

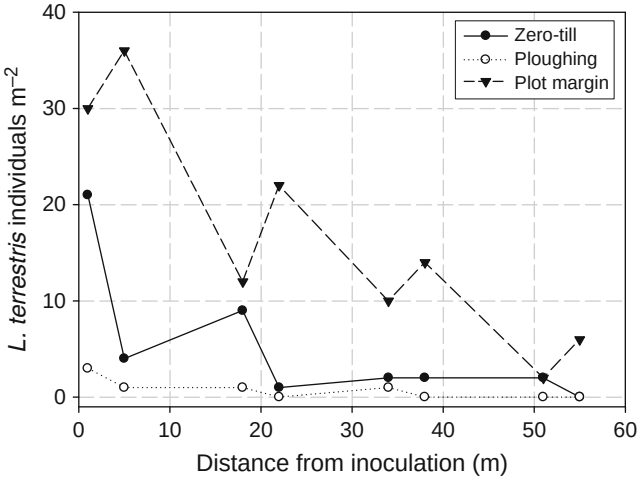

Fig. 6.5 Density of *Lumbricus terrestris* in a Finnish field (Kotkanoja) following colonisation from points of (EIU) inoculation under three types of soil management after 13 years (Nuutinen and Butt in preparation)

6.9 A French Connection

As part of PhD work undertaken by Benjamin Pey, from Nancy, EIUs were used to culture *L. terrestris* and *A. caliginosa*. This experimental work was of some interest, as the type of soil into which earthworms were introduced was an artificially created technosol – a soil with a significant amount of artefacts often referred to as an urban soil (WRB 2006). The particular soil used here was composed of waste materials – composted green waste and paper mill sludge – along with thermally treated industrial soil. Monitoring of this work is ongoing with only initial findings reported (Pey et al. 2010). There is an obvious link here to the earlier EIU work undertaken in the UK, where the soils at Calvert Landfill site were (technically) compacted subsoils and the EIUs were initially fed with paper mill waste or composted green waste was used as a soil ameliorant. Future results from this work will prove to be of some interest.

6.10 Bearing Fruit in New Zealand

In 2005, enquiries were received from a group of horticultural and food researchers operating in Hamilton, New Zealand. Led by Alfred Harris, the group was interested in Kiwifruit production and the likelihood of earthworms playing a more active part in organic farming. The use of EIUs was to be built into more sustainable management and disease prevention in fruit production. The work involved replacing a metre-wide herbicide spray strip beneath the vines with mulch into which a mixture of fungal *Trichoderma* species, acting as bio-control agents (Harman 2006) were introduced to counteract *Armillaria* root rot. Experiments with EIUs then planned to use sufficient numbers of an unnamed Megascolicid earthworm species to spread the *Tricoderma* throughout the mulch. It was also felt that the kiwifruit roots would benefit from inoculation of a deep burrowing earthworm species into the grass/clover sward between the kiwifruit rows. Composted green waste or spent fungal cultures were to be used as food sources for the EIUs. Outcomes from this proposed research, and even if field trials ever took place, remain uncertain. Nevertheless, utilisation of introduced earthworms as vectors for microbial dispersal within EIUs may be a significant application for this technique.

6.11 Future Developments of the EIU Technique

To date, less than ten field trials have utilised the EIU technique (Table 6.2). Of those that were set up, valuable information has been gathered and in at least two instances persistence of the inoculum from this method has occurred whilst other techniques have failed. A number of trials are ongoing and further results will hopefully support greater experimentation with the technique. With increased

Table 6.2 Records of trials utilising the Earthworm Inoculation Unit (EIU) technique

Location	Date	Species	EIU vol (l)	Land type	Objectives	Outcomes	References
Calvert, England	1991	Lt, Al, Ach	4	Landfill cap	Trial technique	Species selection Some modifications	Butt et al. (1997)
Calvert, England	1992	Al, Ach	2	Landfill cap	Earthworm/Tree interactions	Use of mixed species Reduced volume	Butt et al. (1999a, 2004); Moffat et al. (2008)
Coshocton, USA	1992	Lt	4	Agricultural	Colonisation, water infiltration	Unsuccessful	Butt et al. (1999b)
Hallside, Scotland	1996	Lt	3	Ex-Industrial	Soil amelioration	Uncertain	Craven (1995); Bain et al. (1999)
Jokioinen, Finland	1996	Lt	8	Agricultural	Soil amelioration	Successful, ongoing	Nuutinen et al. (2006)
Calvert, England	2003	Al, Ac, Oc	2	Landfill cap	Effect of OM Species interactions	Terminated	Butt (2008); Lowe et al. (2008)
Hamilton, NZ	2005	Megascolicid Spp.	2	Agricultural	Root rot control	Ongoing	Unpublished
Rzeszow, Poland	2006	Lt, Ac	2	Ex-industrial	Soil amelioration	Ongoing	Unpublished
Homecourt, France	2008	Lt, Ac	2	Technosol	Soil amelioration	Ongoing	Pey et al. (2010)

attention placed on the restoration of degraded or unproductive soils, there is a need to ensure that rehabilitation is achieved using the best practicable options. If development of sustainable earthworm populations becomes a critical part of such thinking, then perhaps a technique first considered over two decades ago might be given major (re)consideration.

However, if the EIU technique is to be utilised and of value, a number of factors need to be built into any work undertaken. Most critically, these are selection of earthworm species (determined by site condition and objectives), timing (determined by soil maturity, if a technosol and season, if in a temperate region) and aftercare/monitoring. Although shown to be effective, the EIU technique may not have been explored as fully as possible and warrants further exploitation, for example by further experiments combining earthworm introduction with tree planting. Many years ago, Lee (1995) made reference to the EIU technique as 'mass rearing in plastic bags', but this was only half of the story. Soil inoculation of the EIU contents and the effects the earthworms then have on the substrate are also critical. The EIU technique still has potential, but unless trialled by more researchers under a range of conditions, this may never be fully realised.

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Chapter 7
Controlled Cultivation of Endogeic and Anecic Earthworms

Kevin Richard Butt and Christopher Nathan Lowe

7.1 Introduction

Earthworms have been the focus of specific research efforts and more general field trials for a period in excess of a century. Over his lifetime, Charles Darwin (1881) recorded the activities of earthworms, for example, the way in which one species was seen to gather, curl and draw fallen leaves into its burrow in the soil. Such observations were some of the first that scientifically documented and led to a clearer understanding of the activities of this group. Earthworm activities are multi-faceted and have profound effects on soils, but such details have only become more apparent as scientific investigations have developed. As a group, earthworms are now widely accepted as organisms that perform ecosystem services (MEA 2005), for example, through the processing of organic and mineral materials to assist in pedogenesis. This chapter seeks to describe methods that have been progressed to allow production of specific groups of (temperate) soil dwelling earthworms and demonstrate how their beneficial activities can be harnessed.

7.2 Earthworm Ecological Groupings

Some 3,000 earthworm species are known to science (Sims and Gerard 1999) existing across the biosphere under a range of climatic conditions, from boreal, through temperate to sub-tropical and tropical biomes. Each species is adapted to the environmental conditions under which it is found and may exhibit specific behaviours that permit long-term existence of a population. A number of eminent

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soil ecologists studied the range of earthworm species and sought to group them with respect to morphological and behavioural traits under a number of generalised categories. The most widely accepted of these ecological groupings is that proposed by Bouché (1977) which in its simplest terms provided three distinct categories: *epigeic*, *endogeic* and *anecic*. However, these represent extremes, and intermediate sub-categories, such as epi-anecic, also exist, but for the purposes of this chapter we will use only the basic three group classification, summarised in Table 7.1. Under field conditions, it is thought that synergistic relationships may occur between species within the different ecological groupings (Sect. 7.4.1).

In this chapter, we do not dwell on the cultivation of epigeic species (often referred to in the scientific literature as vermiculture), as this has been explored in great detail over the preceding decades and continues to this day (e.g. Löfs-Holmin 1985; Edwards 1988, 2010). Nevertheless, it is worth mentioning that epigeic species, typified by *Eisenia fetida* (Savigny) (the brandling or tiger worm), live in areas with a very high organic matter content. This material may be formed naturally from leaf litter, but when utilised commercially under vermiculture conditions, it usually consists of available organic material such as animal, industrial or food waste (e.g. Edwards 1988).

Unlike epigeic species, both endogeic and anecic earthworm species require mineral soil in which to exist. Each of these groups will be explored in turn as they exhibit fundamental differences (Table 7.1). Endogeic species are typically smaller than anecics and live within horizontal burrows relatively close to the soil surface (e.g. within 15–20 cm). They are geophagous, deriving their nutrition through consumption of large quantities of soil (relative to body size). Their burrow systems tend to be transient in nature and may frequently be filled by underground cast production as the animal moves through the soil. These species may inhabit the rhizosphere (root zone of plants) and have a relatively intimate association with them. Good examples from temperate regions include *Allolobophora chlorotica* (Savigny) (the green worm) and *Aporrectodea caliginosa* (Savigny) (the grey worm). Such species rarely venture to the soil surface and tend to have pale

Table 7.1 General characteristics of the three major earthworm ecological groupings (after Bouché (1977))

Diagnostic character	Endogeic (shallow working)	Anecic (deep burrowing)	Epigeic (litter dwelling)
Food source	Mineral soil	Surface litter drawn into burrow	Decomposing surface litter
Adult size	Medium	Large	Small–medium
Burrow	Extensive horizontal (to 15 cm)	Large permanent vertical (to 2 m)	None
Reproductive rate	Intermediate	Slow	Rapid
Longevity	Intermediate	Long lived	Short lived
Temperate Examples	*Aporrectodea caliginosa* *Aporrectodea rosea*	*Aporrectodea longa* *Lumbricus terrestris*	*Eisenia fetida* *Dendrodrilus rubidus*

pigmentation. Laboratory and field evidence shows that endogeics are less fecund than epigeic species and produce cocoons which may contain a single hatchling (Sect. 7.3).

Anecic earthworms tend to be larger than those in the other two ecological groupings and exist within a semi-permanent, vertical burrow system (often deeper than 1 m). Typified in temperate soils by *Aporrectodea longa* (Ude) (the black-headed worm) and *Lumbricus terrestris* (Linnaeus) (the lob worm or night crawler), these species are heavily pigmented towards the anterior and dorsal extremities. They tend to form large piles of casts (faeces) at the soil surface and in the case of *L. terrestris*, this may be intimately associated with organic and inorganic materials collected from the soil surface to form a midden (e.g. Butt and Nuutinen 2005). Anecic species are responsible for incorporation of organic matter from the surface into the mineral component of the soil, as witnessed by Darwin. These species are even less fecund than endogeics, tend to be restricted to relatively stable environments and are considered as K-strategists.

7.3 Controlled Earthworm Cultivation

Soil dwelling earthworms have been cultivated under controlled conditions for in excess of 60 years beginning with the seminal work of Evans and Guild (1948). However, in spite of this history, successful and sustainable cultivation of these species appears to remain something of an 'art form' rather than a science and achieved by only a limited number of researchers. The failure to cultivate endogeic and anecic earthworms has resulted in the widespread use of more easily cultivated epigeic species (e.g. *E. fetida*). This reliance on inappropriate species was highlighted by Sizmur and Hodson (2009) who reviewed the impact of earthworms on metal mobility and availability in soils. These authors stated that endogiec species were best suited to determine given effects, but this ecological group was not studied extensively because such earthworms are not commercially available and are difficult to maintain in the laboratory. As a result, the most widely used species were epigeic and commercially available anecic species (e.g. *L. terrestris*). However, even the latter is likely to have been field collected (Tomlin 1983) and may have been kept in cold storage for lengthy periods before sale for experimental use. The widely held assumption that soil dwelling species are difficult to cultivate is, at best, misguided as highlighted by a review of culture techniques for soil dwelling earthworms (Lowe and Butt 2005). This review clearly indicates that this practise (albeit predominantly for temperate species) is both practically achievable and sustainable.

There is little doubt that epigeic species are more easily maintained in greater numbers and produce new generations in less time than soil dwelling species (Table 7.2) and that their widespread use (e.g. in vermiculture and ecotoxicology) has resulted in the development of well-established culture techniques. Epigeic species can be cultured in 100% organic substrates (including animal and vegetable

Table 7.2 Life cycle characteristics of four temperate earthworm species under optimal cultivation conditions

Characteristic	*L. terrestris*	*O. cyaneum*	*A. chlorotica*	*E. fetida*
Mode of reproduction	Obligatory amphimictic	Obligatory parthenogenetic	Obligatory amphimictic	Obligatory amphimictic
Ecological grouping	Anecic	Endogeic	Endogeic	Epigeic
Growth to maturity (days)	112 at 15°C (Svendsen et al. 2002)	168 at 15°C (Lowe and Butt 2008)	84 at 15°C (Butt 1997)	53–76 at 25°C (Edwards 1988)
Cocoon incubation (days)	90 at 15°C (Butt 1991), 50–731 at 15°C (unpublished data)	128 at 15°C (Lowe and Butt 2008)	63–79 (green morph) at 15°C (Lowe and Butt 2007b)	32–73 (Edwards 1988) 12–67
Cocoon viability (%)	83 at 15°C (Butt et al. 1992)	73 (Lowe and Butt 2008)	86 (Lowe and Butt 2007b)	83 (Edwards 1988)
Hatchlings per cocoon	>99% produce a single hatchling (Butt 1991)	$\bar{x} = 1.19$ (20% produced twins, Lowe and Butt 2008)	100% produce a single hatchling ($n = 301$) (Butt 1997)	$\bar{x} = 3.3$ (Edwards 1988)

Table 7.3 Guidelines for sustained culture of four species of temperate soil dwelling earthworms (adapted from Lowe and Butt (2005))

Culture parameter	Anecic		Endogeic	
	A. longa	*L. terrestris*	*A. chlorotica*	*A. caliginosa*
Soil type	Loam (pre-treated to remove macro- and meso-invertebrates)			
Soil depth (cm)	>10	>10	>3	>3
Soil pH	6–7	6–7	6–7	6–7
Soil moisture (%)	25	25	25	25
Food type	Dried and rewetted animal dung (e.g. cattle or horse)			
Food quantity (adult^{-1} month^{-1})	>20 g	>20 g	>10 g	>10 g
Food location	Surface application		Soil incorporation	
Food particle size (mm)	<10	<10	<1	<1
Temperature (°C)	15	15	15	15
Light	24 h dark	24 h dark	24 h dark	24 h dark
Vessel type	Sealed, opaque plastic with ventilation in the lid			
Stocking density (adults l^{-1})	4	3	10	6

wastes) and exhibit a broad tolerance of temperature, moisture and pH levels. Optimal growth rates for *E. fetida* are achieved at 25°C with a moisture content of 60–80% and a pH of 6.5 (Haukka 1987; Edwards 1988; Tripathi and Bhardwaj 2004). However, culture conditions that are optimal for epigeic species cannot be directly applied to soil dwelling species. Temperate anecic and endogeic species require a mineral soil and have different tolerances with respect to temperature, moisture and pH. Table 7.3 provides guideline parameters for establishment of culture environments for four relatively common temperate soil dwelling species. In most cases, these guidelines were derived with reference to the literature, but where this was lacking, values are provided from personal experiences. It is this

disparity in cultivation conditions that is at least partially responsible for a perception that soil dwelling species are difficult to maintain on a sustainable basis. This observation may be exacerbated by researchers in applied fields viewing earthworms simply as a tool that can be utilised to gain results without reference to a species autecology.

Successful production of soil dwelling earthworms relies on frequent monitoring and maintenance of stock to ensure environmental factors are kept within acceptable ranges (Lowe and Butt 2005). Controlling abiotic and biotic factors at optimal levels can greatly reduce length of time for growth to maturity and length of cocoon incubation times and lead to increased earthworm fecundity. For example, reducing food particle size can increase growth rates (particularly in endogeic species), as shown by Lowe and Butt (2003) who fed *A. chlorotica* with milled (<1 mm) or un-milled Separated Cattle Solids (SCS). Reduced particle size increased the mean mass of individuals by 185% with clitellate individuals recorded after 10 and 18 weeks in milled and un-milled treatments, respectively.

All physiological activities of earthworms are influenced by temperature (Löfs-Holmin 1985), so this can be used to manipulate earthworm life cycles. It is widely recognised that increasing temperatures up to critical thresholds can increase growth rates and fecundity (Lowe and Butt 2005); however, reducing temperatures can also play an important role in earthworm cultivation. In temperate species, low temperatures (3–5°C) can be used to inhibit growth by inducing a period of enforced quiescence. At low temperatures, earthworms can be maintained at artificially high densities for long time periods without decreasing survival rates or affecting growth rates when transferred to more optimum conditions at reduced densities. Lowe (2000) maintained in excess of 100 *L. terrestris* hatchlings in a mixture of loam and SCS in a 600-ml vessel at 4 ± 1°C. After 12 months, hatchlings had a mean mass of 76 mg by comparison with 61 mg for newly hatched individuals and showed no significant difference in subsequent growth rates (under optimal conditions). Similarly, low temperatures can also be used to inhibit embryo development. Several researchers have maintained earthworm cocoons at 5°C to slow development and prevent hatching (e.g. Holmstrup et al. 1991).

The maintenance of earthworms at constant temperatures, optimal for growth and fecundity, is advocated in order to overcome the influence of seasonality. However, the temperatures at which earthworms are naturally found are not necessarily the same as those at which they grow most rapidly or are most active. Artificially maintaining species in these 'optimum' conditions can (over time) lead to decreased survival and reduced viability of cocoons. Evans and Guild (1948) indicated that lengthy exposure to temperatures above 15°C led to earthworm fatigue that may be a function of reproductive exhaustion (Butt 1991). Constant relatively high-temperature regimes can in the short term be used to optimise cocoon production, but it is not advisable for long-term studies (greater than 6 months) (Lowe and Butt 2007a).

Constant temperature regimes are usually achieved using temperature-controlled incubators or rooms. This requirement means that space is often a limiting factor and careful thought is required in selection of culture vessels. These should be

Fig. 7.1 Contents of a temperature-controlled incubator: cocoons in Petri dishes (9 cm diam.) on moistened filter paper and earthworms in 750 and 400 ml vessels

easily stacked (but also allow for air to circulate) and small units are preferred to larger ones for ease of handling, sampling (Löfs-Holmin 1983) and maximising storage (Fig. 7.1). The use of small vessels also limits the risk of microbial infection on stock survival. Culture vessels should have sealable lids to minimise moisture loss, but these require small holes (e.g. from a mounted needle) to prevent development of anaerobic conditions. Cocoons are most easily maintained in Petri dishes on moist filter paper. However, as the dishes are not sealed, moisture loss can be relatively rapid and it is therefore advisable to add sufficient water to cover the cocoons to reduce desiccation. Water can be added periodically to ensure conditions for cocoon development remain optimal.

There is no 'one size fits all' approach to cultivation of soil dwelling earthworms. Whilst certain factors such as a permanent darkness regime can be constant for soil dwelling species, other factors are species specific with the overriding influence being ecological grouping. For example, the position of organic matter in the soil profile has a species-specific influence on growth rates and behaviour (Lowe and Butt 2005). In general, anecic species prefer organic matter applied at the soil surface while endogeic species benefit from organic matter incorporated into the soil.

It has been suggested that reasons for perceived problems with sustainable cultivation of soil dwelling species are mainly associated with the time, effort and equipment required to produce successful cultures. However, it may be argued that the greatest barrier is the paucity of information relating to the ecology of species selected for culture (Sect. 7.4.1).

7.4 Why Cultivate Endogeic and Anecic Species?

The requirement for a soil substrate makes the concept of cultivating endogeic and anecic earthworms quite challenging. It would appear to be impractical to specifically create large 'breeding beds', as used with epigeic species (Edwards 1988). Nevertheless, one realistic production method for the required species might be encouragement of naturally occurring populations in field soils. This concept of 'biostimulation', as described by Brun et al. (1987) might work for semi-controlled production of mixed earthworm species required for general (field-related) purposes. This may be achieved simply by artificial feeding of earthworms through addition of organic matter to the soil. For example, an observation in pasture of earthworm density over 1,000 m^{-2} with a biomass in excess of 1 kg resulted from continual (natural) application of cow dung at a location where cattle gathered for milking twice daily (Butt et al. 1993). Alternatively, more controlled earthworm production could make use of smaller (easily handled) cultivation units which might be appropriate where animals are required for specific reasons. Each of these will be examined in further detail within the following sub-sections.

7.4.1 Life History Studies, Species Interactions and Population Ecology

The life history of relatively few earthworms is known in any detail. Edwards and Bohlen (1996) suggested this was an area that warranted further investigation. In recent years, research has begun to reveal more details concerning earthworm reproductive mode (e.g. parthenogenetic or amphimictic), frequency of cocoon production, success of hatching, numbers of hatchlings produced per cocoon and length of time for growth to maturity (e.g. see Table 7.2). From this information, life histories for individual species can be constructed which in turn can be used for model construction which may be of value in predicting population dynamics in given ecosystems (Svendsen et al. 2005; Pelosi et al. 2008).

Further justification for laboratory-based studies can be obtained from earthworm identification guides. For example, a British guide to earthworm (Sims and Gerard 1999) presents information which is severely lacking in places. Records for some very well-known lumbricid species (e.g. *Dendrobaena attemsi*) contain notes such as 'capsules (cocoons) unrecorded' and 'presumably biparental'. Following procedures that have been used for decades (Evans and Guild 1948) and further developed more recently (reviewed by Lowe and Butt 2005), this type of information ought to be relatively easily obtained to provide a more complete view of earthworm life histories.

Having obtained basic information on life history parameters, further experimental work can then be undertaken on a species population dynamics. By its very nature, this will require a consideration of life history stages together with

behaviour and may therefore necessitate the use of culture vessels that are not as simple as those used for examining a single aspect of a species life history. For example, Grigoropoulou et al. (2008) undertook a series of experiments in glass-sided mesocosms (height 80 cm, width 20 cm, thickness 0.8 cm) which considered the effects of adult *L. terrestris* on hatchling behaviour and growth. Use of such vessels allowed a visual inspection of burrow construction and location of the earthworms to be recorded. It also showed position of cocoon deposition which revealed production of short side extensions from the main burrow, which may have been created specifically for purpose and then blocked with casting. To obtain further data on *L. terrestris* adult hatchling relationships, Grigoropoulou (2009) maintained a single adult with different densities of hatchlings in a large (60 × 50 × 40 cm) macrocosm in order to provide realistic conditions for burrow and associated midden construction to try and further elucidate potential functions of these earthworm-created structures (Butt and Nuutinen 2005).

In order to more fully appreciate aspects of earthworm population dynamics, methods for marking earthworms have been developed. Most recently, use of visual implant elastomer (VIE) has been shown to be a reliable option (Fig. 7.2). These tags have been retained in a number of earthworm species for more than 2 years, and had no detrimental effects on growth to maturity, mating and cocoon production in *L. terrestris* which was studied in detail (Butt and Lowe 2007). This technique may prove to be valuable in earthworm age determination, but may also reveal much from studies of population dynamics, in terms of, mark-release-recapture exercises. Gonzalez et al. (2006) used VIE tagging to assess population dynamics of collected *Pontoscolex corethrurus*. Future work might utilise laboratory-reared cohorts of a species, which could be tagged with different colours of VIE in different years to assist understanding of e.g. survival. Used in combination with density manipulation experiments, this type of research has already revealed aspects of *L. terrestris* dispersal and settlement behaviour in woodlands (Grigoropoulou and Butt 2010).

Fig. 7.2 Red visible implant elastomer (VIE) tag in post-clitellum body segments of laboratory-reared *Octolasion cyaneum*

Associations between different earthworm species (often within and between ecological groupings) have been documented from field surveys throughout the scientific literature (Edwards and Bohlen 1996). In addition, negative correlations between species density (within ecological groupings) have been recorded (e.g. Edwards and Lofty 1980). This suggests that earthworm communities are far from random in assemblage and that interactions between earthworm species may have some significance. Under controlled cultivation, Butt (1998) utilised pairings of six species drawn from all three ecological groups and found that under the given conditions, *Dendrobaena (Eisenia) veneta*, an epigeic species, had the greatest effect on depressing growth rates of the other species investigated. Equally, *L. terrestris* and *A. longa* showed a competitive relationship with respect to hatchling growth supporting some of the findings from the field provided by Edwards and Lofty (1980). Further laboratory-based work by Lowe (2000) looked in depth at specific parameters which could have additional effects on earthworm interactions, such as age of interacting organisms (Lowe and Butt 2002) and food particle size (Lowe and Butt 2003). The intensity of these interactions may vary depending on the earthworms involved and as these relationships were recorded from laboratory culture, it may be argued that their greater relevance under field conditions still remains to be tested. Research on earthworm interactions under controlled cultivation conditions has been reviewed in depth by Uvarov (2009).

7.4.2 Ecosystem Improvement

Soil dwelling earthworms burrow in search of a food source, a mate and acceptable abiotic conditions for existence. In doing so, they also pass copious amounts of soil through their digestive tracts and intimately combine organic and inorganic components. Such activities have frequently been reviewed (see Chap. 3), so it is felt that there is no need to undertake the process here. Shipitalo and Le Bayon (2004) documented effects of earthworms on aggregate and water relationships in the soil and summarised literature relating to organic matter-mineral-soil effects mediated by earthworms. This source of reference is thought to provide sufficient background material for the subject developed in this section. Such positive activities of earthworms on 'soil improvement' are therefore widely accepted. It is, however, worth briefly outlining the ecosystem engineering role now ascribed to earthworms, whereby they regulate the soil as an environment for other organisms (including plant roots) by controlling availability of resources e.g. through physical (burrow and cast) formations (Lavelle et al. 1997). In this section, we focus on how controlled cultivation of appropriate species may assist in earthworm-related soil improvement, earthworm–plant interaction and their importance in food webs.

To appreciate earthworm-related activities in soils, a starting point with a complete absence of earthworms may be useful. Such instances can and do occur in anthropogenic soils and have been the subject of much interest over recent decades (see Chap. 6). The development of Earthworm Inoculation Unit (EIU) technology

required fundamental information on controlled cultivation of soil dwelling species. It was this laboratory-based research that enabled field inoculation and subsequent long-term monitoring to occur. In addition, the basic laboratory work on species interactions also allowed for the development and production of mixed species EIUs (Butt et al. 1997). Cultivation of earthworms for soil inoculation should always ensure that appropriate species, determined by site conditions and species requirements, are selected for use. As noted by Kretzschmar (2004), this relies on earthworm behaviour in the athropogenic soil mirroring that of the soil from where they were originally derived. As demonstrated by many species, a degree of behavioural plasticity is therefore required.

In addition to EIU technology, earthworms can be inoculated into soils directly through broadcasting directly on to the soil surface (Chap. 6). Recently, we made use of hatchling *L. terrestris*, cultivated under controlled laboratory conditions for introduction into soils of tree plots on the Isle of Rum, Scotland. Soils within these plots have developed since tree planting on moorland some 50 years ago (Wormell 1968). Under broad-leaved species, such as *Quercus* sp. and *Betula* sp, earthworm communities containing epigeic and endogeic species have developed, which exceed in species diversity and numbers those present on adjacent moorland (Butt and Lowe 2004). The broadcast inoculation of *L. terrestris* in 2007 (Fig. 7.3) was viewed as a way of potentially assisting soil development in this location where natural colonisation of this anecic species was thought to be very unlikely. Further earthworm introductions in areas of more recent tree planting on Rum made use of 'cocoon inoculation'. Here un-hatched, but well-developed, cocoons of *Octolasion cyaneum* (Savigny) and *A. caliginosa* were introduced in soil at known locations where these species were not present. As with the inoculation of hatchlings, monitoring of this work is ongoing but disturbance will be kept to a minimum, with systematic sampling due after a period of 5 years.

Earthworms are significant components in the feeding ecology of numerous invertebrate and vertebrate animals. Major landscape developments, where legally protected species such as Great crested newts (*Triturus cristatus*) and badgers (*Meles meles*) are present, may require that earthworm status is monitored to

Fig. 7.3 Hatchling *Lumbricus terrestris* cultured in the laboratory and ready for broadcast inoculation for soil improvement

ensure an adequate food supply as described by Butt et al. (2003) at Manchester Airport. Here translocation of earthworm-rich turf was considered adequate, but in other situations, a more proactive approach may be required. At a Local Nature Reserve in NW England, steps are underway to create a landscape to encourage feeding and nesting of wading birds. To promote this activity, one suggestion is that appropriate earthworm species should be cultivated in large numbers and inoculated to provide a sustainable community to act as (a part of) the food supply for the desired waders. This project is in development and awaits completion of earthworm monitoring of existing communities to determine if cultivation and introduction is necessary, or if a natural earthworm community will develop to provide a sustainable food source.

7.4.3 Ecotoxicology

Earthworms are both resistant and sensitive to pollutants (Cortet et al. 1999) and are capable of accumulating chemicals at concentrations higher than the surrounding substrate. They are also present in the majority of temperate soils and are relatively sedentary with natural immigration rates of 5 m $year^{-1}$ (Marinissen and Van den Bosch 1992). As a result, earthworms are considered as biological sentinel species (e.g. Stürzenbaum et al. 2009) that can play an important role in ecological assessment of soils.

The last 20 years have seen a rapid expansion in applied earthworm research in ecotoxicology. This area of research has undergone development from standardised acute toxicity tests (e.g. ISO 11268-1) through more ecologically relevant sub-lethal endpoint tests (e.g. ISO Reproduction toxicity test 11268-2). The potential of earthworms as bio-indicators in field-based ecological assessment and in particular bio-accumulation studies is widely recognised (reviewed by Sanchez-Hernandez 2006) and can allow for rapid diagnosis of contaminant effects under field conditions (Lowe and Butt 2007c).

More recently, significant developments have occurred in toxicogenomics (reviewed by Spurgeon et al. 2008) made possible by genomic sequencing of earthworms including two important test species; *Lumbricus rubellus* (Hoff.) (e.g. Owen et al. 2008) and *E. fetida* (e.g. Pirooznia et al. 2007). Changes in gene and protein expression are the first indications of toxicity/stress, therefore measuring gene transcription or protein production (proteomics) may become a key tool in environmental diagnostics (Spurgeon et al. 2008). At the time of writing, the complete genomic sequence for both the aforementioned species is not available. Nevertheless, it is envisaged that once these are produced, that genomic earthworm research will go through a rapid expansion on a scale similar to that seen after the release of the genomic sequences for other model species such as the nematode *Caenorhabditis elegans* and elevate the status of earthworms to an ecologically relevant genetic model organism (Stürzenbaum et al. 2009).

E. fetida has been widely used in acute and chronic toxicity tests and remains the recommended species in several standardised procedures including the ISO reproduction toxicity test. This reliance on *E. fetida* has been attributed to its short life cycle, high fecundity and ease of culture. However, the ecological relevance of employing a species that does not inhabit the mineral soil and has limited distribution (largely restricted to decaying organic matter) has been questioned (Lowe and Butt 2007c). More recently, several workers have stressed the need for a species-specific approach to ecotoxicology and the use of species relevant to the study site (Morgan and Morgan 1998; Van Gestel and Weeks 2004; Svendsen et al. 2005). There is now a general acceptance that, where appropriate, test species from appropriate ecological earthworm groups (Sect. 7.2) are used in ecotoxicological studies as recommended by Bouché (1992). However, due to perceived culture difficulties of soil dwelling species (Sect. 7.3), there remains a heavy reliance on commercially purchased or field-collected earthworms of unknown age, exposure or provenance. The validity of this practise has also been questioned. Lowe and Butt (2007a) demonstrated (under controlled laboratory conditions) that earthworms (*L. terrestris*) purchased from a range of commercial suppliers exhibited marked differences in survival rates and fecundity. We recommended that laboratory-reared earthworms of known age and history ought to be employed in chronic ecotoxicological studies.

It is accepted that genetic differences between populations of the same species may influence the accumulation of contaminants and subsequent chronic endpoints (Lowe and Butt 2007c). The importance of this was highlighted by the recent proposal of genetic lineages by King et al. (2008) in a number of common British earthworm species. Furthermore, Andre et al. (2009) have determined that *L. rubellus* displays a high degree of genetic diversity and that the distribution of genotypes is not uniform across a metalliferous landscape. These researchers suggested that different genotypes may display differential responses or susceptibilities to environmental contaminants. The development of toxicogenomics further magnifies the issue of genetic heterogeneity within test organisms and represents a significant barrier to development of earthworms as a key tool in ecological assessment of soils. We believe that this may be addressed by the controlled cultivation of specific species and strains (or even haplotypes) that can be utilised in field and laboratory-based studies. To maintain genetic consistency, Bouché (1992) proposed that species and strains ought to be cultured and distributed to laboratories to form test cultures. In the long term, we would advocate the development of an 'earthworm bank' that would provide collaborating laboratories with access to a range of laboratory-reared species/strains of known origin, age, pre-treatment and genotype to allow for direct comparison of results. In addition, ecotoxicologists should also consider employing, where appropriate, obligate parthenogenetic earthworms as test species. Parthenogenetic species, such as *O. cyanaeum*, have the potential to provide genetically similar test populations from different geographical locations (Lowe and Butt 2007c) and allow for the development of clonal strains from individual specimens.

7.5 A Future for Cultivation of Soil Dwelling Earthworms

Anthropogenic soil degradation is widely recognised as a serious problem in terms of impacts on human health, the environment and ecosystem function (e.g. Swartjes et al. 2008). The role of soil as a source and sink for carbon has also been highlighted in the context of climate change (e.g. Marmo 2008). Therefore, in the context of this chapter, the ecological application of soil dwelling earthworms at a macro scale (in ecosystem rehabilitation) and at a micro scale (utilising earthworm genomics in ecological assessment) may prove to be of potential significance. However, delivery on this potential may, in the long term, be dependent on the ability of scientific researchers to cultivate the necessary tools—endogeic and anecic earthworms.

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Chapter 8
The Meek Shall Inherit the Burrow: Feedback in Earthworm Soil Modification

Visa Nuutinen

8.1 Introduction

One distinctive feature of earthworm biology from its early start has been a strong emphasis on the study of organism–environment interaction. As an example of that the account of Gilbert White (1789) is worth quoting (Box 8.1). His letter describes the role of earthworms in soils in a remarkably modern tone and in fact so lucidly that it is not easy to produce a fresh and improved introduction to any research paper on earthworm ecology. In the end of his letter, White mentioned that a monograph 'of worms would afford much entertainment and information at the same time, and would open a large and new field in natural history'. While mot making reference to White's suggestion, this was the job Charles Darwin accepted 100 years later (Darwin 1881). His last book was devoted to earthworms and served – along with its many other implications – to link the activity of earthworms to physical soil processes, from local soil profiles to landscape scale. Darwin, thereby, set the foundation for what today is referred to as bioturbation research (Meysman et al. 2006; Wilkinson et al. 2009).

Both White and Darwin recognized the role of earthworms in affecting chemical and physical soil properties and in making the soil favourable for plant growth. Without downplaying the subsequent advancement in earthworm ecology, one could say that fundamentally the leap from Darwin to the prevailing conceptions on earthworms' role in soil ecosystems is not that long. In general, the subsequent findings of earthworm ecology has concurred with the views of the founding fathers while, of course, significantly specifying the physical, chemical and plant growth effects of earthworms (Feller et al. 2003). The recent findings on invading exotic earthworms' influence in North American hardwood forests provide a startling example of earthworm potential in directing soil-related processes in ecosystems – and that

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earthworm influences on plant communities cannot be regarded as universally 'beneficial' (Hendrix 2006).

Since the ideas presented by Jones et al. (1994), it has become customary to describe the ecological role of earthworms in soils as allogenic ecosystem engineering. It seems that in earthworm ecology, the concept has been predominantly employed as an 'outward' referring one, with emphasis on the impacts of soil modification on the environment of earthworms (e.g., Lavelle et al. 2007). However, Jones et al. (1994) already pointed out that the ecosystem engineering is likely to have consequences for the engineers themselves and introduce potentially important ecological and evolutionary feedbacks ('extended phenotype engineering'). The possibility that earthworms change conditions in soil in a way, which may affect the environment's quality for themselves, has received relatively little systematic attention from earthworm ecologists. However, Kretschmar (1983), for instance, did speculate on how the physicochemical changes caused in soil by earthworms can serve to render the soil favourable for their own survival by stabilising the environment. More recently, the feedbacks were discussed by Jouquet et al. (2006) in their paper on different types of ecosystem engineering in soils.

Nevertheless, compared with the comprehensive literature on 'outward' influences of earthworm soil engineering, the feedback effects on individuals and populations have not been widely addressed. This can be seen as an unnecessary restriction, as the strong influences that earthworms can have on soils are almost bound to have consequences on populations themselves and are thus potentially important in understanding their ecology and also evolution. Meanwhile, over the years, the importance of the feedbacks has been sporadically discussed by evolutionary biologists. Recently, this has taken place to an increasing degree within the discussion on niche construction and the related ecological inheritance concepts (Odling-Smee et al. 2003). The aim of this chapter is to review that discussion. Although it consists of diverse and partially opposed views, it holds promise for opening fertile new ground for earthworm ecology. I will also try to provide an example of how the idea of ecological inheritance could be used as an organizing concept in the study of earthworm ecology and behaviour.

Box 8.1. Gilbert White (1789) on the significance of earthworms in soils

'... Earth-worms, though in appearance a small and despicable link in the chain of nature, yet, if lost, would make a lamentable chasm. For, to say nothing of half the birds, and some quadrupeds, which are almost entirely supported by them, worms seem to be the great promoters of vegetation, which would proceed but lamely without them, by boring, perforating, and loosening the soil, and rendering it pervious to rains and the fibres of plants, by drawing straws and stalks of leaves and twigs into it; and, most of all, by throwing up such infinite numbers of lumps of earth called worm-casts, which, being their excrement, is a fine manure for grain and grass. [...] ...

(continued)

the earth without worms would soon become cold, hard-bound, and void of fermentation; and consequently sterile . . .'

Gilbert White 1789. Letter 35 in 'The Natural History of Selborne'

8.2 Earthworm Soil Modification: A Soil Improvement Adaptation?

8.2.1 Viewpoints of Earthworm Adaptations

One interesting and fundamental question is whether the influences of earthworms in soil could be considered as an evolved mechanisms for soil improvement, be that for the benefit of worms themselves or for the benefit of the community or ecosystem as a whole. This issue has been taken up by prominent evolutionary biologists, and it is worth pointing out their views here, particularly as they have received little attention in earthworm ecology literature.

The question was addressed by Williams (1966) – as an example for a more general purpose – in the introduction of his classic book on natural selection and adaptation. Since his text presents the issue with great clarity, the passage is quoted in full in Box 8.2. The value of Williams' account is in setting stringent conditions for when it is justified to interpret earthworms' soil modification as an adaptation with a soil improvement function. As such, the fact that earthworms change the soil conditions or affect plant growth in ways 'beneficial' for themselves or their surroundings gives no right for an argument for design by natural selection. Without evidence for adaptations, which are not explicable in term of design for worm's basic life processes – particularly those relating to nutrition – arguments for design for soil modification are hollow. Williams' own assumption was that the study of earthworm digestive system and feeding would support the view that they are adequately explained by design for nutrition. Any soil improvement effects would be fortuitous, not moulded by natural selection for this function.

Williams emphasized that adaptation is in general an onerous concept. It should only be invoked when truly necessary, and further, the explanation for adaptation is preferably selection among genes or individuals. An advocate of a different viewpoint – group selection or more recently, multilevel selection – has also considered feedbacks in earthworm soil modification. Wilson (1980; pp 95–107) modelled the evolution of indirect effects in what he called structured demes, using a simple model community with earthworms and associated plants and soil microbes. From the results, he concluded that if his theory is valid, Williams' views need to be modified in a number of ways. First, even if the activities of earthworms were for maximizing feeding activity only, they could be 'constrained to behave in the interests of the community'. Second, if earthworm activities improve the environment through indirect effects for the earthworm, it can evolve in a direction that cannot be explained by trophic adaptation alone. Taking sides in this discussion is

beyond the scope of this chapter, but two useful messages can be obtained from it. First, if an adaptive explanation for earthworm soil influences is proposed, it must be made in a disciplined manner. Second, it may be difficult to discuss the feedbacks in earthworm soil modification in an evolutionary context without entering the levels of selection controversy.

Ten years ago a new thread to the discussion was added by Turner (2000) in his treatise on physiology of animal built structures. In Chap. 7 of his book, Turner suggests that the physical changes in soils caused by earthworms are in fact crucial for their subsistence in terrestrial environments. To me, he seems to maintain that the evidence for treating earthworm soil modification as a special soil improvement mechanism has been identified. Turner's account is a carefully built, 20 pages long argument with intricate physiological and soil physical considerations, and here it is possible only to summarize the main points. The argument is importantly based on the observation that for their physiology – for the system of water balance maintenance in particular – earthworms appear ill prepared for life on land. Their nephridia resemble those of aquatic oligochaetes lacking structural adaptations for life on land, and earthworms produce urine as if they were freshwater animals thereby losing considerable amounts of water. Turner's point is that this handicap of internal physiology is compensated by the modification of the physical properties of the soil environment. First, by enhancing soil aggregation, earthworms would lower the matric potential of soil making it easier for them to draw water to their bodies and to replenish the losses caused by the less than optimal excretory system. Further, according to Turner, the network of continuous macropores, which can extend deep into the subsoil and function as efficient percolation routes of water, in its turn would expand the soil horizon favourable for earthworms. In summary, the earthworms would have co-opted the soil as an 'accessory kidney', organ of water balance. The soil would be adapting to the earthworms rather than vice versa.

Turner's interest was not in the origin of the proposed soil modification mechanism and it may well not be possible to track its history in any trustworthy and meaningful way. However, the question of origin is of interest for the discussion of function attached to it. Should the suggested soil co-option mechanism be operational, it may nevertheless not be an adaptation but rather an exaptation as distinguished by Gould and Vrba (1982, their Table 1). The features of the mechanism may not have been in any instance shaped by natural selection for their present role. Instead, for instance, they might have been carried along as exaptations by the ancestral organisms, which invaded the land from freshwater habitats (for earthworm evolutionary origin, see Little 1990). The 'usage' of the soil co-option should then be regarded as its effect rather than its function.

As captivating as the idea of 'accessory kidney' is, it could be useful to still treat it as a working hypothesis for further evolutionarily oriented ecophysiological investigations on the subject. The soils inhabited by earthworms and the worms themselves (their behaviour, population density, etc.) vary widely and the functionality and importance of the co-option is likely to vary accordingly. Comparative anatomy and physiology of the accessory kidney would thus seem worthwhile. For a student of arable soils, it is obvious that earthworms often abundantly populate

and thrive well in soils where their effects on topsoil crumb structure remains moderate due to yearly mechanical disruption of natural soil structure by tillage. Either man with his tillage implements manages to produce a highly functional 'artificial accessory kidney' or the earthworm-moulded crumb structure is not absolutely decisive for the existence of earthworms.

Box 8.2. G.C. Williams (1966) on explaining earthworm adaptations

'It may also happen that incidental effects of individual activities, of no functional significance in themselves, can have important statistical consequences, sometimes harmful, sometimes beneficial. [..]'

The feeding activities of earthworms would be a better example, because here the incidental statistical effects *are* beneficial, from the standpoint of the population and even of the ecological community as a whole. As the earthworm feeds, it improves the physical and chemical properties of the soil through which it moves. The contribution of each individual is negligible, but the collective contribution, cumulative over decades and centuries, gradually improves the soil as a medium for worm burrows and for the plant growth on which the earthworm's feeding ultimately depends. Should we therefore call the causal activities of the earthworm a soil-improvement mechanism? Apparently Allee (1940) believed that some such designation is warranted by the fact that soil improvement is indeed a result of the earthworm's activities. However, if we were to examine the digestive system and feeding behaviour of an earthworm, I assume that we would find it adequately explained on the assumption of design for individual nutrition. The additional assumption of design for soil improvement would explain nothing that is not also explainable as a nutritional adaptation. It would be a violation of parsimony to assume both explanations when one suffices. Only if one denied that some benefits can arise by chance instead of by design, would there be a reason for postulating and adaptation behind every benefit.

On the other hand, suppose we did find some features of the feeding activities of earthworms that were inexplicable as trophic adaptations but were exactly what we should expect as a system for soil improvement. We would then be forced to recognize the system as a soil-modification mechanism, a conclusion that implies a quite different level of adaptive organization from that implied by the nutritional function. As a digestive system, the gut of a worm plays a role in the adaptive organization of that worm and nothing else, but as a soil-modification system it would play a role in the adaptive organization of the whole community. This, as I will argue at length in later chapters, is a reason for rejecting soil-improvement as a purpose of the worm's activities if it is possible to do so. Various levels of adaptive organization, from the subcellular to the biospheric, might conceivably be recognized, but the principle of parsimony demands that we recognize adaptation at the level necessitated by the facts and no higher.

(continued)

8.2.2 Niche Construction and Ecological Inheritance Perspectives

Another fundamental question is involved here: what kind of basic viewpoint one adopts in looking at the interaction between organism and environment? A view that has perhaps dominated in evolutionary ecology is that adaptation is asymmetrical with organisms adapting to their environments and not the other way around (e.g., Williams 1992). This is obviously quite opposite to the reasoning of Turner presented above, which was just one example of an alternative view. For instance, Lewontin (summarised in Lewontin (2000)) has criticised the world view, where the driving force of evolution is a setting, where the pre-existent environment is posing 'problems', which organisms must 'solve'. This asymmetrical cause-and-effect picture does not, according to him, capture the essence of organism–environment relation. A fuller understanding of evolutionary process requires noticing that just as changes in organisms are the effects of natural selection in a given environment, those changes become the causes of changes in that environment. The relationship of organism and environment is that of co-evolution in which both act as cause and effect and the 'fit' between organisms and environments is not a one-way accommodation. The theory of niche construction starts from these premises (Odling-Smee et al. 2003; Laland and Sterelny 2006). Niche construction is defined as a process where organisms through their different life processes modify their own and/or each other's niches (Odling-Smee et al. 2003). Examples of physical modification, which are of special interest here, include construction of burrows, holes, nests, webs and pupal cases. By these activities, niche constructors may change natural selection pressures in the external environment. Although niche construction can be seen as a synonym to ecosystem engineering, compared with the latter, the usage of the niche construction concept has clearly been more evolutionary oriented.

Niche construction theory has developed into a wide encompassing theoretical framework – too wide, say the critics (Keller 2003; Dawkins 2004; Brodie 2005) – with ramifying implications. Here it is possible to point only to some features most important for the present purpose. Niche constructing can be positive, referring to acts that on average increase the fitness of the niche constructing organism. The opposite effects are also possible and they are referred to as negative niche construction. Perturbational niche construction occurs when organisms physically change their environment while relocational niche construction involves modifying selection pressures by actively moving in space, not physically constructing anything. When niche construction affects multiple generations, it introduces a second inheritance system in evolution, ecological inheritance (Fig. 8.1). Incorporating

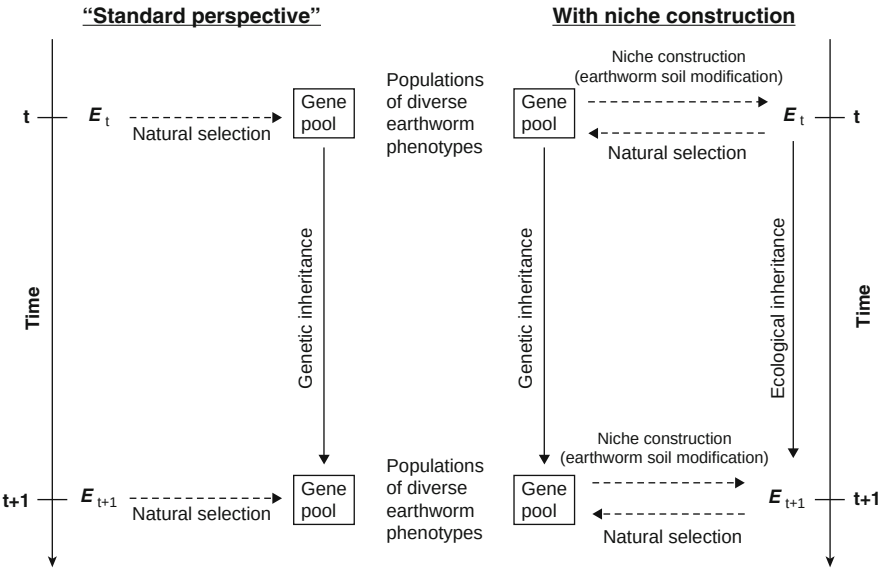

Fig. 8.1 Niche construction and ecological inheritance. *Left*: 'Standard evolutionary perspective': earthworms transmit genes from generation t to generation $t + 1$ with natural selection acting on phenotypes. *Right*: Niche construction perspective: each generation of earthworms inherits from ancestral organism also the modified selection pressures, described as 'ecological inheritance'. Modified from Fig. 1.3 of Odling-Smee et al. (2003)

these ideas in genetic models of evolution can have notable corollaries. Their close discussion is beyond the scope of this chapter, but Hansell (2007) provides a short and simplified example using a litter-feeding earthworm as an example.

The concept of extended phenotype (Dawkins 1999) seems to have affinity with a niche construction view, as it incorporates in evolutionary dynamics the effects of genes upon the environment. Although it has recently been proposed that the two views would be quite concurrent (Hunter 2009), the agreement has not been that complete. Within niche construction theory extended phenotype has been considered too restricted (Odling-Smee et al. 2003, p 30), while according to Dawkins (2004), the parts of niche construction theory which 'work' are already included within the extended phenotype theory. Thus, according to him, the whole concept of niche construction could and should be abandoned. In niche construction theory, earthworms are used as prime examples of niche constructors much on the basis of the ideas of Turner presented in Sect. 8.2 (e.g., pages 374–376 in Odling-Smee et al. 2003). Dawkins (2004) acknowledges that earthworms radically change the environment in which they live and that this causes niche alteration. But he contends that much more work is required before the usage of niche construction could be justified.

Even if one would look critically at niche construction theory's general importance, its value is in paying attention to and detailing the various ways by which organisms change their environment and consequently modify the selection

pressures acting upon them (Keller 2003). For instance, Chap. 2 alone of Odling-Smee et al. (2003) is a mine of fresh insights on the ecology and behaviour of organisms. Thinking of earthworms, the idea of ecological inheritance is intriguing as it is intuitively evident that in earthworms the activities of ancestors can have important consequences for the descendants because of the strong modification of the environment. It is worth noticing that the importance of these types of inheritance processes has also been contemplated in ecology earlier and 'outside' the niche-construction theory framework (Myles 1988; Hansell 1993).

8.3 A Case of Ecological Inheritance in the Dew Worm

8.3.1 Dew Worm Life Style

When earthworms have been used to exemplify niche construction and ecological inheritance, often the 'construction' in mind has seemingly been the worm-worked topsoil in its entirety. However, it is also possible to take a more selective look at the structures that earthworms can create in the soil. In the following lines, the focus will be on extensive burrows that some earthworm species build and maintain. Darwin (1881, p. 112) already noticed that earthworm burrows often are not simple excavations but rather look like 'tunnels lined with cement'. Although he never mentioned the species he was working with, it is likely that he was here referring to the burrows of the deep burrowing, anecic earthworm *Lumbricus terrestris* L., or the dew worm.

Dew worms have spatially distinct living sites in the soil, and mature individuals live in a vertical burrow, which typically is unbranched (e.g., Fig. 1 in Nuutinen and Butt 2003) except for possible side burrow(s) opening to the soil surface. In cultivated boreal clay, burrow depths of about 1 m have been measured (Nuutinen and Butt 2003), but the burrows can extend as deep as 2.5 m (Edwards and Bohlen 1996). The typical smooth, 'cement-like' surface of the burrow results from the physical pressure, secretions and castings produced by the resident worm (see Fig. 7 in Schrader et al. 2007). Burrow surrounding constitutes a drilosphere, which differs from the surrounding soil by its physical (Fig. 8.2a), chemical (Fig. 8.2b) and also microbiological properties (Tiunov et al. 2001). The burrow opens on the soil surface where it is relatively easy to locate because of a midden, which is formed at the opening (Fig. 8.5d). On average, middens were observed at 13 cm distance from one-another and in a highly regular spatial pattern by Grigoropoulou and Butt (2010). Middens are mixtures of litter (collected as food) and surface casts. They constitute hot-spots of high organic matter content and associated decomposer communities (Aira et al. 2009). Although the middens are mini-composts of nutritive importance, they may also have a protective function and possible ancillary, still unidentified functions (Butt and Nuutinen 2005). The dew worm life-style corresponds to what Jouquet et al. (2006) referred to as an 'extended genotype

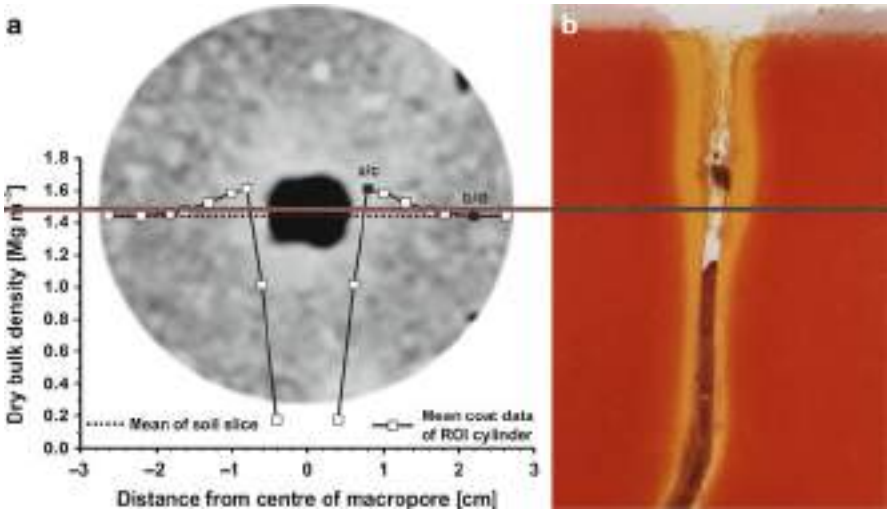

Fig. 8.2 Modification of burrow surroundings by *L. terrestris*. (**a**) Distribution of soil bulk density around the burrow of *L. terrestris* (burrow is the *dark area* in the center scan obtained with X-ray computed tomography). *Source*: Schrader et al. (2007). (**b**) pH change caused in the drilosphere by cutaneous mucus of the resident worm. An individual was allowed to burrow in agar containing methyl red indicator. *Dark light area*: pH 4.4, *area*: pH $\geq$ 6.2. Source: Schrader (1994). Copyright of figures Elsevier

engineer' – type in earthworms, in contrast to an 'accidental engineer' – type represented, for instance, by endogeic earthworms.

Considering its physical nature and dimensions, it is not an exaggeration to call dew worm's 'burrow–midden complex' a construction. Although no one has determined the physical effort and time needed for its completion, it is obvious that the preparation and maintenance of this dew worm home is a notable investment. A question that naturally rises is, could these living sites, and more specially the burrows, be 'recycled' in the population over generations, so that each individual would not necessarily build its own? One clue suggesting that this could occur is the special micro-relief, with miniature hills at dew worm burrow openings, which can develop at sites populated by dew worms (Hazelhoff et al. 1981). Lakhani and Satchell (1970) estimated that longevity of dew worms would be 4–8 years, and it does not seem conceivable that such geomorphic features would develop in one generation.

8.3.2 Indications of Burrow and Living Site Inheritance

It is possible to evaluate the hypothesis of burrow and living site inheritance by following different lines of evidence from the existing knowledge on dew worm ecology. Below I address five points in particular.

1. *Burrow adherence*. Dew worms both mate and collect their food on the soil surface, and in both activities they usually adhere strongly to the burrow by keeping their tail ends firmly attached to the burrow (Fig. 8.3a; Nuutinen and Butt 1997, 2005). This allows a rapid escape below ground and helps to avoid the problems caused by being displaced from the burrow (Michiels et al. 2001). Signifying further the close bond to the burrow, dew worms display homing ability from considerable distance away from the burrow if they have left the burrow completely (Nuutinen and Butt 2005).
2. *Burrows and middens as nursery environments*. Dew worms lay their cocoons in their burrows, usually in the topsoil. Cocoons are typically found partly embedded in the walls of the burrows. However, recently Grigoropoulou et al. (2008) noticed in a laboratory experiment that dew worms can also dig short 'nursery' side burrows where cocoons are laid (Fig. 8.4a). As dew worms are thereby born into the parents burrow or its close vicinity, the likelihood of burrow inheritance is increased in case the burrow is vacated due to death or emigration of the resident individual. Indeed, when Grigoropoulou et al. (2008) had removed adult individuals from experimental boxes, they observed a juvenile worm taking up residence in a vacated burrow (see also Grigoropoulou et al. 2009). Further, middens may be important nurseries. Over 6 months Butt and Lowe (2007) followed in field conditions the abundance of juvenile dew worms in the middens and compared it to the numbers at inter-midden areas. In the combined data, there was a tendency for higher juvenile numbers in the middens (Fig. 8.4b).
3. *Deviations from burrow allometry*. Burrow allometry refers to the linear relationship between burrowing animals' mass and the cross sectional area of the burrows (White 2005). In dew worms, it is not unusual to obtain an individual with disproportionately smaller dimensions from within a burrow with adult dimensions (up to 10 mm in diam.) (Fig. 8.4c). Earthworms are not known to dig burrows wider than their body width, and individuals such as these are evidently either juveniles that have inherited a parental burrow or dispersing individuals that have found a vacant burrow and settled into it.
4. *Settlement preferences*. Relating to (3), in experimental conditions it has been observed that dew worms migrating on the soil surface preferably settle in pre-existing burrows and when such are available, they completely avoid digging a new burrow (Fig. 8.3b; Nuutinen and Butt 2005). If this is a rule in natural habitats too, it would be a factor promoting burrow inheritance and recycling.
5. *Permanence of midden pattern*. I have followed (1993–2009) the distribution of dew worm middens in one square metre quadrats at a forest site in the South-West of Finland. The summary data from one of the quadrats is provided in Fig. 8.5, where the midden numbers range from 15 to 26 middens per square metre (Fig. 8.5c). When all midden observations of the time period are marked on the same plane, it is obvious that their distribution is highly clustered (Fig. 8.5a). When one estimates L-function (Venables and Ripley 1994) for the pattern, it shows statistically significant clustering of points at 7–8 cm distance, which evidently signifies the permanence of the pattern (Fig. 8.5b).

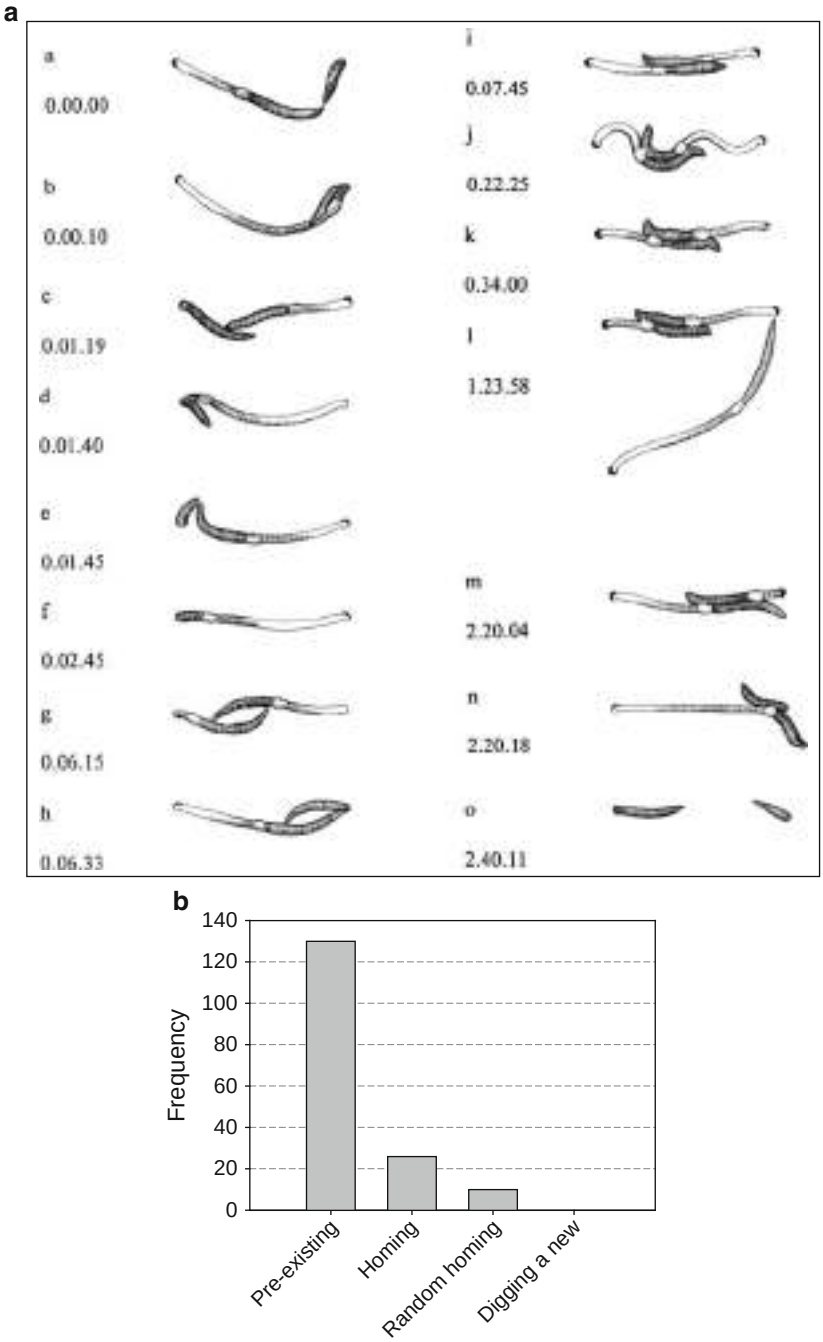

Fig. 8.3 Burrow adherence in *L. terrestris*. (**a**) Mating sequence of *L. terrestris*. Numbers refer to the time from the first contact of individuals. *Source*: Nuutinen and Butt 1997. Copyright Wiley-Blackwell/The Zoological Society of London. (**b**) Settlement preferences of migrating *L. terrestris*

The pattern is then significantly spaced out close to 18 cm distance, which can be taken to represent the inter-midden distance at this site. As the 16-year period of monitoring must considerably exceed the typical life span of the dew worms in the field, the findings suggest that the living sites, possible also the burrows, were passed over generations. It must be stressed, however, that the example given represented the clearest case of space-time clustering of middens and often such clustering is less obvious.

8.3.3 Implications

The above suggests that living site and burrow inheritance may be an important feature in dew worm ecology. It would have many implications, some of which are summarised in Fig. 8.6. Naturally, it would strongly influence the small scale spatial pattern of individual distributions. In natural habitats, the inheritance could promote population persistence as the established housing conditions might help to buffer the fluctuations in environmental conditions. An inheritance system could be a handicap in disturbed habitats, such as frequently tilled soils with repeated destruction of burrows and middens and the unavoidable displacement of juveniles from the nursery environments. This could be one reason for the often observed low numbers of dew worms in ploughed soils compared with direct drilled soils (Chan 2001). The persistence of midden positions would enhance the development of small scale soil heterogeneity and also have effects on plant communities (Milcu et al. 2006). Inheritance of burrows and the long lasting worm impacts on their walls would contribute to development of the distinct drilosphere features. Soil macropore dynamics could also be importantly affected. Darwin (1881, pp. 118–119) concluded that worm burrows must be continuously collapsing for the topsoil not to become a hollow, unsupported space over time. In dew worms, burrow inheritance may be a further factor contributing to a 'burrow balance' in soil. One remark made by Odling-Smee et al. (2003, p. 93) is that niche construction activities lead to the development of elaborate courtship, mating and parental behaviour. It has been pointed out that the evolution of such features could be understood with less complicated theoretical frameworks by simply asking, for instance, how a burrow-dwelling animal evolves in its environment (Wilson 2000). However, in dew worms, the recently discovered nursery burrows and the relatively elaborate pre-mating sequence with mutual burrow 'visiting' could be seen as cases in point.

Fig. 8.3 (Continued) in an experimental set-up. 'Pre-existing': settlement in a pre-existing burrow, different from the burrow from where the worm migrated; 'Homing': backing to its own burrow along the outward track; 'Random homing': apparently random settlement into its own burrow; 'Digging a new': digging of a new burrow and settling there. Data from Nuutinen and Butt (2005)

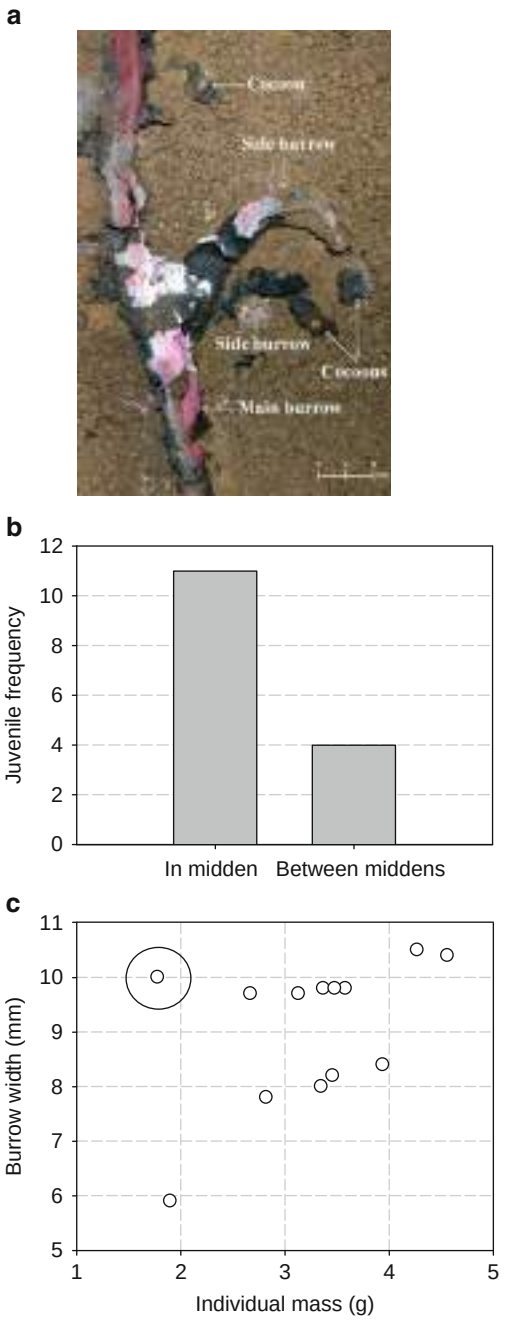

Fig. 8.4 *L. terrestris* burrows and middens as nursery environments. (**a**) *L. terrestris* main burrow (*left*) with side burrows containing cocoons at a depth of 25 cm in an Evans' box. *Source:* Grigoropoulou et al. (2008). Copyright Elsevier. (**b**) Frequency of juveniles observed in middens and in inter-midden areas in a 6-month field follow-up. Data from Butt and Lowe (2007). (**c**) Covariation of *L. terrestris* mass and burrow diameter in data set collected from arable clay in S-W Finland. *Circle* marks a small juvenile individual obtained from a wide burrow. *Source of data:* Nuutinen and Butt (unpublished)

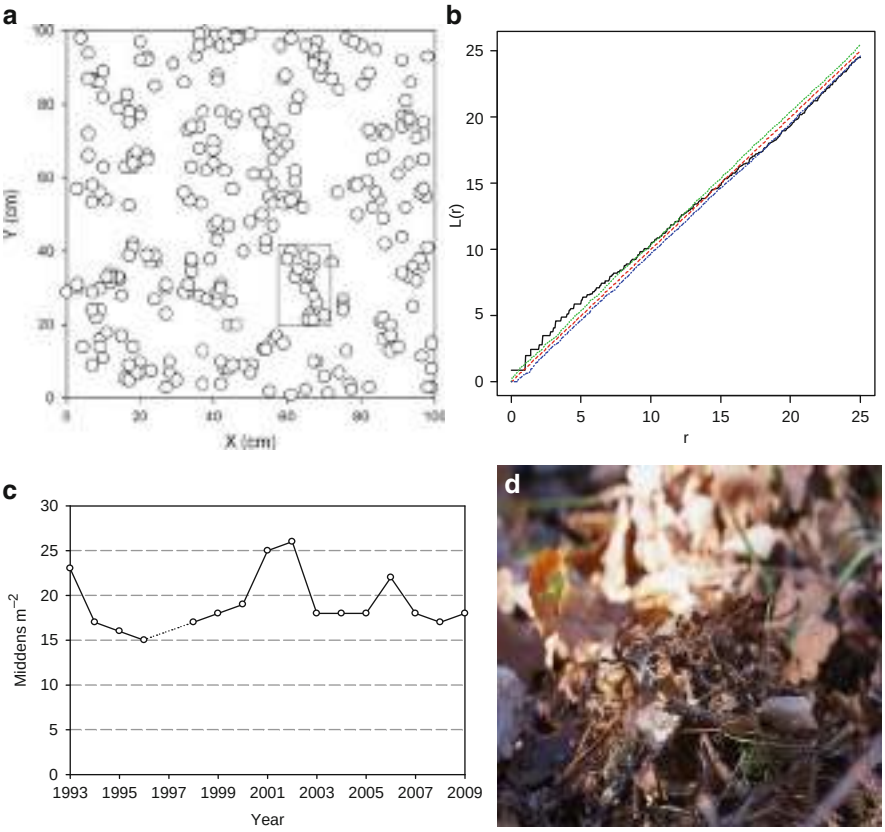

Fig. 8.5 Space-time clustering of *L. terrestris* midden positions in an one square meter study plot in Vaisakko forest, S-W Finland. Midden positions were mapped in spring/early summer over the period 1993–2009 (no monitoring in 1997). (**a**) All midden observations of the monitoring period overlaid. The *inserted box* marks an area where a midden was active at each time instance. (**b**) L-function for the pattern in (**a**) (see text for interpretation). (**c**) Midden density over the monitoring period. (d) The midden marked in (**a**) in the spring 2000 (photo: Risto Seppälä)
Source of data: Nuutinen (unpublished)

8.4 Conclusion

If the study of organism–environment interaction is one of the strengths of earthworm biology, an equally evident feature has been the shortage of evolutionarily oriented investigations. This may have been a deliberate choice and represent caution of researchers, for instance, in front of the onerous question of adaptation. A further reason might be the practical soil management agenda, which provides the motivation for, and also funds, a large proportion of earthworm research. Some importance may have the fact that the earliest phases of earthworm biology had, a little surprisingly, a similar 'non-evolutionary' quality. Quammen (2004) observed

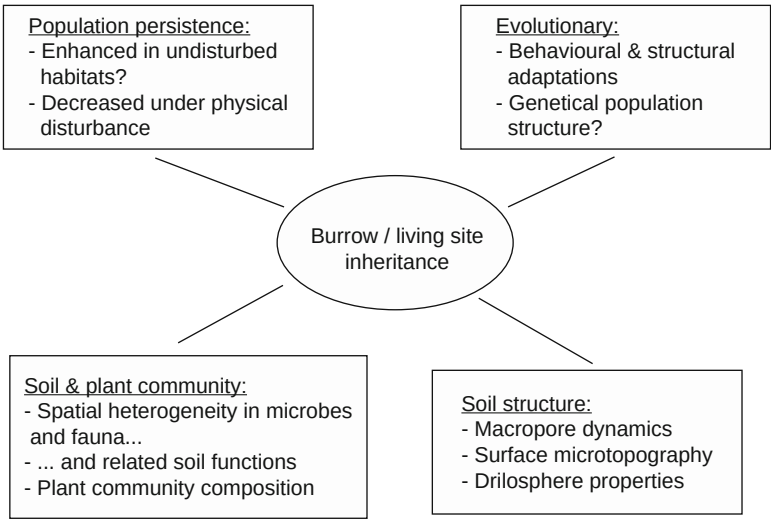

Fig. 8.6 Summary of possible implications of living site and burrow inheritance in the dew worm (not meant to be inclusive)

that Darwin did not mention 'natural selection' or 'evolution' a single time in his book on earthworms. In fact, he did mention 'evolution' once in the introduction of the book (Darwin 1881, p. 6), but in an instance not related to the evolution of earthworms in particular.

However, recently there has been an increase in the study of evolutionarily motivated questions in earthworm biology. Here I think particularly the investigations are on hermaphrodite sexual conflict, which has used common earthworm species such as *L. terrestris* and *Eisenia andrei* as model organisms (Michiels et al. 2001; Koene et al. 2005; Velando et al. 2008). These studies have produced new insights into the biology of earthworms and shown the potential of earthworms in testing hypothesis of general scientific interest. Whatever the eventual values of niche construction and the ecological inheritance concepts will turn out to be, they could presently provide a further useful framework for more evolutionarily conscious earthworm ecology. Niche construction is seen by many as an potential 'growth area' in ecology (e.g., Sherrat and Wilkinson 2009) and within soil science, too, the theory – together with ecosystem engineering and extended phenotype concepts – has obtained increasing interest (Corenblit et al. 2008; Phillips 2009). Considering that in niche construction theory, earthworms are seen almost as its archetypical examples, earthworm ecologists should be able to provide useful input in the discussion. In earthworm ecology, it is routine to estimate earthworm impacts by experimentally cancelling out or enhancing their influences. Without undervaluing the difficulties in designing experiments of feedback processes, the prospects for illuminating empirical studies should be good.

It is not uncommon to see earthworms being described as humble organisms (e.g., Desmond and Moore 1991, p. 628) and elsewhere it has been proposed that

'Blessed are the meek, for they shall inherit the earth' (Matthew 5:5). Be the latter as it may in the human realm, as relates to earthworms, the students of their ecology can take that literally. Over vast expanses of time, the countless generations of earthworms have been born into an environment moulded to varying degree by their ancestors. Identifying and quantifying its blessings could provide much further entertainment and information.

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Chapter 9
Earthworm Interactions with Soil Enzymes

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9.1 Introduction

Beginning with their inhabition of terrestrial ecosystems 600 million years ago, the earthworms have been considerably influenced by climate, soil characteristics, agricultural/industrial activities, and environmental pollution in terms of their population dynamics and biomass in soil. Numerous researches involving soil earthworms provided the information that they stimulate physical, chemical, and biological properties of soil and contribute to soil aeration and drainage through their vital activities such as feeding, burrowing, and casting. These activities have also important roles in transformations of minerals and plant nutrients to available and accessible forms for plant and soil microorganisms and thus improve soil fertility and quality. In this sense, interactions between earthworm activities especially microbiological characteristics of earthworm casts and soil enzymes, which are synthesized by soil microbial biomass and are important indicators of soil fertility, have received an increasing attention in soil science. This chapter considers the relationships between soil earthworms and enzymes in different extents. As a major representative of soil life, the earthworms are significantly influenced by conventional agricultural operations such as intensive tillage and use of chemical fertilizers. Therefore, a special emphasis has been given on the question of how agricultural practices govern the interactions between soil earthworm and enzyme activities.

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9.2 Soil Earthworms

Soil earthworms are the members of the subclass Oligochaeta in the phylum Annelida and are classified into three types, on the basis of their ecological environments: epigeic, endogeic, and anecic. Epigeic species lives above mineral soil layers near the soil surface. Endogeic species inhabit deeper layers (up to 0–20 cm) of soil profile, while anecic species (vertical burrowers) can open deep vertical galleries that may reach up to 1 m depth along the soil profile. Epigeic species function in the mineralization of plant surface residues as anecic species transport the decomposition products of this process to lower soil layers and also increase water infiltration and aeration (Edwards and Bohlen 1996; Karaca et al. 2010a).

Soil physicochemical features such as soil depth, texture, pH, and organic matter content strongly affect earthworm distribution and its activity in soil (Karaca et al. 2010a). For example, soils with coarse structure have better conditions for earthworm survival than those with sandy and clayey soil structure (Guild 1948). Nordström and Rundgren (1974) found that significant relationships were recorded between clay content and populations of *Aporrectodea caliginosa*, *Aporrectodea longa*, *Aporrectodea rosea*, and *Lumbricus terrestris* in grassland, agricultural, and forest soils in Sweden. Khalaf El-Duweini and Ghabbour (1965) found that the increase in sand and gravel content was the reason for decreasing *A. caliginosa* populations. In addition to its direct effect, soil structure has indirect effects on earthworm populations and activities. For example, soil moisture level is an important soil characteristic supporting earthworm population. However, reducing soil conditions occurred after intensive irrigations or rains in the soils with high rate of clay or poor drainage and also high rate of water percolation in sandy soils may have negative influences on earthworm survival. Another crucial factor effective upon the earthworm distribution is the soil depth. The earthworm life and activities in relation to soil depth can change depending on their ecological distribution. Anecic earthworm species are substantially affected from soil depth and densely inhabit lower layers of soil profile. Soil pH has also vital effect on earthworm life. They are mostly present and active between the pH range of 5.0 and 7.4 but their growth is substantially limited within the pH range of 3.5–4.5 and ceases under strongly acidic conditions below pH 3.5 (Satchell 1967). The earthworms feed on soil, organic matter, microorganisms, or mixtures containing at least two of these soil components, thereby the increase in earthworm population is correlated with increasing soil organic matter content, and earthworm addition to soil promotes agricultural–ecological sustainability (Araujo and Lopez-Hernandez 1999). Topography is not directly influential on earthworm distribution in soil (Rossi et al. 1997). However, tillage and plant residue applications to soil can alter their population dynamics (Hubbard et al. 1999).

The relationships between soil earthworms and attributes are reversible, which means earthworm activities may alter soil characteristics. For example, depending on their ecological groups, earthworms have a leading role in transport of organic matter through soil profile. Anecic earthworm species is responsible for downward

transportation of soil organic matter, while endogeic species inhabit near-surface soil and contribute mineralization processes of plant and animal residues (Tomlin et al. 1992). Therefore, inoculation of endogeic earthworm species to soil increases the mineralization of plant-originated organic materials and the nutrients liberated during this process are taken up by the plants. The earthworm activities also redound to formation of water stable aggregates (Marininssen and Hillenaar 1997). Earthworm excretion intensely consists of clay and silt particles and various organic constituents with the size of 210–500 μm giving a strong binding characteristic to the excretion and hence increasing aggregate stability (Chan and Heenan 1995). Soil aggregate stability is also related to the excrement age and increases with increasing excrement age (Decaens et al. 1999). Earthworm excrement reduces soil salinity, improves soil pore volume (Edwards 1998) and water infiltration, and increases the amount of total and available nutrients (Ruz-Jerez et al. 1992), and hence contributes to the crop yield (Haimi et al. 1992). Earthworm inoculation to soil planted with beech seedlings showed that stem and leaf biomasses were higher under earthworm inoculation than that in noninoculated condition. Under soybean growth, applying soil with earthworm excrement increased the rate of plant dry matter between 40 and 70% (Lui et al. 1991) and plant yield 36% (Pashanasi et al. 1996).

9.3 Soil Enzymes

Biochemical reactions occurring in the nature involves enzymes, which are known to be proteins and usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions. Enzymes serve a wide variety of functions inside living organisms and are extensively found in waters, plants, manure, and soil as well. The functionality and resilience of natural and managed ecosystems mainly rely on the metabolic abilities of microbial communities, which are mostly intracellular and extracellular enzyme synthesis mediating critical reactions such as decomposition of organic matter, cycling of nutrients, and breakdown of pollutants. Soils include both intra and extracellular enzymes that can be found as free forms in the soil solution, bound in microorganisms or immobilized extracellularly (McLaren 1975; Tabatabai and Dick 2002). Soil enzymes, like other enzymes in different environments, are affected from the factors such as pH, ion conditions, temperature, and various inhibitors (Burns 1978; Tabatabai and Dick 2002). Plants and microorganisms living in the soil are the sources of both intra and extracellular enzymes (Cashel and Freese 1964; Nishimura and Nomura 1959; Weimberg and Orton 1963, 1964; Estermann and McLaren 1961; Frankenberger and Tabatabai 1982). Moreover, physicochemical and biological soil characteristics (i.e., texture, organic matter content, cation exchange capacity, and microbial biomass) and various inhibitors (such as heavy metals and organic pollutants) have marker effects on soil enzyme productions and activities (Askin and Kizilkaya 2006; Karaca et al. 2010b; Turgay et al. 2010). Extracellular enzymes usually

participate in mineralization process of organic materials, and C, N, P, and S, cycles in soil and their activities are mainly governed by immobilization–stabilization mechanisms such as microencapsulation, cross-linking, copolymer formation, adsorption, entrapment, ion exchange, adsorption, cross-linking, and covalent attachment (Weetall 1975). These mechanisms and enzyme activities are basically controlled by clay type and proportion and also organic matter content of soil (Ladd and Butler 1975; Conrad 1940; Simonart et al. 1967; Ceccanti et al. 1978; Burns 1978; Tabatabai and Dick 2002). In addition, soil characteristics such as quality and quantity of organic matter (Yakupoglu et al. 2007), nutrient level (Kizilkaya et al. 2007a), soil depth, topography (Dengiz et al. 2007), and anthropogenic factors such as heavy metals (Kizilkaya and Askin 2002; Karaca et al. 2000, 2002, 2006, 2010b) and organic pollutants (Turgay et al. 2010) are closely related soil enzyme activities.

9.4 Relationships Between Soil Earthworms and Enzymes

The interactions between earthworms and enzymes in soil can be evaluated in three spatial scales. The microscale interactions occur in earthworm gut or intestine, burrow lining, or casts and contains the changes in enzyme activities pertaining to nutrient characteristics of foods consumed. The level of mesoscale is restricted to the shifts governed by microbial activities that occur in earthworm intestine, excretions, or in soil environment where earthworms survive, whereas macroscale interactions define the changes in soil physico-chemical characteristics due to earthworm activities, affected by agricultural and environmental factors.

9.4.1 Interactions in Microscale

Majority of the earthworms feed on soil and decaying plant remains, which are commonly known as soil organic matter. The consumption characteristics mainly depend upon their ecological levels (epigeic, endogeic, and anecic). Epigeic species (such as *Lumbricus rubellus*) feed mostly on absolute soil organic matter, whereas endogeic (*A. caliginosa*) and anecic (*L. terrestris* and *A. longa*) species prefer the composition of soil and organic matter in the soil (Doube et al. 1997). They can also feed on soil microorganisms such as fungi and bacteria (Schonholzer et al. 1999). Therefore, the composition of the materials and enzyme activities found in earthworm intestine or excrements change depending on their feeding characteristics. In earthworm surroundings, variations in the quantity of organic materials induce fluctuations in enzyme activities of earthworm excrements, which are also related to the type of organic matter and origin of enzymes (Fig. 9.1). In agricultural soils, it was observed that the additions of organic materials with different origins led to increases in extracellular enzymes such as urease, phosphatase, sulphatase, and β-glucosidase, whereas catalase activity involving metabolic activity of aerobic

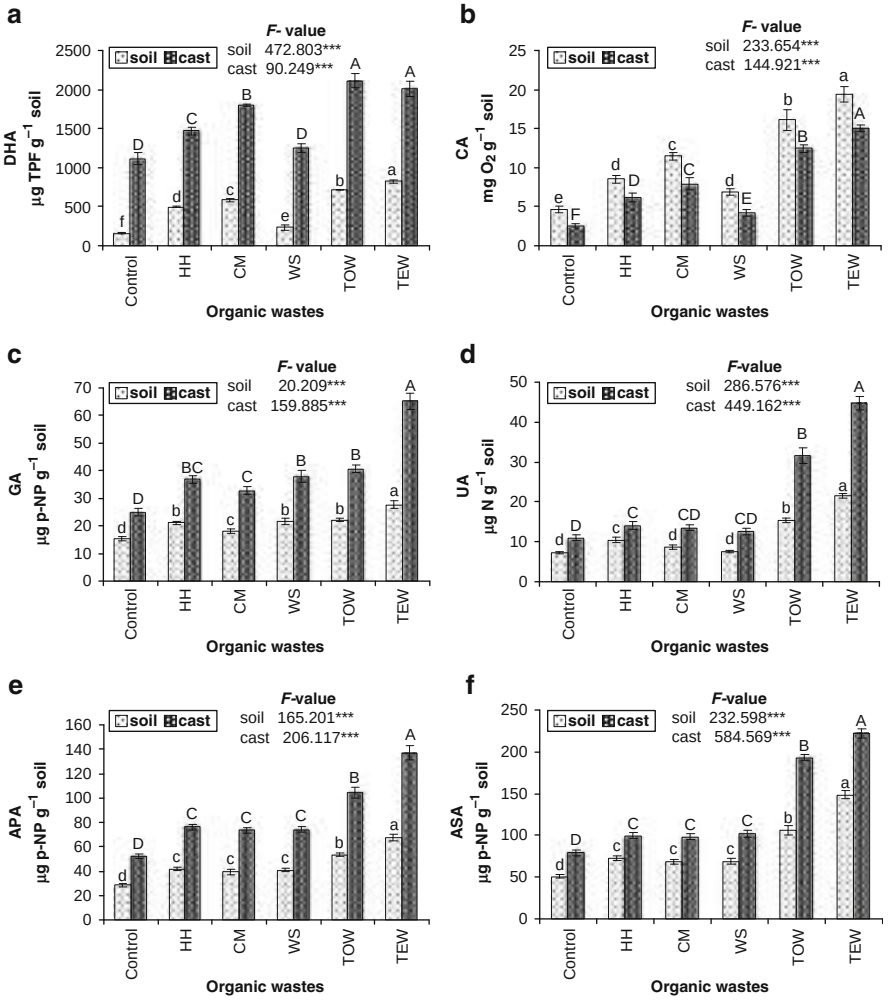

Fig. 9.1 Changes of dehydrogenase (**a**), catalase (**b**), β-glucosidase (**c**), urease (**d**), alkaline phosphatase (**e**) and arylsulphatase (**f**) activity in earthworm cast and surrounding soil. *HH* Hazelnut husk, *CM* Cow manure, *WS* Wheat straw, *TOW* Tobacco production waste, *TEW* Tea production waste (Kizilkaya and Hepsen 2007)

microorganisms decreased in earthworm excrements (Kizilkaya and Hepsen 2007). Moreover chemical composition and C/N ratio of organic materials consumed by earthworms may alter enzyme activities and organic substances with narrow C/N ratios may have more pronounced effect on soil enzyme activities (Zhang et al. 2000; Goyal et al. 2005).

The effect of organic substances on soil enzyme activities is actually related to the fact that organic compounds are substrate sources required by soil microorganisms synthesizing soil enzymes. In the soils with no organic amendments, the earthworms

using available organic materials and microbial biomass exhibited higher enzyme activities in their excrements and burrow walls (Satchell and Martin 1984; Tiwari et al. 1989; Mulongoy and Bedoret 1989; Kizilkaya and Hepsen 2004; Kizilkaya 2008) and also had higher organic C, nutrient content, and microbial populations (Sharpley and Syers 1976; Shaw and Pawluk 1986; Scheu 1987; Daniel and Anderson 1992; Parkin and Berry 1994; Wolter and Scheu 1999; Kizilkaya 2004, 2005). In natural environments, the sources of the enzymes that were measured to be higher in earthworm excrements and burrow walls than that found in feeding materials can be substantially related to the discharge of the enzymes synthesized for intracellular metabolic activities of earthworms and their contamination to earthworm galleries. Furthermore, enzymes are bonded soil organic matter via ionic binding mechanisms resulting enzyme–organic matter complexes (Butler and Ladd 1969). Earthworm excrements densely contain organic materials and their enzymes complex. Earthworms influence both enzyme activities and their kinetic characteristics. (Ekberli and Kizilkaya 2006, Table 9.1). The changes in enzyme kinetics are mainly related to enzyme–substrate affinity formed by the changes in substrate concentration of earthworm excrement.

It has been demonstrated that kinetic and thermodynamic parameters of enzymes could be estimated by substrate concentration, incubation period, and temperature, and substrate concentration has a major effect on the level of enzyme–substrate complex, production rate formed from this complex, and also the return to initial condition (Ekberli et al. 2006; Kizilkaya et al. 2007b; Kizilkaya and Ekberli 2008).

9.4.2 Interactions in Mesoscale

Depending on their feeding characteristics, earthworms can take advantages of microbial populations as well as they ingest soil and organic materials (Schonholzer et al. 1999). This brings microbial community shifts in soil and earthworm digestive system or excrements. In a bacteria/fungi-rich soil, it was found that an anecic species, *L. terrrestris*, fed on bacteria and fungi, and their digestion was fairly lower in earthworm intestine system (Wolter and Scheu 1999). However, the role of soil microbial populations on earthworm feeding ecology is not yet fully understood (Brown et al. 2000). Some enzymes isolated from earthworm intestines were proved to digest miscellaneous organic materials together with various components of soil microflora such as bacteria, fungi, and protozoa, while certain other soil

Table 9.1 Kinetic parameters (V_{max}, K_m, and V_{max}/K_m) of catalase activity in earthworm *L. terrestris* casts, surrounding soil and control (Ekberli and Kizilkaya 2006)

	V_{max} (ml) O_2 (g^{-1} min^{-1})	K_m (ml) O_2 (g^{-1})	V_{max}/K_m (min^{-1})
Control soil	10.25	14.305	0.72
Surrounding soil	11.68	15.547	0.75
Earthworm cast	8.76	12.033	0.73

microorganisms cannot be digested by the earthworms (Brown et al. 2000). The digestivity of microbial populations in earthworm intestine system changes depending on the factors such as structure and biomass of microbial communities, ecological levels of earthworms, and their surrounding environments. It was reported that soil fungal populations existed in feeding environment of *Pontoscolex corethrurus* were also appeared in the excrement of this species without any digestive process (Reddell and Spain 1991a, b), whereas fungal spores in the excrement of *Ulocladium botrytis* had a lower germination rate (Striganova et al. 1989), and the fungi passing through intestinal system of *Pithomyces chartarum* were completely digested and not germinated in the excrement (Keogh and Christensen 1976). In addition, earthworm intestinal microorganisms can die due to the existence of toxin and antibiotic synthesizing fungi, such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., in the feeding environment of earthworms (Morgan 1988). Similar observations were reported for also bacteria and algae (Stamatiadis et al. 1994; Schmidt et al. 1997). Consequently, microorganisms that are not digested in the intestinal system of the earthworm manage to access to the excrement rich in organic matter (Scheu 1991; Schmidt et al. 1999; Kizilkaya and Hepsen 2004). Earthworm excrements and burrows also contain mucus formed by intestinal fluids with higher C and N contents and lower C/N ratio (Scheu 1991; Schmidt et al. 1999; Kizilkaya and Hepsen 2004). For that reason, microbial populations and their activities in the earthworm excrements and burrow walls are usually higher than that in the surrounding soil (Edwards and Bohlen 1996; Tiunov and Scheu 1999). In addition to microbial enumeration techniques, microbial biomass and respiration measurements can be used for indirect evaluation of microbial populations living in earthworm excrements (Scheu and Parkinson 1994). Total microbial biomass of earthworm excrement and its activity was found to be higher than that of the feeding material (Scheu and Parkinson 1994). This also depends on ecological level of the earthworms and organic materials found in their feeding environments (Ekberli İ and Kizilkaya 2005, Fig. 9.2).

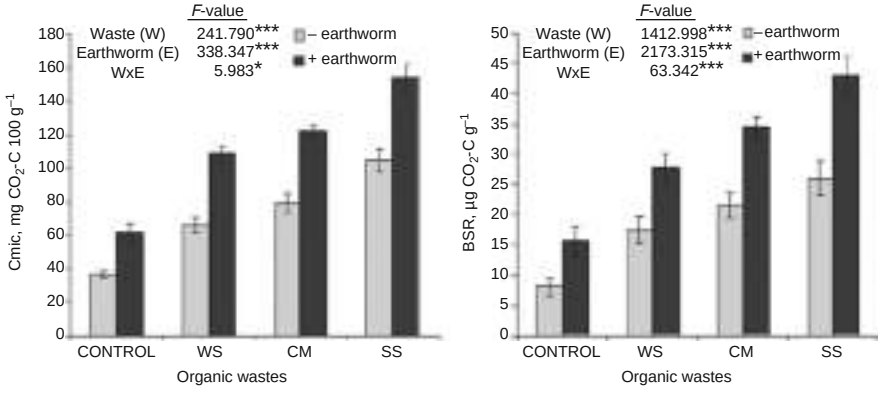

Fig. 9.2 Changes of microbial biomass C (Cmic) and basal soil respiration (BSR) in various organic wastes and earthworm *L. terrestris* L. added soil (Ekberli İ and Kizilkaya 2005)

The sources of intracellular and extracellular enzymes are plant roots and soil microorganisms. Previous researches showed that *Bacillus subtilis* synthesized ribonuclease and alkaline phosphatase (Cashel and Freese 1964; Nishimura and Nomura 1959), and extracellular acid phosphatase and pyrophosphatase were synthesized through cell wall surfaces of *Saccharomyces mellis* (Weimberg and Orton 1963, 1964) meaning the higher the microbial biomass, the higher the enzyme activities, and therefore higher microbial biomass content of earthworm excrements and burrow walls is the reason for higher enzymatic activity.

9.4.3 Interactions in Macroscale

Soil physico-chemical characteristics and especially organic matter content have an important influence on earthworm populations, activities, and their enzyme activities. The organic materials applied for conditioning soil characteristics and increasing plant yield also increase earthworm populations (Killham 1994; Lavelle and Spain 2001) and their enzyme activities (Kizilkaya and Hepsen 2007) independently. In contrast, it has been reported that soil physical characteristics such as aggregation infiltration and porosity were shown to be improved by earthworm activities (Springett 1983; Aina 1984; Kladivko et al. 1986; Joschko et al. 1989; Kooistra 1991) and their excrements remained increased total and available nutrient concentrations in soil (Kizilkaya 2004, 2005, 2008; Kizilkaya and Hepsen 2004, 2007) and depending on these changes, plant root network develops better, soil microbial colonies gain better conditions to be active and proliferate, and finally enzymes produced through plant roots and microbial biomass increase in soil (Crawford et al. 1993; Tabatabai and Dick 2002; Mawdsley and Burns 1994; Naseby and Lynch 2002).

9.4.4 Effects of Agricultural Activities on Earthworm–Enzyme Interactions

Primary agricultural practices such as tillage, pesticide, and fertilization applications have a deep impact on populations and activities of earthworms and enzymes in soil. For example, soil tillage affects earthworm communities building their galleries and burrows in deeper soil layers. When performed once a year, the effect of tillage on earthworm populations was found to be less destructive than that of birds feeding on soil earthworms. However, intensive and frequent tillage activities were found to reduce earthworm populations more severely, while no-till management systems promoted their increase (Edwards and Lofty 1982a; Edwards and Bohlen 1996; Chan 2001; Johnson-Maynard et al. 2007). Seedbed tillage forms aerobic conditions and this stimulates microbial populations causing increasing

enzyme activity and production and eventually mineralization of soil organic matter. However, intensive field traffic brings soil compaction reducing enzyme activities (Kandeler et al. 1999; Mijangos et al. 2006; Jin et al. 2009). Consequently, two important outcomes of soil tillage are general augmentation in soil enzyme activities and reduction in earthworm populations, but then intensive tillage activities have negative impact on both earthworm and enzyme activities in soil.

Fertilizers that are used to address specific nutrient requirements and thus to raise the yield obtained from unit area can be either organic or inorganic. Applications of solid materials obtained from plant and animal originated residues were reported to increase earthworm populations several folds (Leroy et al. 2007, 2008). However, fluid components of livestock wastes may result in a reduction in earthworm populations due to high salt and ammonia content when applied without composting. Most traditional chemical fertilizers influence earthworms indirectly. They generally result in an increase in plant yield, which means an increase in the penetration of organic matter through plant residues that remained in the field after harvest and thus increasing the earthworm populations. Earthworms are very sensitive to ammonia. Therefore, intensive uses of ammonia-based fertilizers often have adverse effects on earthworm populations in agricultural soils (Edwards and Lofty 1982b). The applications of organic fertilizers originated from plant and animal residues were also found to increase soil enzyme activities (Ros et al. 2006). However, fluid livestock fertilizers with higher salt and ammonia contents have no negative effect on soil enzyme activities. Similarly, an ammonia-based fertilizer stimulates soil enzyme activities. This is generally ascribed to the contributory effects of nutrients contained by these fertilizers to the plant development and the microbial biomass proliferation, increasing also enzyme synthesis (Jordan et al. 2003).

The sewage sludges that are used as alternative soil conditioners or organic-nutrient source may also effect earthworm populations and soil enzyme activities (Benitez et al. 1999; Le Bayon and Binet 2006). The extent of this impact changes depending upon the ecological level of the earthworm, the type of enzyme, and the rate of sewage sludge applied. Kizilkaya et al. (2009) noted that when their feeding environment contained 50% or more sewage sludge, the species *Eisenia fetida* was not able to survive because of high concentrations of soluble salts and ammonia. The shifts that are caused from sewage sludge applications in soil enzyme activities are mostly related to soil and sewage sludge characteristics. Some authors reported stimulated soil enzyme activities by sewage sludge applications (Sastre et al. 1996; Banerjee et al. 1997), whereas some others expressed the opposite view, noting that sewage sludge applications inhibited soil enzymes (Knight et al. 1997). These contradictory results in the literature are probably due to the variations in application rates and chemical characteristics of sewage sludge such as C/N ratio and heavy metal content (Tam and Wong 1990). Sewage sludge application to soil was shown to stimulate soil enzyme activity initially but, in the long term, this effect shifted into significant declines enzyme production, concurrent with depletion of soil microbial activity (Kizilkaya and Bayrakli 2005). Similarly, enzyme activity levels of earthworm excrements and burrow walls showed noticeable shifts depending on the rates of sewage sludge applied (Fig. 9.3). Kizilkaya and Hepsen (2004)

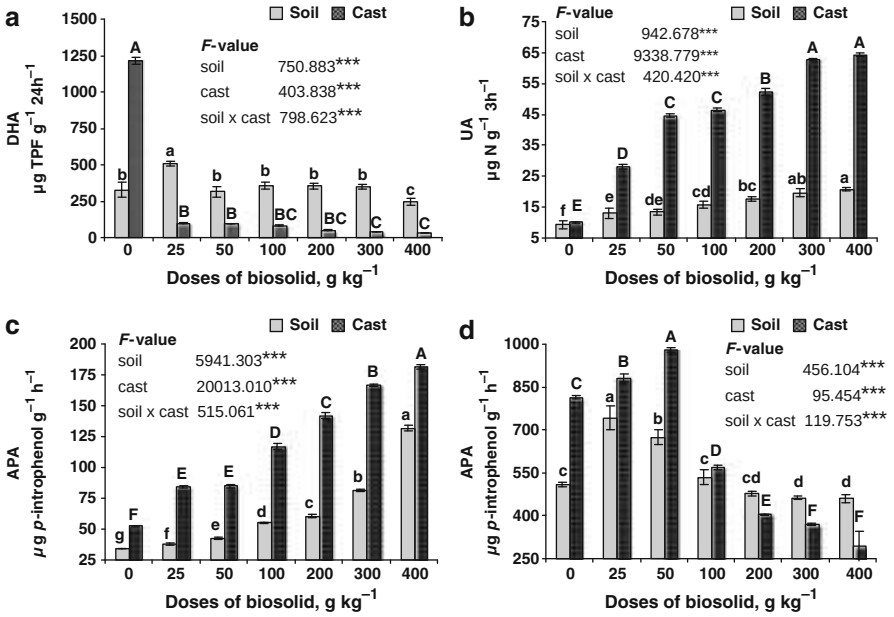

Fig. 9.3 Enzyme activity changes in the excrement of *L. terrestris* and soil under sewage sludge treatments (**a**) *DHA* dehydrogenase activity; (**b**) *UA* urease activity; (**c**) *APA* alkaline phosphatase activity; (**d**) *ASA* arylsulphatase activity (Kizilkaya and Hepsen 2004)

reported that enzyme activities of earthworm excrement were found to be higher than those of control soil (with no sewage sludge amendment). On the other hand, in sewage sludge amended conditions, intracellular dehydrogenase activity of the excrement was lower, whereas urease and alkaline phosphatase activities were higher in earthworm excrement depending on the sewage sludge dose applied to soil. The extracellular enzymes such as aryl-sulphatase were higher under lower application rates and measured to decrease with increasing doses of sewage sludge (Fig. 9.3).

Soil biological systems are negatively influenced by heavy metals (Bååth 1989; Flieβbach et al. 1994; Giller et al. 1998) and in most cases increasing metal concentrations brings about considerable decreases in soil biomass and their enzyme activities (Doelman and Haanstra 1979; Haanstra and Doelman 1991; Kizilkaya et al. 2004; Karaca et al. 2010b). The enzyme activities in soil and earthworm excrements are also influenced from heavy metals that are accumulated in feeding environment of earthworms over natural and anthropogenic processes (Kizilkaya 2008). The natural soil organic matter and adding organic materials (municipal wastes example) to agricultural soils augments both earthworm populations and enzyme activities in their excrement (Killham 1994; Lavelle and Spain 2001; Kizilkaya and Hepsen 2007). However, human originated organic waste products contain many different heavy metals and therefore may inhibit excrement enzyme activities depending on their concentrations (Fig. 9.4) (Kizilkaya 2008).

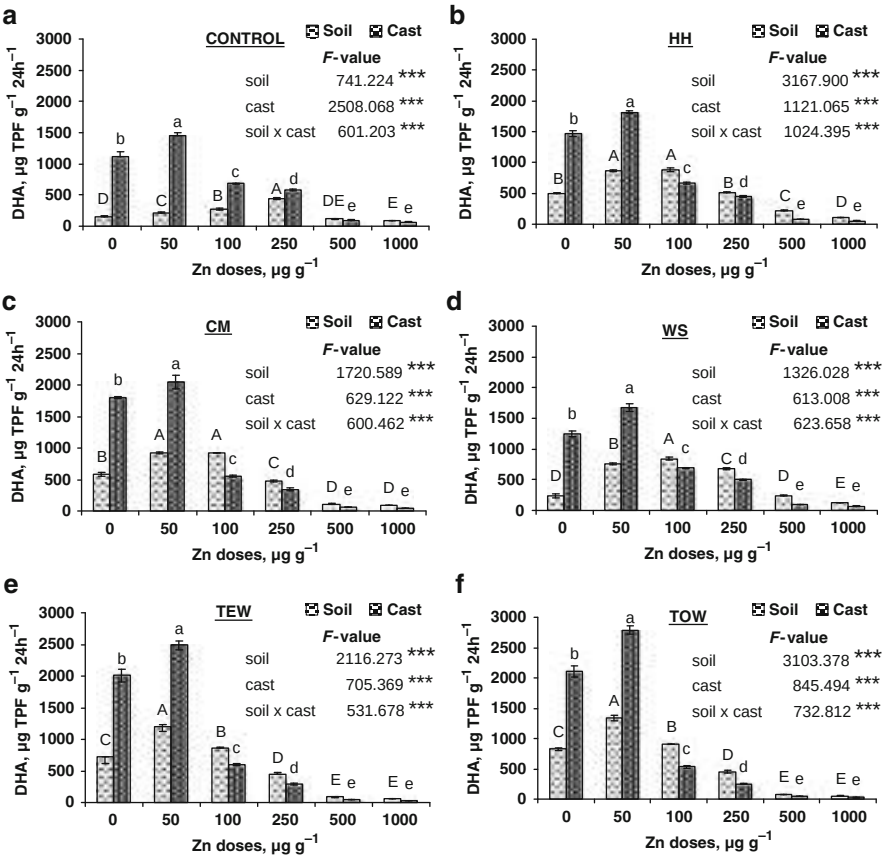

Fig. 9.4 Effect of different organic amendments and increasing levels of Zn on enzyme activities of soil and the excrements of *L. terrestris*. (**a**) CONTROL (nontreated organic wastes); (**b**) *HH* hazelnut husk; (**c**) *CM* cow manure; (**d**) *WS* wheat straw; (**e**) *TEW* tea production waste; (**f**) *TOW* tobacco production waste (Kizilkaya 2008)

Intensive pesticide use is one of the fundamental concerns in developing agricultural countries. The effect of pesticides on earthworm populations is associated with the type of pesticide used (Beiderbeck et al. 1987; Heimbach 1992; Bauer and Römbke 1997). Although lower concentrations of herbicides showed no adverse effect on earthworms, triazine herbicides were found to have a week toxic effect on earthworm survival (REF). The influences of herbicides on earthworms appear indirectly through altering their feeding behaviors and the mineralization of organic materials in soil (Edwards and Thompson 1973). Most fungicides except those including carbamates are toxic to the earthworms. Moreover, insecticides with carbamates, such as carbaryl, carbofuran, and methiocarb, and with organophosphate, such as phorate and also avermectins, are toxic to earthworms, which result in significant depletion in earthworm populations as they penetrate the soil (Edwards

1984). On the other hand, earthworms are capable of bio-accumulating pesticide residues and reduce their concentration in soil (Tarrant et al. 1997).

The shifts that are caused from pesticide applications in enzyme activities are also related to field and incubation conditions (Heimbach 1997). The use of pesticides for plant protection provides increases in yield and plant biomass, which eventually results in advancing enzyme synthesis in the rhizosphere but interactions between pesticides and soil enzyme activities are different in the absence. In such environments, soil microorganisms are adversely affected from pesticides and their residues and decrease their enzyme synthesis. In contrast, microbial populations and their enzyme activities may be expected to be stimulated as the pesticides spread in soil environment are used as carbon and energy source (Sylvestre and Fournier 1979; Niemi et al. 2009). In some cases, pesticides can inhibit soil enzyme activities directly. This inhibitory effect is probably relevant to the fact that some pesticide molecules having similar structure and magnitude with enzyme substrates can be bind to active region of enzymes. Despite the enzyme compartments staying stable, these enzyme–pesticide formations do not behave like regular enzyme–substrate complexes, hamper production formation, and hence cause activity losses. Consequently, pesticides may have either highly toxic or no effect on soil earthworms. This is most probably due to complex interactions between pesticide and soil characteristics, human activities, and environmental factors complicating the evaluation of pesticide effects on earthworm–enzyme interactions.

9.5 Conclusion

The earthworm life in soil is closely related to environmental factors and soil characteristics. They can stimulate microbial activities, nutrient traffic, and physico-chemical characteristics of soil and eventually provide sustainable fertility conditions over their vital activities in soil. The most important contributions of earthworms to soil structure are increasing aeration and water infiltration provided by earthworm burrows and galleries and increasing soil aggregation through their digestive activities and discharges. As a general result of these physical and chemical processes in soil conditions, plant root growth, soil microbial biomass, and activity are also improved well. Soil enzymes originated mostly from plant roots and microbial biomass are more abundantly found in excrements and burrow walls of the earthworms than in the surrounding soil. The stimulatory effect of earthworms on soil enzyme activities can be mostly evaluated through extracellular enzymes such as urease, phosphatase, sulphatase, and β-glucosidase. The intracellular enzyme activities such as dehydrogenase and catalase may show different trends (increases or decreases) depending on the species and ecological level of the earthworm. The increases that especially occur in extracellular enzymes and are derived from earthworm activities are associated with the type and quantity of organic material preferred by the earth worm; enzyme inhibitors such as heavy

metals and also agricultural management practices. Consequently, soil enzyme activities are promoted by earthworm biomass and activities, which will ensure the occurrence of more accurate and rapid biochemical processes governing C, N, S, and P mineralisation and cycles in soil. This information should mean that earthworms have a significant role in agricultural management of plant and animal wastes because of their fundamental contributions to soil biochemical processes.

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Chapter 10
The Impact of Cultivation Techniques on Earthworm Populations

Avril Rothwell, Keith Chaney, and Pat Haydock

10.1 Introduction

Earthworms have been referred to as "ecosystem engineers" because they influence soil stability through the formation and turnover of soil aggregates, mixing of soil horizons, the burial of above-ground litter (Lavelle 1988; Pulleman et al. 2005) and deposition of casts on and within the soil (Hindell et al. 1994). Agricultural production systems have a profound effect on earthworm populations, because mechanical disturbance changes the environment in which they live. These effects include changes in soil temperature, moisture content, soil organic matter content and the availability of food. Earthworms have been suggested as potential indicators of the sustainability of agricultural practises that a farmer might use thereby optimising different farming systems. The main reasons for choosing earthworms as bioindicators of soil health is because of the intrinsic role they play in the soil, the availability of standardised sampling methods and their sensitivity to changes in their environment (Römbke et al. 2005). This chapter will review the effects of conservation tillage and conventional tillage on earthworm populations. Favourable crop management practises which are conducive to increased growth of earthworm populations in under-populated soils will also be referred to, such as crop rotations and crop residue management practises.

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10.2 The Effects of Cultivation and Cropping on Earthworms

The primary aims of cultivation are to provide a suitable seedbed for crop germination and growth, whilst suppressing weeds, controlling soil erosion and maintaining adequate soil moisture. The principal mechanical processes associated with cultivation processes include one or more of the following effects on the soil: bursting, compaction, disintegration, cutting, inversion, mixing and movement. These processes have an immediate impact on earthworms and their habitat. Cultivation by mechanical disturbance completely changes the environment in which the earthworms live, altering the habitat, changing the soil temperature, moisture, availability of food and exposing them to predation. Numerous research has shown that the severity of these effects is dependent on whether it is conventional tillage, utilising the mouldboard plough (MP), or non-inversion tillage (NIT), utilising discs or tines through to zero tillage or direct drilling (e.g. Clapperton et al. 1997; Maillard and Cuendet 1997; Haukka 1998; Emmerling 2001; Hutcheon et al. 2001; Rothwell et al. 2005; Johnson-Maynard et al. 2007). Köller (2003) defines conventional tillage as all tillage types that leave less than 15% of crop residues on the soil surface after planting the proceeding crop. It involves inversion of the soil and to a depth that can vary from shallow ploughing (8–10 cm) to deep ploughing (25 cm) and is in the UK usually to a least 15 cm. The MP inverts the soil by cutting, lifting and turning the furrow slice, burying soil residues to depths of 20–25 cm. Ploughing is then followed by secondary operations such as discing, harrowing and rolling to prepare the seed-bed (Rasmussen 1999). Conventional tillage followed by secondary operations is still the preferred option for many farmers because it has been extensively researched and is perceived as a low risk, tried and tested method. However, research has shown that conventional tillage can result in soil compaction, erosion, reduced water percolation through the soil and has high energy and time requirements.

The increasing awareness of soil health issues has resulted in increased interest in sustainable soil management systems such as conservation tillage. European farmer's interest in the technique has been fluctuating over time, farmers in the UK and Scandinavian countries appear to be pioneering the technique (Lahmar 2008). Conservation tillage as part of conservation agriculture practises (Fig. 10.1) encompasses a wide range of cultivation techniques appropriate to the soil type, crop and weather conditions, including non-inversion, reduced and minimum tillage.

The essential requirement of conservation tillage is that the soil is not inverted. A NIT implement (composed of discs and tines) is designed to lift and loosen the soil and mix residues within the top 5–7 cm of the soil profile. Depending on the working depth and intensity, more or less residues of the previous crop or cover crops remain on the soil surface. Conservation tillage has been defined as any tillage and planting system that leaves 30% or more of the soil surface covered with crop residues after planting (Stinner and House 1989; Köller 2003). It is less intensive and less destructive than ploughing. The major benefits of NIT include improved seedbeds,

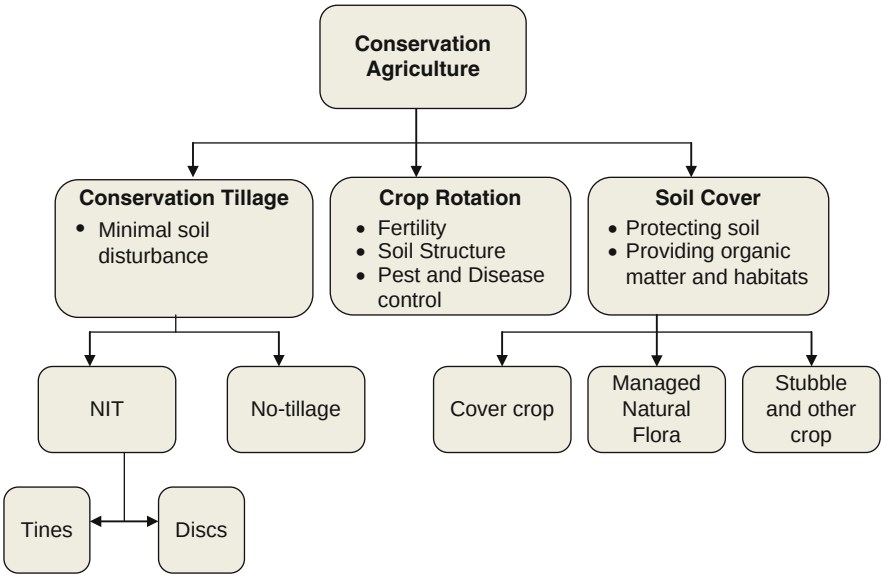

Fig. 10.1 The components of conservation agriculture [adapted from Jones et al. (2006)]

reduction in fixed and variable costs, increase in soil organic matter and aggregate stability, improved soil drainage and increased biodiversity. Provided there is a stable soil structure, soil type is suitable and weather conditions are favourable, then NIT can be adopted. NIT is not suited to all soil types and is most suited to clays, medium loams, chalks and limestone soils (Davies 1988). Conservation tillage processes (such as NIT) in particular have been shown to have a beneficial effect on earthworm populations, in comparison to mouldboard ploughing.

10.3 Earthworm Populations in Cultivated Soil

Earthworms constitute a major proportion of the macrofauna biomass in arable soils (Lee 1985; Edwards and Bohlen 1996) and have a pivotal role to play in maintaining soil health. They may be used as bioindicators of arable land management practises (Suthar 2009). Bailey et al. (1999) examined the value of earthworms through the relative costs and productivity returns of two arable systems, one based on ploughing and seeding, the other on direct drilling. Comparing the relative populations of earthworms, they arrived at a value of between £0.08 and £0.48 per kilo of earthworms. At a minimum earthworm biomass of 125 kg ha^{-1}, this would be equivalent to between £10 and £60 per hectare per year. The intensification of tillage production systems worldwide has resulted in reduced earthworm populations due to the direct and indirect effects of cultivation techniques, primarily removal of soil organic matter, physical disturbance and direct damage.

Earthworms are divided into three ecological categories: epigeics, endogeics and anecics (Bouché 1977). These categories take account of the basic biological and ecological characteristics of earthworms such as burrowing abilities, food preferences, body colour, size and shape (Lavelle 1988). Earthworm nomenclature in this chapter follows Sims and Gerard (1999) and Csuzdi and Zicsi (2003). Abbreviated names of species which are referred to in this chapter and their full names are listed in Table 10.1. The ecological categories of earthworms and some of their characteristics are summarised in Table 10.2.

The earthworm community found in a cultivated soil is the result of many past and present effects and rarely comprises of more than 8–10 species. There are various earthworm associations in agricultural land, varying from 4 to 8 species with biomass ranging from 50 to 120 g m^{-2} with *Allolobophora* species being dominant. *Allolobophora* species are commonly associated with *Lumbricus terrestris* and *Lumbricus rubellus* (Lee 1985).

The actual impact of cultivation of the soil by mechanical disturbance on the earthworm's habitat depends on soil conditions, climatic conditions as well as tillage operations (Edwards and Lofty 1972). Curry et al. (2002) showed that intensive cultivation can reduce earthworm populations in arable fields. Earthworm populations were reduced from 319 individual's m^{-2} and 55 gm^{-2} in a wheat–clover plot to 40–82 individuals and 4–19 gm^{-2} following potato cultivations. Boström (1995) stated that rotary cultivation killed 61–68% of earthworm biomass. Ploughing decreased that population further by 12–19% in ploughed and undisturbed ley.

Earthworms are favoured by conservation tillage and direct drilling compared with conventional methods of cultivation (Table 10.3). However, it is well documented that it takes several years for NIT to enhance the soil environment and

Table 10.1 Earthworm nomenclature and abbreviations

Abbreviation	Earthworm species
A. chlorotica	*Allolobophora chlorotica* (Savigny 1826)
A. caliginosa	*Aporrectodea caliginosa* (Savigny 1826)
A. longa	*Aporrectodea longa* (Ude 1885)
A. nocturna	*Aporrectodea nocturna* (Evans 1946)
A. rosea	*Aporrectodea rosea* (Savigny 1826)
A. trapezoides	*Aporrectodea trapezoides* (Reynolds 1995)
D. octaedra	*Dendrobaena octaedra* (Tetry 1937)
E. hortensis	*Eisenia hortensis* (Gates 1968)
E. tetraedra	*Eiseniella tetraedra* (Michaelsen 1900)
L. castaneus	*Lumbricus castaneus* (Savigny 1826)
L. festivus	*Lumbricus festivus* (Savigny 1826)
L. rubellus	*Lumbricus rubellus* Hoffmeister 1843
L. terrestris	*Lumbricus terrestris* Linnaeus 1758
M. minuscula	*Murchieona minuscula* (Rosa 1906)
O. cyaneum	*Octolasion cyaneum* (Savigny 1826)
O. lacteum	*Octolasion lacteum* (Örley 1881)
O. tyrtaeum tyrtaeum	*Octolasion tyrtaeum tyrtaeum* (Savigny 1826)
S. mammalis	*Satchellius mammalis* (Savigny 1826)

Table 10.2 The ecological categories of earthworms and some of their principal characteristics

Characteristics	Ecological categories of earthworms (Bouché 1977)		
	Epigeics	Endogeics	Anecics
Location on/within the soil	Litter or surface dwellers	Mineral soil dwellers (uppermost 10–15 cm of soil profile) (Paoletti 1999)	Deep burrowers (burrow to a depth of 1–3 m) (Sims and Gerard 1999)
Burrowing strategy	No defined burrowing system	Horizontal non-permanent burrows	Vertical semi-permanent burrows
Feeding strategy	Consume decaying plant residues on the soil surface (Curry 1994)	Consume organic material dispersed in mineral soils (Curry 1994; Shuster and Edwards 2003)	They are surface browsers that come to the surface at night-time and draw litter down into lower soil layers (Paoletti 1999)
Examples	*L. rubellus*, *L. castaneus*, *D. octaedra* and *S. mammalis*	*A. chlorotica*, *A. caliginosa* and *A. rosea*	*L. terrestris* and *A. longa*
Effect of cultivation techniques on the ecological categories of earthworms	Intensive agriculture has a negative impact on this ecological group due to the removal of organic matter (Fragoso et al. 1997)	Ploughing usually shifts the earthworm community towards the shallow burrowing (endogeic) species (Edwards and Lofty 1982; Haukka 1998; Nuutinen 1992; Pitkänen and Nuutinen 1998; Ernst and Emmerling 2009). In arable fields, *A. chlorotica* and *A. caliginosa* are often the dominant species (Edwards and Bohlen 1996). *A. chlorotica* becomes dominant, *A. caliginosa* remains numerous and *A. rosea*, *O. cyaneum* and *L. castaneus* persist (Edwards 1983)	These species require a supply of surface litter and have relatively permanent burrows. These are the species most adversely affected by repeated soil disturbance. Density of anecic earthworms decreases with conventional ploughing (Ernst and Emmerling 2009). Cultivation destroys the continuity of their burrows by cutting them off at plough depth. The low reproductive potential and long life-cycle of a species such as *L. terrestris* means that it is not able to rapidly compensate for high mortality rates

Table 10.3 Research showing the positive effect of conservation tillage versus conventional tillage on earthworm populations

Country	Cultivation techniques	Years established	Effect of cultivation techniques	Crop rotations	Sampling method	Species	Reference
Canada	Conventional tillage using a heavy duty cultivator and zero tillage	25	Significantly more earthworms in zero tillage than conventional tillage	Wheat-fallow rotation	SC	*A. caliginosa*	Clapperton et al. (1997)
Denmark	Direct drilling; ploughing	3	Significant positive effect of direct drilling compared with ploughing. The positive effect was most pronounced on *L. rubellus* and *L. terrestris*, increased by 66 and 9 times respectively	Cereals	EE	*A. caliginosa* *A. chlorotica* *L. terrestris* *A. rosea*	Andersen (1987)
Finland	Ploughing; spring harrowing; autumn harrowing; rotary cultivation; tillage with cultivator and zero tillage	19	Ploughing reduced earthworm populations probably due to removal of surface residues	Barley–oats–wheat	FE	Varied with soil type	Haukka (1998)
France	Mouldboard ploughing (20–25 cm); deep cultivation with a chisel plough (25–30 cm); shallow cultivation with a cultivator (10–15 cm); minimum tillage with a rotary harrow (7–10 cm)	20	Significantly more earthworms in min-tilled plots than mouldboard plots	Winter wheat, rape, winter wheat and maize rotation	FE	*L. castaneus* *L. terrestris* *A. chlorotica* *A. rosea* *O. cyaneum* *Nicodrilus species* (×4)	Maillard and Cuendet (1997)

Germany	Ploughing (25–30 cm); rotary harrow to 10 cm	3	Significantly more earthworms in conservation tillage than conventional tillage	Spring barley–winter wheat	EE & SC	*L. terrestris* most frequent. *A. caliginosa* *A. rosea* *O. cyaneum*	Langmaack (1999)
Germany	Ploughing; layer cultivation (i.e. NIT loosening soil to 30 cm); two-layer ploughing	4	Significantly more earthworms in conservation tillage than conventional tillage	Green fallow–winter wheat with inter-crop-peas-winter wheat with inter-crop-summer barley	ME & SC	*A. caliginosa* *O. cyaneum* most frequent. *L. terrestris* > *A. chlorotica* > *A. rosea*	Emmerling (2001)
Ireland	Potato cultivations (2 × grubbing, ridging, bed tilling, ridging, de-stoning, ridging and planting); ploughing for cereals	6	Earthworms reduced from mean 319 to 40–82 m^{-2}, and 55 to 4–19 g m^{-2}	Winter wheat to potatoes	SC	*M. minuscula*> *A. caliginosa* *A. chlorotica* *A. rosea* > *S. mammalis*	Curry et al. (2002)
UK	Direct drilling; tine cultivation to 8 or 15 cm; ploughing to 20 cm	3	Populations consistently larger in direct drilled than ploughed land. After tine cultivation numbers were similar to those after ploughing	Spring barley and winter wheat	FE & SC	*L. terrestris* *A. longa* *A. nocturna* *A. caliginosa* *A. chlorotica* *A. rosea*	Barnes and Ellis (1979)
UK	Direct drilling; ploughing	8	17.5 and 37.3 times greater abundance in direct drill	Cereals	FE	*L. terrestris* *A. longa* *A. caliginosa* *A. chlorotica*	Edwards and Lofty (1982)
UK	Ploughing; NIT with Väderstad cultivator; NIT with Dutzi cultivator	10	Earthworm biomass and numbers greater in NIT than ploughing	Cereals	FE	*A. chlorotica* *A. caliginosa* *A. longa,* *A. rosea*	Hutcheon et al. (2001)

(*continued*)

Table 10.3 (continued)

Country	Cultivation techniques	Years established	Effect of cultivation techniques	Crop rotations	Sampling method	Species	Reference
						E. hortensis *E. tetraedra* *L. castaneus* *L. festivus* *L. rubellus* *L. terrestris* *O. cyaneum* *O. tyrtaeum tyrtaeum* *S. mammalis*	
USA	Chisel plough to 20 cm; no-tillage	3	Significantly more earthworms in no-tillage than conventional tillage	Spring pea, wheat and barley	SC	*A. trapezoides*	Johnson-Maynard et al. (2007)
USA	Chisel disc and no-tillage	3	Significantly more earthworms in no-tillage compared with chisel disc	Maize–soybean, continuous soybean and continuous maize	SC	*A. trapezoides*	Jordan et al. (1997)
USA	Disc, chisel; autumn ploughing; Spring ploughing; min-till using rotary cultivators	30	Six times more earthworms in discing (2.5 cm) than ploughing	Pea–winter wheat rotation	SC	*A. trapezoides*	Wuest (2001)
USA	Ploughing and no-tillage	115	Significantly more earthworms in no-tillage than conventional tillage	Continuous maize/ continuous grass/ continuous wheat/ maize–wheat–red clover rotation/ continuous soybeans	SC	*A. trapezoides* *A. caliginosa* *L. terrestris*	Jordan et al. (2004)

EE Electrical expulsion, *FE* Formalin extraction, *SC* Soil cores, *ME* mustard extraction

provide a habitat which is beneficial to earthworm populations, for populations to become established and their effects on soil properties to accumulate (Table 10.3). It is not only the direct effects of cultivation technique that effect earthworm populations, but the indirect effects of cultivations also affect earthworms. The indirect effects of cultivations, which are likely to affect earthworm populations, include variability in surface soil temperature and moisture due to the absence of a permanent vegetative layer and decreased food supply, as well as crop residue management.

10.3.1 The Effect of Crop Rotations on Earthworm Populations

Soil cultivation coupled with the removal of the litter layer from the soil surface may be responsible for the decline in earthworms, especially deep-burrowing species (Söchtig and Larink 1992). This was reiterated by Curry and Byrne (1997) who studied the role of earthworms in straw decomposition in a winter cereal field. They concluded that a possible reason for the reduced earthworm influence on straw decomposition over time was the decline in numbers of *L. terrestris* due to arable cultivation. Earthworm populations benefit from tillage practises which return a high proportion of crop residues to the soil, particularly when the residues remain on the soil surface. Arable soils to which organic matter is added can support large earthworm populations despite repeated cultivations. If annual crops are continuously cultivated, the food sources for earthworms will decrease and reduce earthworm populations (Boström 1995). In such circumstances, fields with annual crops would contain less earthworm biomass than grass leys. In conventionally tilled soil, there is a rapid depletion of soil organic matter and accelerated crop residue decomposition (Eriksen et al. 2009). This has been attributed to increased microbial respiration as a result of the topsoil being aerated by ploughing (Emmerling 2001); this can lead to a decrease in food supply for many earthworms, in particular endogeic earthworms. Edwards (1983) found that as the amount of organic matter decreased with continual arable cropping, the species most dependent upon it, *L. terrestris* and *Aporrectodea longa*, became less common. Cultivation favoured some species, such as *Allolobophora chlorotica*, by mixing organic matter in the upper soil layers. *Aporrectodea caliginosa* remained numerous and *Aporrectodea rosea*, *Octolasion cyaneum* and *Lumbricus castaneus* persisted.

There are certain types of plant litter that earthworms prefer and they consume these before consuming less desirable litter, with quality rather than quantity being an important factor (Gallagher and Wollenhaupt 1997; Curry 2004). The quality of plant litter is positively related to the quantity of hemicellulose, nitrogen and other nutrient elements and negatively to its concentrations of lignin and cellulose (Lavelle and Spain 2001). Litter from grass and herbaceous plants have ratios of carbon to nitrogen close to or less than 20:1. Litter with a carbon to nitrogen ratio of more than 60:1 is unpalatable and unfavourable to earthworms (Curry 2004).

However, if the food supply is limited, the absence of disturbance will not benefit earthworms (Curry 2004). Schmidt et al. (2001, 2003) showed that wheat–clover inter-cropping systems supported larger earthworm populations than conventional wheat monocropping systems. Their research suggested that the combination of the absence of ploughing and the presence of a suitable food supply increased earthworm populations. It was concluded that cereal–legume inter-crops supported large earthworm populations primarily because of the quantity, quality and continuity of the food supply. Fraser and Piercy (1998) studied the effect of cereal straw management practises on lumbricid earthworm populations and one of the treatments involved growing cereals and undersowing them with clover. The experiment was conducted for 4 years and in the final year there was a large increase in earthworm numbers, biomass and species. They attributed this to the clover plants providing a moist and cool microclimate conducive for earthworm survival, and the high protein content of the clover plants providing a rich food source. Pižl (1992) also found very small earthworm populations under wheat and maize fields, with larger populations in clover and peas. These crops returned more crop residues to the soil and permitted the temporary development of larger populations. Therefore, in the absence of other constraints, it is likely that the carrying capacity of most habitats could be increased by increasing food supply (Curry 2004).

10.3.2 The Effect of Crop Residue Disposal on Earthworm Populations

The various ecological groups of earthworms have different burrowing and feeding habits and respond differently to agricultural practises. Anecic species, such as *L. terrestris*, depend on their vertical burrows, which they leave to feed on organic material on the soil surface. Epeigic species (e.g. *L. rubellus*) feed on organic material on the soil surface. Endogeic species (e.g. *A. caliginosa*) feed on soil they ingest while burrowing. Therefore, in agricultural soils, earthworm growth and reproduction are often limited by the availability of food. Consequently, cropping regimes significantly influence earthworm community structure and populations density (Shuster and Edwards 2003). In conventional tillage systems, crop residues are incorporated into the soil, decreasing their availability for earthworms feeding on the surface. Therefore, the method of crop residue disposal after harvesting is likely to influence earthworm abundance. Ernst and Emmerling (2009) demonstrated that cultivation techniques modified the soil organic carbon distribution within the soil. Soil organic carbon was increased in the topsoil under reduced tillage compared with ploughing. Arable soils which have had organic matter added, for example straw, can support large earthworm populations despite repeated cultivations. A field experiment established by Fortune et al. (2005) investigating the effect of NIT vs. conventional tillage utilising a MP plus straw chopping vs. baling and removing on earthworms. After 4 years, earthworm numbers and biomass

increased, especially in NIT where straw was incorporated. These findings were supported by Fraser and Piercy (1998), who compared earthworm numbers under different stubble management regimes. They found that after 4 years, earthworm densities in plots where stubble was burned or removed had lower populations than plots where residues were incorporated (150 earthworms m^{-2} vs. 400 m^{-2}, respectively). Eriksen et al. (2009) concurred with the above findings that the addition of crop residues to tilled soil could alleviate the negative effects of tillage and help maintain more stable earthworm populations. However, they went onto state that the response of earthworm populations to crop residues depended on the residue characteristics. They found that earthworm populations were larger in long-term no-tillage plots than ploughed plots, but crop residue management did not affect earthworm populations possibly due to the high C:N ratio of the material, a residue with a low C:N ratio might have been more favourable.

10.4 Conclusion

Earthworms are sensitive to cultivation techniques and consequently may be used as bioindicators of soil health. Conservation agriculture is not a single prescriptive system, but it is a set of principles applied carefully and intelligently to a variety of different soil types, crops, climates and farm objectives. Earthworms are sensitive to cultivation techniques and consequently may be used as bioindicators of soil health. Research conducted worldwide has revealed that conservation tillage practises

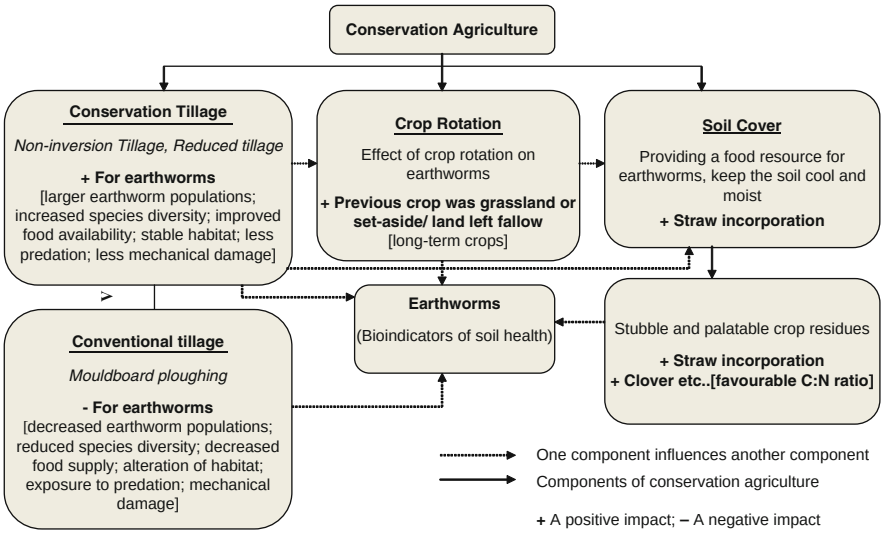

Fig. 10.2 Overview of the impact of cultivation techniques on earthworm populations (Rothwell 2007)

have been shown to provide a favourable environment for increased earthworm populations when compared with conventional tillage practises, due to factors such as decreased soil disturbance and an accessible palatable food supply, additional factors are summarised in Fig. 10.2. However, as the majority of research has been conducted in the United States, Canada and Australia, more research regarding the impact of cultivation techniques, crop rotations and crop residue management on earthworm populations within Europe is required.

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Chapter 11
Assessing the Role of Earthworms in Biocontrol of Soil-Borne Plant Fungal Diseases

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11.1 Introduction

Earthworms called as "the intestines of the earth" by the Aristotle are soft-bodied, bilaterally symmetrical, elongated, metamerically segmented invertebrates belonging to class Oligochaeta of phylum Annelida. They are found in a variety of environments and soil types wherever there is adequate moisture and organic matter available. Among the total known species, about half are terrestrial earthworms. They feed on organic matter of soil and make large burrows, thus rendering soil more pervious to rains and plant fibers and augment water holding capacity of soil. Through feeding, burrowing and casting, earthworms alter the physical, chemical, and biological properties of the soil organic matter. Changes in physical properties of organic matter due to earthworm activity include improved aggregation, stability, and porosity. Chemical properties in soil processed by earthworms include organic matter dynamics in terms of quality and quantity, nutrient cycling, chemical forms of nutrient in soil and their availability to plants. Earthworms also modify microbial and invertebrate activity, abundance, biomass, species composition, and diversity

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(Lavelle et al. 1998; Aira et al. 2007a, b; Lazcano et al. 2008). Microbial community of earthworm gut and cast are known to be potentially active and can digest a wide array of organic materials and polysaccharides including cellulose, sugars, chitin, lignin, starch, and polylactic acids (Zhang et al. 2000; Aira et al. 2007a; Vivas et al. 2009). Earthworms have been described as being one of the main groups of soil engineers in tropical and temperate ecosystems because they change the structural properties of soil and thus influence soil microorganisms, SOM regulation, and plant growth (Lavelle 1997). Since the earthworms are more near the root zone of plants (rhizosphere) where there is rich organic matter, drilosphere (the earthworm influenced soil volume and microbiota) and rhizosphere are interdependent.

Earthworms are very suitable candidate for biological decomposition of organic materials. Decomposition of organic material using earthworms is called as vermicomposting and finished product vermicompost. This technique has been extensively used to process several different types of residues including organic and industrial waste. Moreover, there are several reports showing the positive effect of vermicompost application on growth and yield of plants (Maize, Gutie´rrez-Miceli et al. 2008; Rice Tejada and Gonzálezb 2009; Garlic Suthar 2009). The realization of harmful effects of excessive use of agrochemicals and fertilizers has generated the need of alternative approaches to maintain the soil fertility in sustainable manner. In recent decades, scientists the world over have advocated low cost, low input, and ecology-based approaches for the sustainable agriculture. The vermicomposting is one such approach that can play a significant role in building up of soil fertility and improving soil health for sustainable agriculture. In addition, the potential role of vermicompost in soil-borne plant fungal disease control has also been established (Rivera et al. 2004; Asciutto et al. 2006; Sahni et al. 2008a). The physical and chemical properties of vermicomposts (Albanell et al. 1988; Orozco et al. 1996) make them suitable to be used as disease-suppressive substrates (Szczech 1999; Rodríguez Navarro et al. 2000). Indiscriminate use of fungicides for disease control and their effect on the environment have necessitated interest in the search for eco-friendly alternatives. Due to encouraging results with vermicompost, researchers are now making efforts to understand the mode of action of vermicompost in suppressing various fungal plant diseases. Consequently, several mechanisms have been proposed to explain the suppression of plant diseases by application of vermicompost. Earthworms play a vital role in creating the optimum conditions for the beneficial organisms to establish and reproduce which compete with and dominate the more harmful microbes (Manandhar and Yami 2008). In a recent study, Gopal et al. (2009) concluded that there was an amplification of general and plant beneficial microorganisms when coconut leaf litter [amended with 10% (w/w) cow manure] was converted to vermicompost by the indigenous strain of *Eudrilus* sp.

Considering currently available information, the potential role of earthworms in regulating the diversity of micro flora in soil systems per se and in controlling plant fungal pathogens in particular may be important and calls for further research. The scope of present review is to evaluate the role of earthworms in controlling

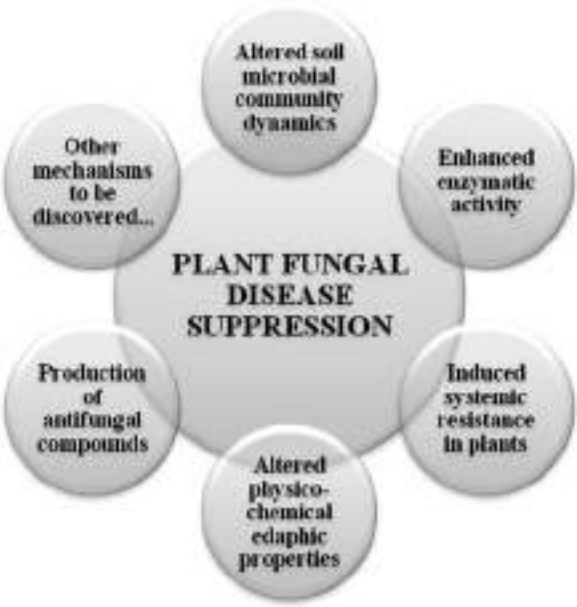

Fig. 11.1 Earthworm-mediated mechanisms of plant fungal disease suppression

soil-borne plant fungal diseases as they are among the most common soil-borne diseases. Although abundant literature is available on this subject as a consequence of increasing awareness on importance of soil micro flora in agro ecosystem processes, the mechanism of suppression of fungal activity is yet to be fully understood due to its complexity. We have tried to discuss various mechanisms, with a functional approach rather than descriptive one, by which earthworm activity can render the environment less disease-favorable and the host plant less susceptible. In this article, however, it is not our aim to merely review the literature but to carry out an in depth analysis of various factors responsible for suppression of fungal activity due to vermicomposting. Further, we have attempted to identify key areas where sincere research efforts are still required and make strategies for manipulating vermicomposting process in such a way that it could be more efficiently utilized in controlling soil-borne fungal plant pathogens (Fig. 11.1).

11.2 Soil-Borne Plant Fungal Diseases

Among the four major groups of plant pathogens (Agrios 2005), only two are the key players in the soil: fungi (true fungi and oomycetes) and nematodes. Only a few groups of bacteria are soil-borne, probably because of the fact that nonspore forming bacteria cannot survive well in soil for long periods. Fungi are the most

important soil-borne pathogens; therefore, they will be the focus of this review article. Fungal soil-borne pathogens are capable of manifesting in plants a large number of diseases and symptoms. The losses due to such pathogen are most of the times underestimated and unnoticed. These soil-borne pathogens are quite frustrating to the plant pathologists and farmers because they often survive in soil for several years due to their capability of producing resilient survival structures such as sclerotia, oospores, and chlamydospores. These survival structures can withstand high or low temperatures and desiccation. Many diseases caused by soil-borne pathogens are difficult to predict, detect, and diagnose. In addition to this, the soil environment is extremely complex, making it a challenge to understand all aspects of diseases caused by soil-borne fungal pathogens (Koike et al. 2003). The most common soil-borne fungal pathogens are probably rots that damage below ground plant tissues (e.g., seed decay, damping-off of seedlings, root, and crown rots). Vascular wilt initiated by root infection is also frequently observed in the field. A few soil-borne fungi cause foliar disease with symptoms and damages appearing on above ground plant parts.

11.3 Biocontrol of Soil-Borne Fungal Diseases

Biological control is defined as the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state by one or more organisms, except man, accomplished naturally or through manipulation of the environment, host, or antagonists or by mass introduction of one or more antagonists (Singh 2002). Biocontrol approaches have been in practices since the ancient times. As early as 5000 BC, in China, it was a common practice to place bamboo poles between ant nests and fruit trees to make it easier for ants to prey on citrus scales. In the 1750s, the mynah birds were transported from India to Mauritius to control locusts by the British and French. Although, the progress in biocontrol research has yielded several effective products, there are still many obstacles in their wide application. According to Van Lenteren (1995), biological control is practiced in just 5% of the estimated 741,290 acres of greenhouses worldwide. The key factors affecting adoption of biological control are efficacy, predictability, and cost (Parrella et al. 1992; Van Driesche and Heinz 2004). In addition, the microbial control efficacy is influenced by various abiotic and biotic factors such as soil microbial biomass and activity, soil moisture, temperature, and nutrients in host and pathogen interactions (Shaukat and Siddiqui 2003; Landa et al. 2004; Bae and Knudsen 2005). This is one of the reasons why inconsistent control of plant diseases under diverse field conditions is often achieved. Also, there are apprehensions that application of microbes as biocontrol agents in large densities could probably have negative effects on the indigenous rhizosphere microbiota (Scherwinski et al. 2007). In view of this, the use of organic amendments to decrease the incidence of disease caused by soil-borne fungal

pathogens (reviewed by Bonanomi et al. 2007) seems to be more holistic approach as it also improves soil structure and fertility (Cavigelli and Thien 2003; Padmavathiamma et al. 2008). Application of vermicompost, one of a spectrum of techniques of organic amendments, has been proposed to control soil-borne fungi (e.g., *Phytophthora*, Szczech and Smolinska 2001). Sahni et al. (2008b), while studying the collar rot disease incited by *Sclerotium rolfsii*, demonstrated that substituting the soil with different amounts of vermicompost showed significant reduction in mortality of chickpea compared with control. In a more recent study, Elmer and Ferrandino (2009) concluded that augmenting earthworm populations can suppress *Verticillium* wilt of eggplant, and strategies that increase earthworm numbers may contribute to disease suppression.

11.4 Mechanisms of Soil-Borne Fungal Disease Suppression Due to Earthworm Activity

Earthworms can greatly affect fungal communities in soil by influencing spore germination and creating microsites favorable or unfavorable to fungus development (Brown 1995; Tiunov and Scheu 2000). The overall effect of earthworms on soil processes, however, is not uniform and varies with their ecological category and species (Brown 1995). Since the earthworm activity in soil is influenced by several factors, often a variation in disease suppression is observed. The possible mechanisms suggested to be involved in disease suppression by earthworm activity include the influence on: (1) soil microbial communities; (2) enzymatic activity, (3) production of antifungal compounds, (4) soil physicochemical properties, and (5) systemic resistance in plants (Fig. 11.1). One mechanism may or may not be exclusively responsible for carrying out desired level of disease suppression as it is a complex process influenced by several other factors.

11.4.1 Changes in Microbial Community Dynamics

Earthworms can affect soil microbial community directly and indirectly by various mechanisms such as comminution, burrowing and casting, grazing, and dispersal. These activities modify the soil's physicochemical and biological status and may cause drastic shifts in the density, diversity, structure, and activity of microbial and faunal communities within the drilosphere (Brown 1995; Fracchia et al. 2006; Lazcano et al. 2008). According to McKellar and Nelson (2003), the disease suppression is most likely a property of the microbial community as a whole and not the result of any single species. The common suggested methods by which earthworms can modify fungal populations include (1) ingesting certain fungal species, (2) affecting the germination of ingested spores in such a way that certain

fungi may have higher, lower, or no germination, once these spores exit the earthworm, (3) changing fungal successional patterns, dispersing selected fungi, altering substrate quality, by grazing on particular fungal species, reducing fungi's inoculums potential, and (4) reducing surface dispersal of certain fungal spores by burying leaves and other plant materials contain spores and hyphae (Brown 1995).

Earthworm digestion can increase the availability of nutrients for micro-organisms, rising microbial numbers in casts (Parthasarathi and Ranganathan 1999). In a recent study, earthworms increased microbial biomass during vermicomposting of pig slurry independently of the rate of application of pig slurry (Aira et al. 2007a). It has been reported that intense microbial activity can limit the development of pathogenic populations in various pathosystems such as wilts due to *Fusarium oxysporum* (Steinberg et al. 2007) or damping-off due to *Pythium aphanidermatum* (Grunwald et al. 2000) or *Rhizoctonia solani* (Diab et al. 2003). Elevated microbial activity, a feature usually observed in vermicompost, results in increased competition between compost inhabiting microbes and the fungal pathogen for root exudates components, which are essential for the germination of fungal propagules. In addition, earthworms have been reported as potential vectors for actinomycetes antagonistic to pathogenic fungi by Jayasinghe and Parkinson (2009). These researchers have concluded that the selective consumption of *Streptomyces* by earthworms and the ability of certain *Streptomyces* in worm casts to spread into surrounding soil may increase the population size of some *Streptomyces*, and some *Streptomyces* species may become dominant species in the forest floor.

The disease suppression can be the result of activities of certain microflora or of local environmental conditions. According to Weller et al. (2002), suppressiveness can be definitively established or temporarily acquired depending on the pathosystem and the environmental conditions (biotic and abiotic). In a more recent review, Garbeva et al. (2004) discussed that plant type, soil type, and agricultural management regime are the main drivers of soil microbial community structure, which have implications for disease suppressiveness. The disease suppression is usually categorized as "general" or "specific" based on the absence or presence of information about the mechanisms involved. It has also been suggested that during decomposition of organic matter in soil, the soil ecosystem is subjected to "oligotrophication", and the ratio of oligotrophic (K-strategist) to copiotrophic (r-strategist) organisms changes during microbial succession (Van Bruggen and Semenov 1999). The range of this ratio has been attributed to general disease suppression (Van Bruggen and Semenov 2000).

The role of resident microbial communities in affecting pathogen responses to plants and subsequent disease development is also very crucial. In a study, McKellar and Nelson (2003) have shown that communities of compost-inhabiting microorganisms colonizing cottonseeds within the first few hours after sowing in a *Pythium*-suppressive compost play a major role in the suppression of *Pythium ultimum* sporangium germination, seed colonization, and damping-off. They attributed the *Pythium* suppression to fatty acid metabolism by these seed-colonizing bacterial consortia. The biosynthesis of cutin (the major component of plant cuticle) is dependent on fatty acid oxidases, acyl-activating enzymes, and acyl transferases.

The cuticle, being a physical barrier to pathogen entry into host plant, is considered as first line of defense against pathogen. Therefore, the fatty acid metabolic pathways play significant roles in pathogen defense in plants (reviewed by Kachroo and Kachroo 2009).

Since the earthworm activity significantly influences the soil type and microbial community by one or several mechanisms, no doubt, it can be important in rendering the soil disease-suppressive. Although, in past few years, substantial amount of information has been generated by the researchers, we are yet to understand the role of exact biotic factors in underlying direct disease suppression due to earthworm activity. In this context, case-by-case study shall be more appropriate for agricultural soil to know the microbes or the combination thereof that are mechanistically involved and their activities that are important for the suppressive effects. Agricultural management can be directed toward maximizing the quality of the soil microbial community in terms of disease suppression, if it is possible to shift soil microbial communities (Van Bruggen and Semenov 2000).

11.4.2 Enzymatic Activity

The organic matter decomposition by earthworms is mediated by several enzymatic activities. In the earthworm gut, various enzymes (of microbial or earthworm origin) are secreted, along with intestinal mucus in different quantities, which can have variable impact on the soil microbes. One of the major enzymatic activities influenced by earthworms in soil is chitinase activity, which play a significant role in suppression of soil-borne fungal pathogens. Therefore, use of chitin metabolizing enzymes and chitin synthesis inhibitors, either singly or in combination to arrest the chitin metabolism in fungi has attracted the attention of several researchers. Chitinases are the digestive enzymes that hydrolyse the β1–4 glucosidic bonds of *N*-acetylglucosamine residues in chitin. Chitinase is essential for the degradation of chitin, a major component of the fungal cell wall (Thomas and Cole 1999). Chitinolytic bacteria like Actinobacteria and *Streptomyces* species are reported to degrade the chitinous cell wall of plant fungal pathogens through production of chitinases and antibiotics (Kawase et al. 2006; Yu et al. 2008). Recently, Yasir et al. (2009) investigated the bacterial communities and chitinase gene diversity of vermicompost to clarify the influence of earthworms on the inhibition of plant pathogenic fungi (*R. solani*, *Colletotrichum coccodes*, *P. ultimum*, *Phytophthora capsici*, and *Fusarium moniliforme*) in vermicompost. Results of their study revealed that the diversity of chitinase gene increased in vermicompost. Chitinase gene sequences from vermicompost libraries have been grouped mainly with chitinases from *Streptomyces*, *Aeromonas*, *Lysobacter*, and *Salinispora*. In addition, the chitinolytic isolates were most active against the target fungi. This suppressive activity of fugal pathogen may be due to the synergistic effect of chitinases and antifungal compounds produced by the isolates (Yu et al. 2008). Moreover, *Streptomyces* has also been reported to produce certain volatile

substances that caused suppression of growth of *R. solani*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea*, as well as reduction of the incidence and/or severity of leaf light/seedling blight of rice caused by *R. solani*, leaf blight of oilseed rape caused by *S. sclerotiorum*, and fruit rot of strawberry caused by *B. cinerea* (Yu et al. 2008).

The activation of various enzymes due to earthworm activity in soil is also attributed to increase in microbial biomass as reported by Aira et al. (2007a), which can have important role in suppression of fungal pathogen. Apart from chitinase activity as discussed above, there might be several other enzymes responsible for suppression of fungal pathogen through cell wall degradation or other means in soil under the influence of earthworms. More studies are necessary to elucidate the operative mechanisms of suppression of fungal pathogen through such enzymatic activities.

11.4.3 Production of Antifungal Compounds

11.4.3.1 Antibiotics

In the process of organic matter decomposition, earthworms also produce or facilitate production and secretion of several bioactive chemical substances that exhibit not only plant growth promoting activity but also improves the soil suppressiveness to fungal pathogens. It is well established that the gut and casts of earthworms tend to be much more microbiologically active than the surrounding soil. The current literature contains a considerable body of experimental evidences of presence of the actinomycetes in earthworm gut. Yasir et al. (2009) reported that aqueous extract of vermicompost significantly inhibited the spore germination of *F. moniliforme in vitro*. They related this antifungal activity to increased population of actinobacteria (mainly *Streptomyces* species), which produce approximately two-thirds of the known microbial antibiotics (Hoster et al. 2005; Yu et al. 2008). In fact, approximately 60% of the antibiotics used for agricultural purposes are obtained from *Streptomyces* species (Tanaka and Mura 1993). The antibiotics produced by *Streptomyces*, such as polyoxins and nikkomycins, are structurally very similar to UDP-GlcNAc, an active monomer of chitin. Due to this structural similarity, antibiotic can mimic the action of UDP-GlcNAc and bind to chitin synthase. This would block the active site of the enzyme and thus make the enzyme unavailable to the monomer for polymerization. Thus, the synthesis of chitin would be blocked. In case of fungi, the observed effect on species exposed to polyoxins and nikkomycins is swelling and bursting of growing hyphae (Deshpande 1998).

11.4.3.2 Phenolics

In a study, Singh et al. (2003) reported that aqueous extract of vermicompost was highly effective in inhibiting the spore germination of several species of *Alternaria*,

Curvularia, and *Helminthosporium*. They attributed this antifungal activity to induction of phenolic acids (gallic and chlorogenic acids) synthesis in plants, which was maximum in pea plants treated with 4% aqueous extract of vermicompost. Although phenolic acids are the preexisting biochemical compounds present in low quantity in the plants (Agrios 1997). Application of vermicompost in the soil increased the level of phenolic acids in pea plants. Several phenolic acids are already reported as antifungal and also confer resistance either by being directly antifungal (De Vecchi and Matta 1989) or indirectly by inducing the synthesis of phenolics following postinfectional responses in the hosts (Harborne 1988). Gallic acid itself, not being antifungal, is converted into antifungal gallotannins (Salisbury and Ross 1986). Chlorogenic and protocatechuic acids are highly antifungal (Agrios 1997; Tamari and Kaji 1954). Recently, Hussin et al. (2009) have observed significant antifungal activity of methanolic extract of *Barringtonia racemosa* against *Fusarium* sp., which contained two different phenolic acids (gallic acid and ferrulic acid) and four different flavonoids (naringin, rutin, luteolin, and kaempferol).

11.4.3.3 Antimicrobial Peptides

Another emerging approach of plant disease control is the use of antimicrobial peptides (AMP) which have captured the attention of researchers in recent years because of their efficiency in fighting against pathogens (reviewed by Keymanesh et al. 2009). These peptides are found in nature and have been recovered from a wide array of organisms. A substantial number of plants and animals have been manipulated with antimicrobial peptide-encoding genes, and several pesticides and drugs have been produced based on these peptides. The mode of action of killing microbial cells by such AMPs is multitargeted and highly effective and involves rapidly killing the microbe by drilling a molecular "hole" in its membrane, then inactivating and destroying its genome. Since the primary target of membrane-active, positively charged AMPs is the cell membrane and not specific receptors or substrates, these peptides usually show their activity against a broad spectrum of pathogenic microorganisms, and there is less probability of resistance arising by variation of its metabolic pathways (Misra 2005). Interestingly, earthworms also possess several AMPs as a part of their innate immune system. Lumbricin-1 obtained from *Lumbricus* is a proline-rich antimicrobial peptide of 62 amino acids showing antimicrobial activity *in vitro* against fungi, Gram positive, and Gram negative bacteria without hemolytic activity. In the tiger worm *Eisenia fetida*, three very short antibiotic peptides, F-1, F-2, and OEP3121, have been isolated and identified (Zhang et al. 2002; Liu et al. 2004). These are described as exhibiting an activity against Gram positive, Gram negative bacteria, and fungi. Inhibition of fungi requires 5–20-fold larger concentrations of antimicrobial peptides than bacterial inhibition (Alan and Earle 2002; Alan et al. 2004). In view of these reports, it would be incorrect to completely correlate antifungal activity of vermicompost to the presence of AMPs. However, this could be one of the plausible mechanisms

involved in antifungal activity of vermicompost against phytopathogens which requires further investigation.

11.4.4 Physicochemical Properties of Soil

Soil suppressiveness is often attributed to activity of soil microorganisms or microbial metabolites. However, physicochemical properties of soil, including pH, organic matter, and clay content can also contribute to the suppression of plant diseases directly or indirectly through their influence on soil microbial activity. It is therefore important to know the influence of soil physicochemical properties on disease suppression. Although one set of physicochemical attributes of soil considered as suppressive for a disease may be conducive for other one. It is therefore equally important to understand the physicochemical characteristics of soil which are unfavorable to the specific disease development.

Earthworm activity significantly alters the physicochemical properties of the soil. During the process of vermicomposting, earthworms hasten the fragmentation of particulate matter by grinding muscular gizzard with the help of enzymatic secretions such as amylase, cellulase, protease, lipase, chitinase, and lichenase. This results in a loosely aggregated, granular material whose stability depends on organic matter and moisture concentrations and bacterial and fungal polysaccharides structure (Kale 1993; Subler 2002). Although the amount of nutrients in a finished vermicompost certainly varies according to the initial feedstock, the nutrient content of the digested waste is usually lower than that of the raw material.

There are some abiotic indicators which have been directly correlated with earthworm activity leading to soil suppressiveness. Considerable work in this regard has been carried out. In a study on the control of *R. solani* in vermicompost-amended substrates, a temperature-dependent response was observed (Rivera et al. 2004). It is also reported that increasing phosphorus rates above the level needed to grow the crop can increase the severity of *Fusarium* wilt in cotton and muskmelon (Jones et al. 1989). There are many reports indicating increased phosphorus concentration during vermicomposting due to loss of dry matter (Elvira et al. 1998; Ghosh et al. 1998; Chowdappa et al. 1999). Most literature indicates an association between the level of available soil phosphorous and crop disease development (reviewed by Ghorbani et al. 2008). The optimum level for individual crops will vary from soil to soil and disease to disease, so should be determined. Subsequent careful monitoring and management of available phosphorus and its balance with other nutrients could then be considered in an overall strategy for crop disease management (Ghorbani et al. 2008). Atiyeh et al. (2000), while studying changes in biochemical properties of cow manure during processing by earthworms (*Eisenia andrei*) reported that earthworms enhanced nitrogen mineralization and increased the rates of conversion of ammonium–nitrogen into nitrate. It is well established that the form of ammonium or nitrate may influence plant disease incidence. Matocha and Vacek (1997) demonstrated that the ammonium form of

nitrogen reduced the severity of the *Phymatotrichum omnivorum* in cotton, while the form increased plant mortalities. The ammonium form assimilated in plants is rapidly nitrate converted to amino acids, whereas nitrate can be stored. This stored form of nitrate in plants is considered as the main source of nutrients for pathogens (Lampkin 1999). However in an another study, nitrate forms of nitrogen fertilizer suppressed *Fusarium* wilt of tomato, while the ammonia form increased disease severity (Woltz and Jones 1973), which is in contrast to earlier mentioned reports. Ammonium ions are absorbed by the roots through exchange with H+ ions that are released to the surrounding medium, thus decreasing soil pH (Agrios 1997). It encourages diseases, which are favored by low pH. The vermicomposting process generally neutralizes the pH of the substrate through $CaCO_3$ secretions in the worm gut that have an alkalizing effect on the feedstock (Senapati 1993), thereby reducing the population of fungal pathogen active at low pH. It is also established that vermicomposted material has a larger surface to volume ratio (Haimi and Huhta 1987) that enhances aeration. Poor soil aeration caused by poor soil structure, soil type, or waterlogging has been found to be associated with the development of cavity spot (*Pythium* spp.) disease in carrot (Hiltunen and White 2002). Oyarzun et al. (1998) investigated three major pea root rot pathogenic fungi and their relationship with various abiotic and biotic soil factors. Their results revealed that capacity of the soil to allow soil-borne pathogens to produce disease by *Thielaviopsis basicola* was positively associated with soil pH, organic matter content, and C/N ratio. The amounts of soluble K, P, Mg, and total C and N in soil, individually, were higher with increasing conduciveness to *Fusarium solani* f. sp. pisi. Elvira et al. (1998) reported significant reductions of total K by the end of the vermicomposting process, which they attributed to its high water solubility and leaching of the windrows.

It is evident from foregoing discussion that earthworms play a significant role in soil suppressiveness through altered soil physicochemical properties but due to intricate nature of mechanisms involved in the process, it is difficult to clearly establish the soil abiotic factors contributing to it. The physicochemical attributes of suppressive soil have been reported to vary considerably with the type of pathogen. It is necessary to continue investigations for increasing the knowledge of these mechanisms to establish the definite role of earthworms in this process and the possibilities of their incorporation in environmentally friendly and sustainable crop productions.

11.4.5 Induced Systemic Resistance in Plants

Enhancement of natural plant-defense mechanisms to provide resistance against pathogens is a potential approach for plant protection (Van Loon 1997). Considerable work has been carried out in understanding the phenomenon of induced resistance in a number of crops and its possible application in crop protection against diseases. Systemic resistance is also induced in plants in response to compost treatments. Hoitink et al. (1997) demonstrated that composts and compost

teas indeed activate disease resistance genes in plants. These disease resistance genes are typically "turned on" by the plant in response to the presence of a pathogen. These genes mobilize chemical defenses against the pathogen invasion, although often too late to avoid the disease. Plants growing in compost, however, have these disease-prevention systems already running (Hoitink et al. 1997). Induced resistance is somewhat pathogen-specific, but it does allow an additional way to manage certain diseases through common farming practices.

It has been suggested that the earthworm activity can influence aboveground plant defense levels (Stratmann 2003). Via decomposition, earthworms can increase nitrogen availability in the soil, resulting in the plant investing more in growth and less in direct defense compounds. Goldstein (1998) reported that composts and compost extracts activate disease resistance genes in plants. These genes are normally activated in response to the presence of a pathogen; they mobilize chemical defenses against the pathogen invasion. Plants growing in compost may have these disease-prevention systems already activated (Sullivan 2001). A recent study has shown that earthworms can increase lipoxygenase gene expression in the leaves (Blouin et al. 2005). Lox is the first enzyme in the pathway that results in the signaling hormone Jasmonic acid, which is responsible for systemic defense induction (Stratmann 2003). Thus, the increase in lipoxygenase gene expression caused by earthworm activity in the soil can influence aboveground plant defense responses directly. Induction of resistance in the host plant by the application of the compost and compost tea has been reported (Hoitink et al. 1997; Zhang et al. 1998). Recently, Siddiqui et al. (2009) while evaluating the effect of compost tea produced from agro-waste such as rice straw and empty fruit of oil palm composts on *Choanephora cucurbitarum*, the causal pathogen of wet rot disease of okra demonstrated the increased concentration of polyphenol oxidase, peroxidase, and phenylalanine ammonia lyase in plants treated with compost tea. Enzymes such as polyphenol oxidase and peroxidase have been considered to be responsible for the oxidation of phenolic compounds into antimicrobial quinones in plant cells infected by phytopathogens and thus conferring disease resistance during incompatibility reactions (Chittoor et al. 1999).

11.5 Conclusion

Earthworms are one of the most important groups of soil invertebrates involved in decomposing organic matter thereby potentially contributing to soil fertility. It has been established that earthworm activity in the soil creates a favorable environment for growth of plant beneficial microbes and suppresses the growth of pathogenic microbes. Based on the literature analysis, it can be convincingly concluded that earthworm activity can play a significant role in suppressing soil-borne fungal disease by directly or indirectly influencing soil microbial community dynamics, physicochemical edaphic properties, and inducing systemic resistance in plants. Therefore, it appears that the application of earthworm-worked material

(vermicompost) could be a practical strategy for managing soil-borne fungal diseases in sustainable manner. However, disease suppression caused by earthworm activity has been related directly or indirectly with several other biotic and abiotic factors of the soil. Consequently, variability in disease suppression is often encountered with field application of vermicompost. Proper understanding of mechanisms involved in disease suppression under the influence of earthworm activity can minimize this variability and improve the efficiency of this process. Considerable research efforts have been made in this regard during past few decades. Several indicators of suppressive soil have been proposed, which can be correlated with earthworm activity. Yet, concerted research efforts are required to better identify pathogen-specific attributes of the fungal disease-suppressive soil by carrying out case-by-case investigations with the help of modern scientific tools and devise strategies for vermicomposting in such a pattern that these attributes can be manipulated in order to maximizing the potential of earthworms.

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Chapter 12
The Use of Vermicompost Products to Control Plant Diseases and Pests

Yurdagul Simsek-Ersahin

12.1 Introduction

Intensive uses of agrochemicals in conventional cropping systems have caused irreversible effects on soil and water ecosystems i.e., pollution of surface and fresh water resources, and endangered food safety. Inefficient organic waste management practices, i.e., burning and land filling, have also contributed adverse environmental problems. Since 1960s, increased social consciousness has urged humanity to develop viable and eco-friendly sustainable management methods for both agronomic production and management of biodegradable organic wastes. In this respect, vermiculture and culturing/utilization of earthworms have introduced a wide variety of efficacious environmentally safe low-input processes manageable at different scales for a variety of product aims. Vermicomposting provides biological conversion of biodegradable organic wastes into economically high-valuable products (Edwards and Neuhauser 1988; Chaoui 2003). Vermicomposting has been extensively utilized both in industrialized and in less industrialized countries during the last 40 years (Edwards 1995). Vermicomposting produces both worm meal used as animal protein for poultry and fishery and vermicompost. Vermicomposts have proved to be more effective as organic fertilizers and bio-control agents with respect to their counter parts, thermophilic aerobic composts (Szczech 1999; Dominguez et al. 1997; Edwards and Arancon 2004). In addition to use solid vermicompost for inhibition of plant pathogens (Simsek-Ersahin et al. 2009) and pests, development of liquefied vermicompost products, i.e., vermicompost extracts/teas, has been increasingly utilized during the last decade (Yardim et al. 2006; Zaller 2006; Edwards et al. 2009). Both forms of vermicompost products have great deal of potential for crop production and protection in sustainable organic cropping systems.

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The present chapter focuses on comprehensive utilization of vermicompost products, either solid or liquefied, for the inhibition of a variety of plant diseases and pest attacks. Although the mechanisms of inhibition have not been fully described, the application as a natural/biological control approach has great potential. The disease inhibition is mostly suggested to be related with the diverse microflora, especially increased variety and number of antagonists enriched in solid and liquefied vermicompost products.

12.2 The Use of Vermicompost Products for Plant Disease and Pest Control

Although there is extensive research on the suppressive effect of aerobic compost products on plant diseases, in container based or field scale, there is less of vermicomposts. The major reason for that is that the potential of vermicomposting approach for plant production and protection was discovered later than composting. Second, utilization of a vermicomposting operation requires a well-balanced and stable maintenance conditions that make vermicomposting a more delicate process than aerobic composting. Third, because of the complexity in yielding large amounts of high-quality vermicompost, field applications of vermicompost for agricultural purposes are quite scarce.

Excessive and frequent applications of chemical pesticides in conventional agriculture induced "biological resistance" in crop pathogens and pests. Hence, logarithmically much higher doses are now required to suppress them for the growth of high-yielding crops which have become more susceptible to pests and diseases (Patriquin et al. 1995). Vermicompost application has a great potential to reduce the use of chemical pesticides and fertilizers; hence, significantly to cut down on the costs of food production.

12.2.1 Application of Solid Vermicomposts for Plant Disease Control

There has been considerable increase on the literature within the last two decades proving the efficacy of vermicompost products to protect plants against various diseases and pest attacks (Edwards 1999). Up to date, solid vermicomposts in laboratory, greenhouse, and field proved to have a great potentiality in suppression of disease incidences caused by pathogens such as *Pythium*, *Phytophthora*, *Fusarium*, *Rhizoctonia*, and *Verticillium*. Disease suppression ability of vermicomposts produced from various waste streams, i.e., animal manures (Szczech and Smolinska 2001), separated dairy solids (Kannangara et al. 2000), cattle manure (Szczech 1999), sewage sludge (Szczech and Smolinska 2001), and a mixture of vegetable

wastes, bark (*Salix* spp.), and cattle manure (Simsek-Ersahin et al. 2009) were tested on *Phytophthora nicotianae*, *Fusarium oxysporium* f.sp. *lycopersici*, and both *F. oxysporium* and *Rhizoctonia solani*, respectively. Disease suppression ability of small applications of some commercially produced vermicomposts was examined on attacks caused by fungus *Pythium* in cucumbers, *Rhizoctonia* in radishes in the greenhouse, *Verticillium* in strawberries and by *Phomposis* and *Sphaerotheca fulginae* in grapes in the field (Edwards and Arancon 2004).

Earlier applications of solid vermicomposts for plant disease control mostly were carried out as pot amendments in which plant growth media was substituted with low rates of solid vermicomposts in the greenhouses to examine their potentiality for inhibition of soil-borne fungal root diseases. Szczech et al. (2002) mentioned about earlier works by Szczech (1999), Szczech and Smolinska (2001), and his co-workers. In those works, solid vermicomposts mostly produced from animal manures tested for their inhibition efficacy on fungal root pathogens. Studies carried out by Szczech and his co-workers showed that vermicomposts produced from cattle manure can suppress some soil-borne plant pathogenic fungi. Those vermicomposts added to container media significantly reduced infections of tomato plants by *P. nicotianae* and *F. oxysporum*. The vermicomposts from cattle manure protected about 52% more plants than control peat medium, the most effective were pure vermicomposts. Those results correlated with those reported earlier by Szczech and his co-workers with vermicomposts produced from cattle manure-inhibited club rot caused by *Plasmodiophora brassicae*. Szczech and Smolinska (2001) mentioned that potato plants treated with vermicompost were also less susceptible to *Phytophthora infestans* than plants treated with inorganic fertilizers, and the loss during storage due to dry and wet putridity was lower than that in controls. Edwards et al. (2004) also mentioned about the suppression of tomato late blight caused by *P. brassicae*, *P. nicotianae*, and tomato *Fusarium* wilt by *Fusarium lycopersici* via vermicompost applications reported by Nakamura (1996).

Following that, vermicomposts produced from household waste and organic industrial residuals were also tested for their inhibition efficacy on the plant pathogens in the same manner as vermicomposts from cattle manure. A comparative study was performed by Szczech and Smolinska (2001) to determine the suppressiveness of vermicomposts produced from animal manures and vermicomposts from sewage sludge against root rot of tomato plants caused by *P. nicotianae* var. *nicotianae*. They also studied the effect of the vermicomposts on total microflora and density of the pathogen in container media amended with vermicompost. They reported that although vermicomposts produced from sewage sludge strongly decreased the population of *P. nicotianae* in potting media; they were not suppressive against *P. nicotianae*. The decrease in the population of *P. nicotianae* in the media was suggested to be due to the high concentration of zinc in the vermicomposts. On the other hand, the vermicomposts from manures did not decrease the density of *P. nicotianae* in potting media; however, they were suppressive on the disease. They suggested that the suppression effect of the vermicomposts produced from manures against the pathogen were fungistatic rather than fungitoxic; however, other possible suppression mechanisms derived from chemical factors of the

vermicomposts, i.e., their pH value was not excluded. The fungistatic effect of soil amended with mature vermicomposts from municipal wastes on the infection of citrus seedlings by *P. nicotianae* earlier was reported by Widmer et al. (1998). Widmer et al. (1998) stated that fungistatic effect of soil amended with mature vermicomposts has reduced the infection of susceptible citrus seedlings by *P. nicotianae*, however, not inhibited the development of the pathogen in the soil. Hoitink and Grebus (1994) earlier explained that propagules of *Phytophthora* generally do not decline rapidly in suppressive soils. The propagules of *Phytophthora* depend on exogenous sources of nutrients and are sensitive to microbiostasis sustained by the activity of soil microflora.

Rodríguez-Navarro et al. (2000) incorporated vermicompost into the growth media and reported general suppression of fungal diseases of gerbera plants cased by soil-borne pathogens of *R. solani*, *Phytophthora drechsleri* and *F. oxysporium*. Chaoui et al. (2002) also demonstrated the disease suppression efficacy of substitution of low rates (10–30% by volume) of vermicompost into commercial bedding mixtures (MM360) in greenhouse. Chaoui et al. (2002) demonstrated significant reduce in disease attacks by fungus *Pythium* in cucumbers and *Rhizoctonia* in radishes in greenhouse. Substitution of small quantities into sterilized MM360 was adequate to induce *Pythium* suppression, and that was attributed to the presence of less aeration in the soil that might lead to a greater competition between *Pythium* and beneficial microorganisms for resources, since *Pythium* is reported to be suppressed through general microbial suppression (Craft and Nelson 1996). However, small vermicompost substitution rates (10% by volume) provided disease suppression on *Pythium*; however, the largest substitution rate (40% by volume) could sustain suppression on *Rhizoctonia*. They indicated the presence of *Trichoderma* spp. in the vermicompost, known as a bio-control agent for *Rhizoctonia*. They also achieved efficient suppression levels on disease incidences caused by *Verticillium* in strawberries and *Phomposis* and *S. fulginae* in grapes in field conditions. They stated that the ability of pathogen suppression disappeared when the vermicompost was sterilized, convincingly indicating that the biological mechanism of disease suppression involved was "microbial antagonism." Their results supported the statement earlier made by Edwards (1998) that earthworms promote microbial activity and diversity in organic wastes to levels even greater than those in thermophilic composts. Therefore, vermicomposts proved to have an even greater potential for suppression of plant diseases than aerobic composts, probably due to stimulatory effects of earthworms on soil microbial activity (thereby encouraging competing microorganisms).

Punja et al. (2002) evaluated the disease suppression potential of three composts (greenhouse waste, windrow dairy solids, and vermicompost dairy solids) and commercially available biological control agents (BCA) to reduce disease incidences of *Fusarium* root and stem rot, caused by *F. oxysporum* f.sp. *radicis-cucumerinum*, and *Pythium* damping-off and crown rot, caused by *P. aphanidermatum*. They reported that all three composts reduced root and stem rots to some degree, and autoclaved compost lost its suppression effect suggesting the microbial antagonism involved.

A comprehensive study on potentiality of a variety of disease control methods was carried out by Rose et al. (2003) against root and stem rot of cucumber caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum*. Crab/shrimp shell chitin; three composted media (greenhouse compost, windrow-composted dairy solids, and vermicomposted dairy solids); the BCA *Pseudomonas chlororaphis* strain 63-28, *Trichoderma harzianum* (RootShield Drench), *Streptomyces griseoviridis* (Mycostop), *Gliocladium catenulatum* (Prestop Mix) and *T.* (*Gliocladium*) *virens* (SoilGard); and the fungicides thiram or benomyl were added at seeding time to the rock wool block system followed by inoculation with either of the pathogens. The addition of windrow-composted dairy solids and vermicomposted dairy solids reduced plant mortality, but to a lesser extent than greenhouse compost. Plant mortality averaged 33.3 and 36.7% for windrow-composted dairy solids and vermicomposted dairy solids, respectively. From 3 to 4 weeks after seeding, however, the pathogen population recovered from compost-amended plants declined by 68%, whereas that recovered from nonamended plants declined by only 22% over the same time period. The population of *F. oxysporum* recovered from compost-amended plants declined by an additional 15% from 4 to 5 weeks after seeding, while the pathogen population recovered from nonamended plants increased by only 1%. When the composts were autoclaved, the disease-suppressive activity was significantly reduced, suggesting involvement of competing microorganisms in suppression of the pathogen. Rose et al. (2003) underlined the potential use of rock wool as the growth medium to sustain enhanced disease-suppression ability. They also pointed out the benefit of combined approaches enhancing disease control efficacy of *Fusarium* root and stem rot in greenhouse-grown cucumbers.

Rivera et al. (2004) mentioned the studies reported by Rivera, Wright, and their co-workers. They conducted considerable amount of work on disease suppression potentiality of vermicompost products to reduce disease incidences of soil-borne pathogens. Rivera et al. (2004) carried out the simultaneous evaluation of vermicompost as plant growth promoter and bio-control agent on damping-off tomatoes and African daisy (*Gerbera jamesonii*) caused by *R. solani*. The microbial composition of the plant growth media amended with vermicompost at rates of 0, 25, 50, 75, and 100% (by volume) was explored as well. Vermicompost incorporation at 20% rate reduced the incidence of *Rhizoctonia* root and crown rot on African daisy, and the addition of 25–100% of vermicompost promoted seedlings growth and prevented damping-off caused by *R. solani*. Thirty-six microorganisms were isolated, 13 of which were antagonistic to *R. solani* in vitro. They concluded that vermicomposts can be included in the development of effective alternatives to control tomato damping-off, and it may be an effective tool to promote seedlings growth as well.

Asciutto et al. (2006) evaluated the growth and suppression effect of vermicompost mixed with substrate at 75, 50, and 25% by volume on the disease incidence of damping-off of patience plants (*Impatiens walleriana*) caused by *R. solani*. Treatments with 100–75% of vermicompost showed important increases of leaf area, plant height, and fresh and dry weight of aerial and subterranean organs. They stated that crop protection was dose dependent, in which only 75% vermicompost diminished the incidence of damping-off caused by *R. solani*.

Stephens and Davoren (1997) from Australia have tested influence of presence of earthworms which are found in higher numbers under permanent pasture in southern Australia. They utilized *Aporrectodea trapezoides* and *Aporrectodea rosea* on subterranean clover and perennial ryegrass, grown in a red-brown earth soil artificially infested with *R. solani*. In infested soil, the presence of *A. trapezoides* (at a number equivalent to 300 m^{-2}) was associated with a significant reduction in the percentage length of roots containing *Rhizoctonia* lesions and a significant increase in shoot weight, root weight, and root length of both subterranean clover and perennial ryegrass. The results demonstrated the great potential of earthworms to reduce the deleterious effect of *R. solani* on the growth of the pasture plants.

Recently, Sahni et al. (2008) have compared the suppression effectiveness of two different approaches, in which two nonconventional chemicals $ZnSO_4$ and oxalic acid and the bio-control agent *Pseudomonas syringae* were applied as foliar sprays and seed coating, respectively, with the combination of vermicompost substitution in the potting soil against collar rot of chickpea caused by *Sclerotium rolfsii*. Vermicompost substitutions provided significant reduction in mortality of chickpea compared to control, but the suppression was more effective with treatments in which preinoculation with the nonconventional chemicals as foliar sprays were applied against the pathogen. Sahni et al. (2008) indicated the possible synergistic effect of both vermicompost and nonconventional chemicals increasing the plant vigor by altering host plant nutrition and induction of plant defense mechanisms, similar to systemic acquired resistance (SAR).

Simsek-Ersahin et al. (2009) compared the disease suppression efficiency of mature vermicompost (9 months old) with *T. harzianum* on damping-off of cucumber seedlings caused by *R. solani*. The vermicompost, produced from apple scabs, potato, and tree bark (*Salix* spp.), not fortified with *T. harzianum* achieved the same suppression level with that by the vermicompost fortified with *T. harzianum*. Simsek-Ersahin claimed that an antagonist bacterium observed during in vitro studies with water extracts of the vermicompost was responsible for that specific suppression mechanism on damping-off of *Rhizoctonia* in cucumber seedlings (Figs. 12.1 and 12.2).

12.2.2 Application of Solid Vermicomposts for Plant Pest Control

Edwards et al. (2004) briefly summarized studies reported by Arancon et al. (2002); Arancon (2004) has provided strong evidence that solid vermicomposts sometimes repel hard-bodied pests. Low vermicompost application rates in the field, or substitutions of vermicomposts into plant growth media in the greenhouse, have been shown to suppress numbers of plant parasitic nematodes (Arancon et al. 2002) and pest arthropods (Edwards et al. 2006, 2007; Yardim et al. 2006).

Fig. 12.1 An example of specific suppression by a bacterium against *Rhizoctonia solani*. Bacterium grows over the hyphae of *R. solani* (Unpublished data by Simsek-Ersahin)

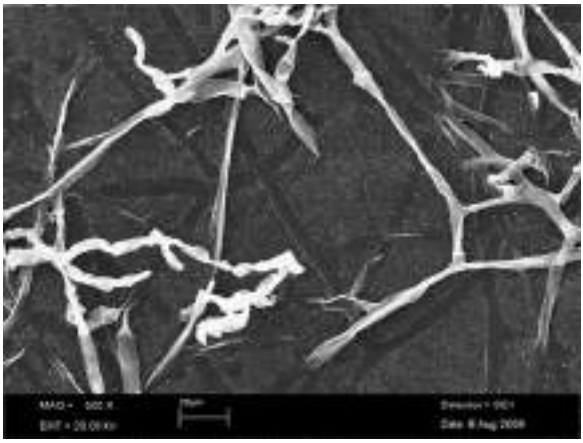

Fig. 12.2 An electron (SAM) photography of a hyper-parasitic bacterium over-growing and totally destroying the hyphae of *R. solani* (Unpublished data by Simsek-Ersahin)

12.2.2.1 Suppression of Plant Arthropod Pests

Various forms of organic matter (OM) applied at field conditions were known to cause a decrease in populations of arthropod pests and the resultant crop damage (Patriquin et al. 1995). Applications of OM and conventional thermophilic composts have suppressed attacks by pests such as aphids and scale insects (Culliney and Pimentel 1986; Yardim et al. 2006). Edwards and Arancon (2004) mentioned the studies of Biradar et al. (1998), Ramesh (2000), Rao et al. (2001), and Rao (2002) on suppression effects of vermicompost amendments on arthropod plant pests. Those earlier reports revealed that vermicomposts suppressed attacks and damages by jassids, aphids, and spider mites (Rao 2002) and psyllids (Biradar et al.

1998). Biradar et al. (1998) reported a clear correlation between the amounts of vermicomposts in the medium in which *Leucaena leucocephala* was grown and the degree of infestation by the psyllid *Heteropsylla cubana*. Ramesh (2000) reported suppression effect of vermicompost in groundnut plots attacked by sucking pests. Rao et al. (2001) stated that treatment of groundnut plots with vermicomposts decreased both incidence and overall densities of leaf miner (*Aproaerema modicella*). He also reported significant decreases in attacks by the jassid (*Empoasca verri*) and the aphid (*Aphis craccivora*) and changed predator population densities, in response to field applications of vermicomposts.

Edwards and Arancon (2004) in the Soil Ecology Laboratory at OSU, in the USA, carried out some detailed and elaborate greenhouse studies on the effects of vermicompost additions on arthropod populations and subsequent plant damage for tomatoes, pepper, and cabbages. They have demonstrated that low amendment rates of solid vermicomposts, 20 and 40% (by volume) in commercial plant growth medium MetroMix 360 (MM360), significantly decreased the populations and damage by aphids (*Myzus persicae*), mealy bugs (*Pseudococcus*) and cabbage caterpillars (*Pieris brassicae*). Cabbage seedlings grown in 100% MM360 lost more leaf area than those grown in the same media containing 20 or 40% vermicompost (Arancon et al. 2003). George Hahn, doing commercial vermicomposting in California (California Vermiculture), USA, claimed that vermicompost products repelled many different insect pests. His explanation is that this is due to production of enzymes "chitinase" by worms which breaks down the chitin in the insect's exoskeleton (Munroe 2007). Similar reports support the idea that suppression potentiality of vermicomposts on arthropod plant pest populations could lead to viable pest management methods.

In another study of Arancon et al. (2005), commercial vermicomposts produced from food waste have proved to be significantly suppressive on infestations and damages by aphids, mealy bugs, and cabbage white caterpillars. They amended vermicomposts into MM360, at rates of 100% MM360 with 0% vermicompost, 80% MM360 with 20% vermicompost, and 60% MM360 with 40% vermicompost to grow peppers, tomatoes, and cabbages. The substitution rates of 20 and 40% vermicomposts significantly suppressed populations of aphids (*M. persicae*) and mealy bugs (*Pseudococcus* spp.) in peppers, and mealy bugs in tomatoes. Vermicompost substitutions significantly decreased losses of dry weights of peppers after aphid and mealy bug infestations, and shoot dry weights of tomatoes after mealy bug infestations. The decreased losses in leaf areas of cabbage seedlings in response to the cabbage white caterpillar (*P. brassicae*) infestations were significant.

Arancon et al. (2007) continued to examine vermicomposts, produced commercially from food wastes, for its capacity to suppress populations and damage to plants, by two-spotted spider mites (*Tetranychus urticae*) on bush beans and eggplants, mealy bugs (*Pseudococcus* spp.) on cucumbers and tomatoes, and aphids (*M. persicae*) on cabbages in greenhouse. They had treatments in which vermicompost mixed at rates of 0, 10, 20, 40, and 80% with the MM360. Almost all treatments suppressed the arthropod pest populations and decreased pest damage

significantly. They stated that vermicomposts made the plants less attractive to the pests, and also had considerable decrease in pest reproduction over time.

Yardim et al. (2006) evaluated the effects of food waste vermicompost on populations of adult-striped cucumber beetles (*Acalymma vittatum*) and spotted cucumber beetles (*Diabotrica undecimpunctata*) on cucumbers and larval hornworms on tomatoes (*Manduca quinquemaculata*) in greenhouse and field experiments. In the field, cucumber and tomato plants were grown, with two different application rates (1.25 and 2.5 t ha^{-1}) of food waste vermicompost comparatively with inorganic fertilizer. Field cucumber beetle populations were suppressed significantly on cucumber plants treated with food waste vermicompost at both application rates. In the greenhouse, cucumber and tomato plants were grown in MM360 substituted with 0, 20, or 40% food waste vermicompost. Both the 20 and 40% vermicompost substitutes decreased damage by cucumber beetles to cucumber foliage and hornworms to tomato foliage, significantly.

12.2.2.2 Suppression of Plant Parasitic Nematodes

There is a great deal of literature on suppression effect of OM amendments into soils, providing satisfying levels of decrease in populations of plant parasitic nematodes (Akthar and Malik 2000). The literature showed that conventional thermophilic composts can suppress plant parasitic plant nematode populations (Gutpa and Kumar 1997; Khan et al. 2001a, b). Compared to OM and thermophilic compost amendments, there are only a few scientific reports on suppression effect of solid vermicomposts on populations and attacks of plant parasitic nematodes.

Morra et al. (1998) showed that applications of solid vermicomposts as soil amendments provided partial suppression on populations and attack of *Meloidogyne incognita*. Arancon et al. (2002, 2003, 2005, 2007) have conducted a great deal of work on solid vermicompost applications for control of plant parasitic nematode populations. They utilized solid vermicomposts in field treatments, ranging from 2 to 8 kg ha^{-1}, to tomatoes, peppers, strawberries, and grapes. They demonstrated significant success in suppression of plant parasitic nematodes. These researchers compared suppression capacity of vermicomposts produced from paper waste, food waste, and cattle manure of plant parasitic nematodes in field conditions and reported significant suppression. Arancon et al. (2003) investigated effects of vermicomposts on plant parasitic, fungivorous and bacterivorous nematode populations in grape and strawberry in field. Commercially produced vermicomposts derived from recycled paper and supermarket food waste were applied at the rates of 2.5 or 5.0 t ha^{-1} for the grape crop and 5.0 or 10 t ha^{-1} for the strawberry crops. Soils from all the vermicompost-treated plots contained smaller populations of plant parasitic nematodes than soil from inorganic fertilizer-treated plots. Conversely, populations of fungivorous nematodes and, to lesser extent, bacterivorous nematodes increased in the vermicompost-treated plots in comparison with that treated with inorganic fertilizers. They stated that the greater influence of vermicomposts on fungivorous than bacterivorous nematode populations is possibly due

to the fact that fungi serve as a major source of food for earthworms, and earthworms facilitate dispersal of fungi by excreting fungal spores in their casts as reported earlier by Edwards and Fletcher (1998).

12.3 Application of Vermicompost Extracts for Plant Disease and Pest Control

Since, compost extracts are much easier to handle and apply to crops than solid composts, which are bulky and heavy and need soil incorporation, these extracts are increasingly being applied, as soil drenches or soil and foliar sprays, to promote plant growth and control plant diseases and pests, especially in organic production systems. Potentiality of vermicompost extracts as fertilizer and bio-control agents has been recognized after the extensive studies carried on thermophilic compost extracts. Hence, production and application of vermicompost tea is a considerably new developing area; there are few reports presenting solid scientific data on the issue. Utilization of vermicompost extracts/teas as bio-control agents has accelerated within the last decade. There is still a big gap in methodology for production and uses of vermicompost teas in terms of optimal dilution and application rates with respect to targeted plant pest or pathogen (Edwards et al. 2007).

12.3.1 Application of Vermicompost Extracts for Plant Disease Control

Growth effect of aqueous extracts from vermicompost on ornamental plants showed similar patterns as with the addition of auxins, gibberellins and cytokinins through the soil (Tomati et al. 1988). There are only a few literatures available that have investigated the quality of vermiliquids and their use for disease and pest control. Pesticide spray was significantly reduced where earthworms and vermicompost were used in agriculture (Singh 1993). Edwards et al. (2004) mentioned that the study of Nakasone et al. (1999) reported that aqueous extracts of vermicomposts inhibited the mycelial growth of *Botrytis cinerea*, *Sclerotinia sclerotum*, *Corticium rolfsii*, *R. solani* and *Fusarium oxysporum*. Latter, Szczech and his co-workers, Rodríguez-Navarro et al. (2000), and Zaller (2006) showed that the aqueous extracts of vermicomposts depress soil-borne or foliar plant pathogens and pests.

Singh et al. (2003) conducted a study in which the effect of foliar application of the aqueous vermicompost extracts against powdery mildews of pea and balsam caused by *Erysiphe pisi* and *Erysiphe cichoracearum*, respectively, was examined. Aqueous vermicompost inhibited significantly in vitro spore germination of several saprophytic and phytopathogenic fungi (*Alternaria solani*, *A. brassicae*, *A. alternata*, *Curvularia penniseti*, *Curvularia maculans*, *Curvularia palliscens*, *Curvularia* spp.

Helminthosporium speciferum, *Helminthosporium penniseti*). However, under field conditions, post-inoculation treatment offered better protection than preinoculation to pea from *E. pisi*, and the effect of aqueous vermicompost on balsam against powdery mildew was effective in both pre- and post-inoculation treatments where the latter was better. However, this variable effect is difficult to explain. Those results have indicated the high potentiality of the use of vermicompost as a viable alternative method of controlling plant diseases. Singh et al. (2003) suggested that the foliar application of aqueous extracts are to be utilized for the farmers as an easy, cheap, and eco-friendly system of crop protection with high-yielding capacity.

Zaller (2006) examined effects of foliar sprays with aqueous vermicompost extracts on growth, yields, morphological and chemical fruit quality, and their disease suppression potentiality on natural infection with late blight disease (*P. infestans*) on three tomato varieties. However, he reported decreased susceptibility of tomato leaves, stems, and fruits to natural infection by *P. infestans* in which only half as many vermicompost sprayed plants showed clear signs of *P. infestans* infection as compared with water sprayed control. On the other hand, he stated no difference between the vermicompost extract or water control treatments with respect to the severity of the infection. Zaller (2006) indicated the high potentiality of vermicompost products in organic farming not only as a substitute for peat in potting media but also as foliar sprays for fertilization and biological disease control.

Manandhar and Yami (2008) conducted a field study comparing the potentiality of aqueous extracts of aerobic composts and vermicomposts on foot rot disease of rice caused by *Fusarium moniliforme*. They applied four treatments as aerated vermicompost tea (ACTV), nonaerated vermicompost tea (NCTV), aerated compost tea (ACTC) and nonaerated compost tea (NCTC). They reported that ACTV showed statistically significant maximum control efficiency followed by ACTC, and the least effect was obtained by NCTC. Following that field trial, treatment of the rice seeds with compost tea revealed highest efficiency of ACTV in reducing the number of affected seeds followed by ACTC and NCTV, and the least effective was the NCTC. They concluded that the effectiveness of compost extract seems to depend on many factors including method of preparation (aerated or nonaerated), extraction time, the type of compost used (compost or vermicompost) and crop applied. Maturity of the compost extracted and consistency in resultant effects of compost extract were suggested to be more variable among all factors. They pointed out the need for field evaluations on specific crops and specific disease organisms over a long period to account for year-to-year variations in disease dynamics before making recommendations to farmers for compost tea use in plant protection systems.

As Zaller (2006) discussed the ongoing debate between researchers on which preparation method of compost teas, produced by either anaerobically or aerobically, is more effective on its disease suppression efficacy. There are reports supporting either of the claims. Zaller (2006) mentioned that anaerobically prepared extracts produced from spent mushroom substrate were more effective in inhibiting apple scab than aerobically treated ones. Manandhar and Yami (2008)

mentioned that Weltzien (1989) promoted the anaerobic method of compost extract preparation. Those who are in favor of anaerobic compost extract preparation claimed that the likely disease-suppressive effect of anaerobic method of compost extract preparation to be due to a metabolite produced by anaerobic microorganisms in the extract.

On the other hand, there is evidence supporting that aerobically produced compost extracts are much more effective and aerobic microbes dominate compost extracts (Hoitink et al. 1997). Edwards et al. (2006) have demonstrated clearly that aerobically produced vermicompost teas are much more stable and effective than those produced without aeration. The research team in Ohio Soil Laboratory, in OSU, identified microorganisms and plant growth regulators such as hormones, humic acids and fulvic acids present in the vermicompost tea as the most probable factors for growth increases in tomatoes. Most published research on the use of compost tea for disease suppression utilized passive compost teas. Although passive compost tea is not aerated, it is not necessarily anaerobic unless additional technology and nutrients are added. If a passive tea turns anaerobic, it can putrify, rather than ferment, which may produce phenols and alcohol that can harm plants and beneficial soil microorganisms (Edwards et al. 2006).

As stated by Yami and Shrestha (2005), the vermicompost acts like a buffer for plants where soil pH is too high or low, making soil nutrients available to the plant. Vermicompost is biologically active and the castings are much higher in bacteria, OM, available nitrogen, calcium, magnesium, phosphorus and potassium than soil itself. Yami and Shrestha (2005) assessed greater diversity of beneficial microorganisms including nitrogen fixing bacteria in the vermicompost than in the compost. Worms play a vital role in creating the optimum conditions for the beneficial organisms to establish and reproduce. These "beneficial" organisms outcompete with and dominate the harmful microbes. Yami and Shrestha (2005) described an abundance of oxygen and nitrogenous compounds (urea, proteins, and NH_3) in the vermicast and mucus secreted from the external tissues of the worms. Zaller pointed out that the underlying mechanisms in use of vermicompost extracts for plant protection are not clearly understood, but involvement of induced resistance is mostly considered as claimed by Fokkema (Zaller 2006). The possible disease suppression mechanisms for vermicompost products were discussed in detail in Sect. 16.4.

12.3.2 Application of Vermicompost Extracts for Plant Pest Control

The researchers at Ohio State University, USA, claimed that some form of agitation or aeration is needed during production of vermicast teas to be used as plant growth promoter or plant disease control agent. They showed successful transformation of the microbial activity and diversity, enzymatic activity, and the key nutrients from solid vermicomposts into the teas (Edwards et al. 2007).

12.3.2.1 Suppression of Plant Arthropod Pests

The experiments on the plant arthropod pests were carried out on the two-spotted spider mite: *Tetranycus* spp. and *M. persicae* by Arancon et al. (2007). Either thermophilic or vermicompost tea were sprayed onto the tomato seedlings and then the pests were released into the cages where the seedlings were placed. The control efficiency of the vermicompost tea outperformed that of thermophilic compost tea in experiments with both plant pests. Although the vermicompost tea has significantly prevented the plant damages caused by both pests, thermophilic compost tea has no effect on control of both pests. These results are in accordance with those performed on control efficiency of solid vermicompost on the same group of plant pests as spider mites and aphids (Arancon et al. 2007).

Edwards et al. (2009) recently published a report aimed to reveal the effects of "aqueous vermicomposts" extracts, on numbers and damage by green peach aphids (*M. persicae*), citrus mealybugs (*Pseudococcus citri*), and two-spotted spider mites (*T. urticae*) infesting tomatoes and cucumbers in greenhouse. The ratio of vermicompost to water was 1–5 (v:v) yielding a 20% aqueous solution. The effects of vermicompost extracts at dilutions of 20, 10, and 5%, respectively, were applied to the plants as soil drenches to field capacity at germination and thereafter at weekly intervals. All the vermicompost extracts suppressed pest establishment on the plants and rates of reproduction of all three pest species, significantly. They also caused some of the pests on the plants receiving the higher extract application rates to die after 14 days of treatment. The higher the rate of extract application, the greater was the suppression of the pests. They concluded that the most likely cause for the unacceptability of the plants to pests and decreased reproduction and mortality was the uptake of soluble phenolic materials from the vermicompost extracts into the plant tissues. These compounds are known to make plants unattractive to pests and decrease pest rates of reproduction and survival.

12.3.2.2 Suppression of Plant Parasitic Nematodes

Some earlier studies on application of vermicast tea for inhibition of damages by plant pests were carried out on plant parasitic nematodes (Arancon et al. 2002) and pest arthropods (Arancon et al. 2005; Edwards et al. 2007; Yardim et al. 2006). The researchers demonstrated control efficiency of vermicast tea on the root knot nematodes and arthropod pests, i.e., aphids and spider mites. They performed number of greenhouse and field experiments to test the suppression efficiency of vermicompost tea as soil drench in tomato seedlings infested with the eggs of *Meloidogyne hapla*. They reported considerable decrease in the number of root knot galls on tomato roots and significant growth effect on the seedlings as well. Those results were in accordance with those stated earlier by Arancon et al. (2003) who revealed the control efficiency of solid vermicompost on plant parasitic nematode attacks.

12.4 Mechanisms of Plant Disease Suppression by Vermicompost Products

Overall research on applications of vermicompost products, either solid or liquefied, to control plant diseases has shown that suppression effect has a biological nature rather than chemical (Szczech 1999; Simsek-Ersahin et al. 2009), similar to that of compost products. An overwhelming body of evidence in the literature indicated that microbial communities, stimulated by conventional aerobic compost amendments, are responsible for disease suppression (Hoitink and Fahy 1986; Hoitink et al. 1997; Hoitink and Krause 1998; Hoitink and Boehm 1999; Krause et al. 2001; Scheuerell et al. 2005). Proposed disease suppression mechanisms for control of plant pathogens via application of conventional composts are defined within two types as general and specific suppression mechanisms that include microbial antagonism, nutrient release, induced host resistance, and abiotic inhibitory factors of disease suppression. Edwards et al. (2004) stated that it is likely that these two mechanisms of suppression also apply to vermicompost products. They suggested that "general suppression mechanism" would be the more predominant case for vermicomposts, since vermicomposting greatly enhances both biodiversity and number of microorganisms. Earthworms are known to promote microbial activity and diversity in organic wastes to levels even greater than those in thermophilic composts (Edwards et al. 2004). Thus, that enhanced suppression ability of the vermicomposts on plant pathogens is mostly attributed to its microflora, since suppressiveness mostly disappeared after autoclaving as reported in the literature (Szczech and Smolinska 2001; Simsek-Ersahin et al. 2009).

One of these disease suppression mechanisms termed as "general suppression" has been defined by nutrient competition, antibiosis, in which beneficial organisms secrete antibiotics that directly inhibit the pathogen, hyperparasitism/direct parasitism (one organism feeding on another), and possibly induced systemic plant resistance (Hoitink and Grebus 1994). That suppression mechanism has been described based on the concept that a high biodiversity of microbial populations act as biocontrol agents that creates conditions such as fungistatis (Serra-Whittling et al. 1996) unfavorable for plant pathogens to develop. Suppression of nutrient-depended plant pathogens such as *Pythium* and *Phytopthora* was explained by general suppression mechanism (Krause et al. 2001; Chen et al. 1988; Mandelbaum and Hadar 1990). Hoitink and Grebus (1994) suggested that this mechanism is also effective in suppression of human pathogens, such as coliform bacteria and other fecal pathogens. The scientific explanation behind this concept is that agronomically beneficial microorganisms block the pathogens' excess to plant roots by occupying all the available sites. This concept is based on "soil-foodweb" studies pioneered by Dr. Elaine Ingham of Corvallis, Oregon, USA (http://www.soilfoodweb.com).

One of the factors defined within "general disease suppression" by organic amendments is the concept of induced systemic resistance (ISR). Induced systemic resistance in plants triggered by compost applications is suggested to be possibly derived from the presence of some antibiotics and actinomycetes in vermicomposts

which increases the "power of biological resistance" (Zhang et al. 1998; Pharand et al. 2002). However, this explanation is the least mutually accepted claim among all those proposed for general disease suppression mechanisms. The concept of ISR is based on the idea that the response of plants growing in the soil contributes to suppressiveness to disease incidences. That occurs, in case of soil-borne pathogens, when the rhizosphere is inoculated with a weakly virulent pathogen. After being challenged by a weak pathogen, the plant develops the capacity for future effective response to a more virulent pathogen. In most cases, adding mature high-qualified compost products to a soil induces disease resistance in many plants (Zhang et al. 1998; Pharand et al. 2002).

The other factors defined within general disease suppression context are the antibiosis and competition. Antibiosis is the secretion of antibiotics by some beneficial organisms and direct parasitism by other beneficial microorganisms on pathogens. Application of vermicompost products for soil-borne plant disease and pest control aims to create soil conditions with all these factors present. Therefore, adding vermicompost to soil greatly enhances the numbers and diversity of competitors, inhibitors, and predators of disease organisms, as well as the food sources on which these beneficial organisms depend. The food for beneficial organisms is more readily available in vermicomposts comparing with the aerobic compost and other organic wastes. The food in OM and waste products require the preliminary process through the growth of other organisms, such as soil predators and microorganisms, before it becomes available for these beneficial microorganisms (Edwards et al. 2004).

Competition is the most commonly defined factor within the context of general disease suppression mechanism in use of composts, produced by either aerobic composting or vermicomposting. Therefore, the level of disease suppressiveness is typically related to the level of total microbiological activity (active microbial biomass) in a soil. The larger the soil's active microbial biomass, the greater the soil's capacity to use carbon, nutrients, and energy, lowering the nutrient availability to pathogens. In other words, when most soil nutrients are tied up in microbial bodies, the competition for readily available mineral nutrients gets a higher level. Competition context suggests that limited nutrients are a key for general suppression. Hence, with an abundance of free nutrients, commonly seen in case of unmature aerobic compost products, the pathogen can prosper (Hoitink and Grebus 1994). Virtually, any treatment to increase the total microbial activity in the soil will enhance general suppression of pathogens by increasing competition for nutrients.

In case of deficiency of readily available nutrients in soil environment, microbial associations with mychorrhizal fungi and bacteria that live on and near the roots become vital for plants. These fungi scavenge nutrients for the plant to use. In return, the plant provides carbon in the form of sugars and proteins to the microbes. This symbiotic system supports the beneficial organisms and the plant, but generally excludes the pathogens that would attack the plant. The earthworms have been long known to transmit and propagate the mychorrhizal fungi in soil (Turk et al. 2006). Among the most beneficial root-inhabiting organisms, mycorrhizal fungi can cover plant roots, forming the structure named as "fungal mat." The mycorrhizal

fungi protect plant roots from diseases in several ways such as forming a physical barrier, antibiotic secretion, increasing nutrient uptake capacity of plant roots. The physical barrier provides a physical exclusion more likely to the invading pathogens such as soil insects and nematodes rather than bacteria or fungi. However, some studies have shown that nematodes can penetrate the fungal mat. Mycorrhizal fungi can produce a variety of antibiotics and other toxins that act against pathogenic organisms. They also increase the nutrient-uptake capacity of plant roots. For example, improved phosphorus uptake in the host plant has commonly been associated with presence of mychorrhizal fungi. When plants are not deprived of nutrients, they are better able to tolerate or resist disease-causing organisms. Mycorrhizal fungi can also change the amount and type of plant root exudates. As the root exudates change, pathogens dependent on stimulation by certain exudates cannot locate the plant for penetration (Turk et al. 2006).

The second type of disease suppression mechanism is named as "specific mechanism" in which only a narrow range of microorganisms facilitate the suppression or one organism directly suppresses a known pathogen. These are cases where a biological control agent is introduced into the soil for the specific purpose of reducing disease incidence (Hoitink et al. 1997). That mechanism is suggested to be responsible for suppression of pathogens such as *R. solani* and *S. rolfsii*. Control of *Rhizoctonia* and *Sclerotium* is problematic because of their large propagules (Krause et al. 2001; Scheuerell et al. 2005; Termorshuizen et al. 2006). Their propagules, assuring them to be less reliant on external energy or nutrient sources, make them "nutrient-independent pathogens" and un-susceptible to microbial competition. Control of these two pathogens is carried out through "specific" beneficial organisms such as *Trichoderma* and *Gliocladium* that will colonize the harmful propagules and reduce the disease potential. *R. solani* is highly competitive as a saprophyte, but it cannot colonize low-cellulose mature compost while i.e., *Trichoderma* spp. is capable of colonizing fresh as well as mature compost (Chung et al. 1988). The beneficial fungus *Trichoderma* locates *Rhizoctonia* through a chemical released by the pathogen and then attacks it. Fungal strands of *Trichoderma* entangle the pathogen and release enzymes that dehydrate *Rhizoctonia* cells, eventually killing them. The decomposition level of OM in compost-amended substrates has a major impact on disease suppression (Hoitink et al. 1997).

12.5 Mechanisms of Plant Pest Control by Vermicompost Products

Mechanisms in which vermicompost suppress the plant pest attacks are still speculative as stated by Edwards et al. (2004). Two of the mechanisms that have been suggested by researchers for decreasing pest attacks are based on differential availability of mineral nutrients to plants and on changes in the balance of available nutrient elements that could affect aspects of plant morphology and physiology in ways that could render plants more resistant to pest attacks (Patriquin et al. 1995).

The change in the nutrient characteristics and balances of plants in response to vermicomposts is possibly derived from the phenol contents of plant leaves. Organic nitrogen being released more slowly from organic amendments i.e., vermicomposts than from inorganic fertilizers makes plants less susceptible to arthropod attacks (Patriquin et al. 1995). Arancon et al. (2007) described the possible mechanisms for the arthropod suppression by solid vermicomposts including the form of nitrogen available in the leaf tissues, the effects of vermicomposts on micronutrient availability, and the possible production of phenols, by the plants after applications of vermicomposts, making the tissues unpalatable. They hypothesized that decreased insect pest numbers and damage on plants grown with vermicomposts, in both greenhouse and field experiments (Yardim et al. 2006), could be attributed at least partially to changes in the form of N, a controlled slower release rates of mineral nutrients and particularly by the production of phenolics through the use of vermicomposts. Further research is needed to support this hypothesis and to further identify mechanisms by which vermicompost suppress arthropod pest feeding and reproduction.

Edwards et al. (2004) described a number of possible mechanisms for the decrease in populations of plant parasitic nematodes by solid vermicomposts. He indicated the possibility of those predatory–prey interactions, decreasing populations of plant parasitic nematodes. For that, vermicomposts might increase numbers of omnivorous nematodes or arthropods that selectively prey on plant parasitic nematodes, or they might also promote the growth of nematode-trapping fungi as well as species of fungi that attack and destroy nematode cysts and affect populations of plant parasitic nematodes. Another possibility for that is the possibility of *Rhizobacteria* colonization on roots and killing plant parasitic nematodes by producing enzymes and toxins that are toxic to them. He also mentioned the possible effects of biotic interactions and abiotic factors provided by vermicomposts. By that, vermicompost might contain compounds that might affect the survival of nematodes such as hydrogen sulfide, ammonia, and nitrates during vermicomposting.

Overall, the costs of vermicomposts, proved to be effective at the low application rates (Arancon et al. 2002; Yardim et al. 2006), are much lower than those of nematicides and insecticides. In addition, vermicomposts are a natural way to add nutrients and plant growth regulators to the soil, control diseases, and prevent soil contamination resulted from pesticide applications. Hence, use of vermicomposts for arthropod pest and parasitic nematode control has great potentiality, specifically for organic, production in horticulture and agriculture.

12.6 Important Factors of Disease Suppression Mechanisms by Vermicompost Products

Hoitink et al. (1991) stated that composts must be adequately stabilized and analyzed for maturity to assess the carrying capacity or the potential of a soil mix to support sustained microbial activity that will carry out “general suppression.”

By that, primary factor for sustaining suppression effect was designated as the unique active microbial biomass of the vermicast produced through worm digestive system, secreting mucus that amplifies both the variety and total biomass of bacteria, fungi (Szczech 1999), and actinomycetes (Szczech and Smolinska 2001) in vermicast, and promoting microbial competition (Edwards and Arancon 2004). The fact that the suppression impact was lost both in vitro and in vivo conditions upon heat sterilization of both the water extracts of the vermicompost (Simsek-Ersahin et al. 2009) and the vermicompost itself (Krause et al. 2001) signifies the microbiological nature of the suppression effect by the vermicompost.

The second important factor sustaining disease suppress effect of vermicompost is the decomposition level of the product. Decomposition level of the organic material, such as compost products, was stated to have a substantial effect on both composition and activities of the BCA (Hoitink and Boehm 1999). Similarly, additional curing period of vermicompost is thought to intensify the activities of naturally occurring BCA (Simsek-Ersahin et al. 2009). Third, proportion of the vermicompost used in potting mixes was also defined to be co-related with the proportion of the vermicompost used in the potting mixtures, reaching absolute suppress level at 20% (v/v) (Szczech 1999; Simsek-Ersahin et al. 2009). Noble and Coventry (2005) stated that compost amendment rates of at least 20% (v/v) were normally required to maintain consistent disease suppress effect, particularly in peat-based media. Simsek-Ersahin et al. (2009) compared the effectiveness of vermicast, cured within 9 months, with *T. harzianum* for control of damping-off of cucumber seedlings caused by *R. solani*. They had treatments in which pots amended at four different rates (0, 10, 20, 30%, (v/v)) with vermicompost fortified with either *T. harzianum*, the known bio-control agent, or antagonistic activity toward *R. solani*. They concluded that amendments of vermicompost not fortified with *Trichoderma* provided a consistent and efficient suppressiveness against the pathogen even at lower vermicompost amendment rates (20% (v/v)).

Literature on suppression of plant diseases by aerobic and vermicompost products showed that a strong correlation exists between the composition of the microflora of the compost product and pathogen type. Some pathogens like *Rhizoctonia* require presence of some specific antagonists not consistently colonized in all types of OM. Simsek-Ersahin et al. (2009) suggested that the suppressiveness of the vermicompost on *Rhizoctonia* was due to specific disease suppression mechanism rendered by an antagonist bacterium isolated from the water extracts of the vermicompost (Figs. 12.1 and 12.2). The control of pathogens such as *Pythium*, *Fusarium*, and *Phytophthora*, instead, has often been related to general suppression due to compost amendments. Feedstock origin (Termorshuizen et al. 2006), compost maturity, and application rate (Serra-Whittling et al. 1996) are the most important factors for predicting compost suppressivity. Phytotoxicity occurs only rarely with composts and is limited to immature materials (Widmer et al. 1998) and very high application rates (Erhart et al. 1999; Szczech and Smolinska 2001).

Although, use of vermicompost products for plant disease and pest suppression has a high potentiality, the underlying mechanisms for suppressiveness have not been defined explicitly, and very little is known about the relationships between the

microbiological characteristics of vermicompost and disease suppression. Compost amendments produced from either aerobic composting or vermicomposting are mostly suppressive; however, some variable responses have been cited. None of the microbiological parameters that consistently predict vermicompost suppression has been elucidated. A promising parameter for prediction of vermicompost suppressivity is fluorescein diacetate hydrolytic activity (FDA) suggested by Chen et al. (1988) which includes several soil enzymes (nonspecific esterases, proteases, lipases) related to OM decomposition. FDA has been found in several studies to positively correlate with soil disease-suppressive capacity toward *Pythium* (Craft and Nelson 1996; Stone et al. 2004), but was unreliable in other cases (Erhart et al. 1999).

12.7 Conclusions

Along with increasing concerns on adverse side effects of agrochemicals and improper organic waste management methods, alternative plant disease and waste management techniques have been widely searched and studied since 70s. Vermiculture, as a new industrial domain, provides a wide variety of alternatives for biodegradable organic waste management and agrochemical-free food production. Vermicomposting has tremendous potential to use a wide range of feedstocks of on- and off-farm wastes including those generated in agriculture, food processing, wood processing, sewage treatment, industrial, and municipal wastes. The vermitechnology is still under development and being utilized all over the world for mid- to large-scale vermicomposting of many organic wastes and several companies have come up in the last decade in US, Canada, New Zealand, Japan, France, and less-industrialized ones i.e., India, Cuba, Philippines, Argentina. Vermicomposting is an excellent example of the practical use of biotechnology. Vermicomposting involves highly complex biological processes, including many species of bacteria, fungi, and actinomycetes, which convert a low-valued material into a higher valued product. The product, also named as vermicastings/vermicast, could be utilized in either solid or aqueous forms and has proved to be an excellent organic fertilizer in agriculture and horticulture production.

There has been extremely limited amount of work carried out to determine the impacts of vermicompost products on soils and crops. There is strong need to optimize the production and use of vermicompost products to improve crop quality without compromising food safety. The research conducted on vermicompost applications, in either solid or aqueous forms, for plant protection purposes proved them to be effective on suppression of pathogens and pest attacks. There is no doubt that the benefits of vermicompost products far outweigh their drawbacks; however, the impact of this technique on pathogen populations and disease suppression remains unpredictable. Also, there is still a huge gap in development of reliable guidelines to predict the impact of any form of, solid or liquefied, vermicompost products on specific plant diseases or pest attacks. Desirable vermicompost

characteristics and their assessment for disease and pest suppression are still under development. Suppression variability and inadequate understanding of variables and parameters that affect it are the principal factors limiting the wider utilization of vermicompost products for disease suppression purposes. Vermicompost application rate, feedstock types, maturity level, production method, target pathogen, and active microbial biomass in vermicomposts are among the sources of variability.

It is necessary to continue studies for increasing the knowledge of disease suppression mechanisms and parameters that consistently predict the suppressive ability and the possibilities of their incorporation in environmentally friendly and sustainable crop production systems. In future, plant disease control, presently provided mostly by agrochemicals, will be complemented or replaced by new disease control technologies emerging from the basic knowledge of plant–microbe interactions within the context of OM amendments. However, before a general use of vermicompost products in crop protection, nontargeted effects and social concern should be minimized or counteracted. If vermiproducts (worms, vermicastings, liquefied extracts) are able to substitute "agrochemicals" in agriculture and horticulture production, it would certainly help generate "truly sustainable food production systems" providing "chemical-free food" for people in future.

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Chapter 13
Vermicompost as a Biological Soil Amendment

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13.1 Introduction

Today, the viability of using earthworms as a treatment technique for numerous waste streams has been well established. Vermicompost is considered as an excellent product since it is homogeneous, has desirable esthetics, reduced level of contaminates, plant growth hormones, higher level of soil enzymes, and greater microbial population, and tends to hold more nutrients over a longer period without adversely impacting the environment. Earthworms while ingest organic waste and soil, consume heavy metals through their intestine as well as through their skin, wherefore concentrating heavy metals in their body (Hand et al. 1988; Logsdon 1994; Singh and Sharma 2002).

A growing awareness of some of the adverse economic and environmental impacts of agrochemicals in crop production has stimulated greater interest in the utilization of organic amendments such as compost or vermicompost for crop production (Follet et al. 1981). Utilization of earthworms may be an answer as an ecologically sound, economically viable, and socially acceptable technology (Sharma et al. 2005).

Vermicomposting as a principle originates from the fact that earthworms in the process of feeding fragment the substrate thereby increasing its surface area for further microbial colonization (Chan and Griffiths 1988). During this process, the important plant nutrients such as nitrogen, potassium, phosphorus, and calcium

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present in the feed material are converted through microbial action into forms that are much more soluble and available to the plants than those in the parent substrate (Ndegwa and Thompson 2001). Earthworms are voracious feeders on organic waste and while utilizing only a small portion for their body synthesis they excrete a large part of these consumed waste material in a half digested form. Since the intestine of earthworms harbor wide ranges of microorganisms, enzymes, hormones, etc., these half digested substrates decompose rapidly and are transformed into a form of vermicompost within a short time (Edwards and Lofty 1972). Earthworm prepares organic manures, through their characteristic functions of breaking up organic matter and combines it with soil particles. The final product is a stabilized, well-humidified, organic fertilizer, with adhesive effects for the soil and stimulator for plant growth and most suitable for agricultural application and favorable environmentally. Biochemical changes in the degradation of organic matter are carried out through enzymatic digestion, enrichment by nitrogen excrement, and transport of organic and inorganic materials. About 5–10% of ingested material is absorbed into the tissue for their growth and metabolic activity and rest is excreted as vermicast. The vermicast is mixed with mucus secretion of the gut wall, and of the microbes and transformed into vermicompost (Edwards and Lofty 1972). The decomposition process continues even after the release of the cast by the establishment of microorganisms. The studies on the effect of vermicomposting on some components of organic waste showed that vermicompost enhances degree of polymerization of humic substances along with a decrease of ammonium N and an increase of nitric N (Cegarra et al. 1992).

Vermicompost is a peat-like material with high porosity, aeration, drainage, water holding capacity, and microbial activity, (Edwards and Burrows 1988), and has large particular surface area that provides many microsites for microbial activity and for the strong retention of nutrients. The plant growth regulators and other plant growth influencing materials, that is, auxins, cytokinins, humic substances, etc., produced by microorganisms have been reported from vermicompost (Atiyeh et al. 2002; Muscolo et al. 1999). The humic materials extracted from vermicomposts have been reported to produce auxin-like cell growth and nitrate metabolism of carrots (*Daucus carota*) (Muscolo et al. 1999).

13.2 Characteristics of Vermicompost

The nutrient status of vermicompost produced with different organic wastes is: organic carbon 9.15–17.98%, total nitrogen 0.5–1.5%, available phosphorus 0.1–0.3%, available potassium 0.15, calcium and magnesium 22.7–70 mg per 100 g, copper 2–9.3 ppm, Zinc 5.7–11.5 ppm, and available sulfur 128–548 ppm (Kale 1995).

Several researchers have compared vermicasts with the surrounding soils and reported their results (Lavelle 1978). The vermicasts have been reported with a higher Base Exchange capacity and are rich in total organic matter, phosphorus, potassium, and calcium with reduced electrical conductivity, large increase in

oxidation potential, and significant reductions in water-soluble chemicals which constitute possible, environmental contaminants. Vermicompost is rich in microbial diversity, population, and activity (Subler et al. 1998), and vermicast contains enzymes such as *proteases*, *amylases*, *lipase*, *cellulase*, and *chitinase* which continue to disintegrate organic matter even after they have been ejected. The chemical analysis of casts shows 2 times the available magnesium, 5 times the available nitrogen, 7 times the available phosphorus, and 11 times the available potassium compared to the surrounding soil (Bridgens 1981). The vermicompost is considered an excellent product since it is homogeneous, has reduced level of contaminants, and tends to hold more nutrients over a longer period without impacting the environment (Ndegwa and Thompson 2001).

13.3 Potential Application of Earthworms and Vermicompost in Plant Growth

Being rich in macro- and micronutrients, vermicompost has been found an ideal organic manure enhancing biomass production of a number of crops (Vasudevan and Sharma 1997; Hidalgo 1999; Pashanasi et al. 1996). The importance of vermicompost in agriculture, horticulture, waste management, and soil conservation has been reviewed by many workers (Edwards et al. 1995; Riggle and Holmes 1994; Kaviraj and Sharma 2003). Darwin (1881) stated that the earthworms prepare the ground in an excellent manner for the growth of fibrous-rooted plants and for seedlings of all kinds. The beneficial effect of earthworms on plant growth may be due to several reasons apart from the presence of macronutrients and micronutrients in vermicasts and in their secretions in considerable quantities. It is believed that earthworms produce certain metabolites, vitamins, and similar substances into the soil which may be the B or D group vitamins (Nielson 1965). The use of earthworm compost in commercial production was advocated by Martinez and Gomez Zambrano (1995).

Edwards et al. (1995) reported that in a Rothamsted study with 25 types of vegetables, fruits, or ornamentals, earthworm casts performed better than compost or commercial potting mixture amendments. The beneficial effects of earthworm cast utilization in other horticulture settings have also been reported (Hidalgo 1999). Fresh casts often contain high ammonium levels, but rapid nitrification results in stable levels of both nitrogen forms due to organic matter protection in dry casts (Decaëns et al. 1999). Nutrients in casts are initially physically protected, but this is reduced as the aggregate structure weakens over time (McInerney and Bolger 2000). In addition to increased N availability, C, P, K, Ca, and Mg availability in the casts is also greater than in the starting feed material (Orozco et al. 1996). Earthworm cast amendment has been shown to increase plant dry weight (Edwards et al. 1995) and plant N uptake (Tomati et al. 1994). Cantanazoro et al. (1998) demonstrated the importance of the synchronization between nutrient release and plant uptake and showed that slower release fertilizers can increase plant yield and reduce nutrient leaching. Soil quality is affected by soil aggregates

and these aggregates often determine the retention and movement of water, diffusion of gasses, growth and development of roots in the soil.

13.4 Metals and Agrochemicals Accumulation from Soil by Earthworms

Earthworms ingest large amount of soil and are therefore exposed to heavy metals through their intestine as well as through the skin, therefore concentrating heavy metals from the soil in their body (Morgan and Morgan 1999). Earthworms may serve as bioindicators of soil contaminated with pesticides, that is, polychlorinated biphenyls, polycyclic hydrocarbons (Saint-Denis et al. 1999), and heavy metals (Spurgeon and Hopkins 1999). Lead, cadmium, zinc, and copper are accumulated and under some environmental conditions, bioconcentrated in earthworms (Cortet et al. 1999). It is presumed that in many cases zinc is the critical toxic metal for these organisms (Spurgeon and Hopkins 2000). Mortality and fecundity of earthworms as bioindicating organisms may serve as reliable, but ultimate and time-consuming, indices of environmental pollution (Morgan and Morgan 1999).

Suppression of labile aluminum in acidic soils by the use of vermicompost extract was observed by chelation combined with pH-induced precipitation (Mitchell and Alter 1993). The same authors in 1992 also reported that in solutions above pH 6.0, a 98% reduction of total aluminum was obtained due to chelation (Alter and Mitchell 1992). Ireland (1977) reviewed the effect of various pesticides and heavy metals on earthworms. This will bring down the risk of entry of these pollutants into plant system and then into sequential food chain. When worms are used for this purpose, they should be prevented from entering into food chain as they are found to concentrate very high levels of these toxins in their tissue.

13.5 Plant Growth Trials Using Vermicomposts

The potential of vermicompost for plant growth was raised by Edwards and Burrows (1988). Various animal, agricultural, and industrial wastes were vermicomposted, including pig, poultry, and cattle manure, potato, brewery, paper, and mushroom wastes. Plant trials were carried out on ornamental shrubs, vegetables, and bedding plants, using a commercial plant growth medium as a control. Because most of the castings tended to be alkaline (pH $>$ 7), it was necessary to dilute with peat for some trials.

Early plant growth was reported to be better with vermicompost than in the commercial growing medium, and seeds germinated faster for most plant species grown in vermicompost. After transplanting into pots, the ornamentals grew better in vermicompost/peat mixtures than in the commercial growth medium. Also, several of the flowering plants flowered much earlier.

Overall, Edwards and Burrows (1988) concluded that vermicompost mixed with peat or other materials makes superb plant growth media and that there could be significant commercial potential. Edwards and Burrows also noted that the paper waste vermicompost was one of the best feed stocks in terms of consistency of product.

Subler et al. (1998) confirmed that the trend in trials for plants grown in container media was that the optimum responses normally occurred when worm castings constituted 10–20% of the volume of the mix. They believed that the substantial growth effects that were observed were more than simply a function of the mineral nutrient content of the castings. They considered that the effects might also be related to enhanced micronutrient availability, the presence of plant growth regulators, or the activity of beneficial microorganisms in the castings. However, that does not deny the fact that vermicomposts do contain nutrients in forms that are most readily taken up by the plants such as nitrates, exchangeable phosphorus, and soluble potassium, calcium, and magnesium (Edwards and Burrows 1988).

It has also been noted (Edwards and Burrows 1988; Subler et al. 1998) that vermicomposts tend to differ from composts in that they normally have higher nitrogen levels with more of that nitrogen in the nitrate rather than the ammonium form.

Several of the plant growth trials undertaken by Edwards' group have been discussed by Atiyeh et al. (2000a, b). Noticing that the germination of tomato, pepper, and lettuce were very low in pit/perlite mixtures, Atiyeh et al. (2000b) substituted some of the peat/perlite mixtures with equal amounts of vermicomposts. This enhanced germination rates greatly, comparable to the germination obtained in a commercial medium that already contained a starter nutrient fertilizer in its formulation. The researchers also made a key observation that vermicomposts still boosted growth rates even when additional fertilizers were applied. That is, their effects must be due to more than just nutrient values. Tomati et al. (1994) used earthworm castings as a propagation and growing substrate for ornamental plant production and found a promotion of root development and a reduction in fertilizer use in plants grown in substrates containing castings.

While the researchers have demonstrated that the addition of vermicomposts to growing media normally produces beneficial effects on plant growth, the reasons why the effects happen are still not yet fully understood. The earthworms certainly fragment the organic waste substrates stimulate enhanced microbial activity and increase rates of mineralization, rapidly converting the wastes into humus-like substances (Atiyeh et al. 2002). A decrease in the carbon from fulvic acids and an increase in the percentage of the carbon from humic acids are seen in the vermicomposting process.

13.6 Disease and Pest Suppression

The beneficial effects of worm casts on plant growth can be put down largely to increased microbial populations that produce plant growth hormones. Those hormones are believed to be adsorbed on to the humates produced during the

vermicomposting process. Edwards and Arancon (2004) noted that beneficial effects were not simply confined to plant growth. They were apparent on the incidence of plant diseases and pest attacks from plant parasitic nematodes, insects, and mites.

Edwards and Arancon (2004) and Chaoui et al. (2002) have shown on their research that relatively small applications of commercially produced vermicomposts significantly reduce attacks by *Pythium* sp on cucumbers, *Rhizoctonia* sp on radishes plants in the greenhouse, *Verticillium* on strawberries, and *Phomopsis* and *Sphaerotheca fulginae* on grapes. The pathogen suppression was almost eliminated if the vermicomposts were sterilized prior to use. Edwards and Arancon (2004) consider it most likely that the effects arise through microbial antagonism.

Low applications of vermicomposts have also been found to affect the populations of plant parasitic nematodes. Vermicomposts from paper waste, food waste, and cattle manure, applied at 2–8 tons per acre to soils planted with tomatoes, peppers, strawberries, or grapes, gave a consistent and significant suppression of plant parasitic nematodes.

The incorporation of small proportions of vermicompost was found to reduce arthropod pests (aphids, mealy bugs, spider mites) on tomatoes, peppers, and cabbage and the extent of crop damage caused by them.

Szczech and Smolinska (2001) investigated the effect of vermicomposts on the growth and infection of tomato seedlings by *Phytophthora nicotianae*. While vermicomposts produced from animal manure significantly reduced the infections in the seedlings, vermicomposts from sewage sludge offered no protection.

13.7 Accumulation of Heavy Metals

Heavy metals include several elements which have a biological function or are toxic to some organisms. According to Lee (1985), the most important environmental pollutants are lead (Pb), cadmium (Cd), mercury (Hg), zinc (Zn), copper (Cu), nickel (Ni), antimony (Sb), and bismuth (Bi). Although many other elements are involved, but most attention has been given to the first two. Since earthworms ingest large quantities of substrate they are particularly susceptible to accumulation of pollutants which may be passed to other animals directly (e.g., predation by birds or mammals), or indirectly via plant uptake of earthworm products from the soil. The main issues are toxicity and the rate and means of heavy metal accumulation in earthworms.

Beyer (1981) and Ireland (1983) have reported that earthworms can accumulate heavy metals from both contaminated and noncontaminated environments. Ireland (1983) states that Cd does not appear to concentrate on earthworm tissues indefinitely, and the ratio decreases with increasing Cd concentration, unlike Pb, which appears to accumulate continuously.

Graff (1982) examined the accumulation of heavy metals in *Eisenia fetida* and *Eudrilus eugeniae* before and after feeding on compost made from municipal garbage. The heavy metal contents before and after feeding were: for *E. fetida*, Cu

4–29, Zn 140–640, Pb 3–14, Cd 2–9, Hg 0.1–14; for *E. eugeniae*, Cu 17–55, Zn 165–360, Pb 10–72, Cd 4–6, Hg 1–15. These data indicate that the earthworms are extracting the heavy metals from the compost and are concentrating them in their tissues.

13.8 Potential for Transmission of Pathogens

Earthworms feeding on sludge may be potential vectors of a wide range of parasitic and pathogenic organisms (Lee 1985; Satchell 1983). It has been determined that passage of organic material through the gut of an earthworm can reduce numbers of some microorganisms and increase numbers of others (Satchell 1983). Spores and cysts of some parasites pass unharmed through the gut of earthworms while some pathogens are reduced.

Brown and Mitchell (1981) reported that *E. fetida* feeding on a growing medium inoculated with *Salmonella eneritidus*, reduced populations of this enteric pathogen by 42 times, compared to controls, after 28 days with the greatest rate of reduction of pathogen in the first 4 days. Satchell (1983) reported the findings of Day (1950) and Bruzewitz (1959) that two species of Enterobacteriaceae, *Serratia marcessens* and *Escherichia coli*, which inoculated in soil were killed when ingested by the earthworm *Lumbricus terrestris*.

13.9 Effect of Worm Castings on Crop Yields

There is little scientific literature on the subject of the usefulness of vermicompost on plant growth (Edwards and Burrows 1988).

During the passage through the gut of the earthworm, ingested material is mixed and has its physical, chemical, and biotic components altered, but very little material is actually digested by the worm, and the structure and composition of the casts is dependent on the composition of the food source (Edwards and Burrows 1988; Buchanan et al. 1988). Organic materials differ greatly in their nutrient content; processing by the earthworm can change the form of these compounds but has very little effect on the total amounts contained. The physical structure of the casts also depends on the source material; however, the final product usually comprises finely mixed and relatively stable aggregates with good structure, porosity, and moisture-holding capacity (Edwards 1981; Lee 1985). The composition of casts from earthworms feeding on sewage sludge can be expected to have a different composition to those produced by earthworms feeding on unamended soils.

Casts produced from soil have increased nitrate and exchangeable calcium, magnesium, potassium, and phosphorus than the original soil (Lunt and Jacobson 1944). Other chemical and physical changes in earthworm casts compared to parent

soil are given by Zhang and Schrader (1993) and changes in microbial populations are covered by Satchell (1983).

Edwards and Burrows (1988) also compared the nutrient contents of several organic wastes before and after being worked by earthworm: all had increased nitrate, soluble P and exchangeable potassium, calcium, and magnesium when worm-worked. They found that emergence and growth of range of seedlings in pots was frequently enhanced in these worm-worked compared to unworked media. Fresh earthworm casts may contain high salt soluble concentrations, especially of Na^+, sufficient to damage plants. Stark et al. (1978) found that leaching cast with water reduced these salts to tolerable levels while still retaining most of the plant beneficial nutrients.

Handrek (1986) compared the porosities, salinities, nutrient contents, pH values, and trace elements of several vermicomposts and potting mixes. Vermicomposts varied widely in total nutrient content: most had negligible amounts of soluble N-nitrates but had ample amounts of P and some had high concentrations of Zn and Cu.

Few reports deal with field trials involving the application of vermicompost. Kale et al. (1992) studied vermicompost in a rice paddy in India. Significant increases in the colonization of soil by microbes (including N-fixers, Actinomycetes, spore formers, and Mycorrhizae) occurred in the experimental plots compared to the control plots without added vermicompost. Higher levels of total N in the experimental plot where vermicompost was added was attributed to higher counts of N-fixing microbes. Lee (1985) mentions findings by Khan (1966) that the growth of maize on a loamy soil in Pakistan was enhanced by the addition of casts of *Metaphire posthuma* and that their effect was greater than was obtained with the addition of farmyard manure. In India, Reddy (1988) compared the growth of an ornamental shrub, *Vinca rosea* and rice, *Oryza sativa*, in soils with or without the casts of *Pheretima alexandri*. Those *V. rosea* plants in casts grew better and produced flowers and fruits earlier than plants in soil alone. Rice growing for 4 months in pots with highest concentrations of added casts grew best, the whole plant lengths (means) being 81.5 cm in soil mixed with casts compared to 62.8 cm in soil alone.

13.10 Detrimental Effects of Earthworms

Despite the many documented and putative beneficial effects of earthworms on soil structure, nutrient dynamics, and plant growth, some aspects of earthworm activities are considered undesirable (Edwards and Bohlen 1996; Lavelle et al. 1998; Parmelee et al. 1998). Detrimental activities include removing and burying of surface residues, which would otherwise protect soil surfaces from erosion; producing fresh casts that increase erosion and surface sealing; increasing compaction of surface soils; depositing castings on the surface of lawns and golf greens, where they are a nuisance; dispersing weed seeds in gardens and agricultural fields;

transmitting plant or animal pathogens; riddling irrigation ditches, making them less able to carry water; increasing losses of soil nitrogen through leaching and denitrification; and increasing loss of soil carbon through enhanced microbial respiration.

It is the net result of positive and negative effects of earthworms that determines whether they have detrimental impacts on ecosystems (Lavelle et al. 1998). Obviously, an effect such as mixing of organic and mineral soil horizons may be considered beneficial in one setting (e.g., urban gardens) and detrimental in another (e.g., native forests). The undesirable impacts of exotic species are central to assessing the risks associated with their introduction and spread.

13.11 Interpretation of Findings

Some microbial and enzyme activities are occurring within the gut of the earthworm that (1) enhances the breakdown of cellulose material, and (2) conveys some property, or properties, to the breakdown product (casting) that are generally beneficial to plant growth. The research team at Ohio State University has demonstrated that it is not just because of the relatively high levels of nutrients and micronutrients within castings. The explanation may be more deeply linked to the richer microbial calories conveyed through the castings, or could be due to the relatively high humification, and specifically the levels of humic acid associated with the castings (Tucker 2005).

In general, researches have shown that blending vermicomposts with traditional growth media has shown positive effects on plant growth, particularly on root growth but also on shoot and leaf growth and fruit and flower production as well. Some experiments have shown that the application of vermicompost produces poorer growth than that produced in the controls. The scientific evidence is less strong with regards to vermicompost having any positive effects on seed germination. Some researchers have found it may inhibit germination slightly, though once germinated the plants can then pick up and forge ahead in the vermicompost.

Effects on plants have been seen with as little as 5–10% of vermicompost added to the growing media. An addition of around 20–40% vermicompost is considered to provide the optimum blend. Then there appears to be a turnaround for concentrations above 40% with the higher rates impacting negatively on plant growth (Tucker 2005).

13.12 Conclusion

Some species of epegic earthworms can live in decaying organic waste materials and convert it to odor free fine particulate materials high in available nutrient (Marsh et al. 2005; Suthar 2006, 2007).

The utility of epigeic earthworm for successful degradation of organic wastes is well documented for different industries such as paper and pulp (Elvira et al. 1997, 1998; Reddy and Shantaram 2005). Compared to thermal composting, vermicomposting with earthworm often produces a product with a lower mass, lower processing time, and humus content; phytotoxicity is less likely; more N is released; fertilizer value is usually greater; and additional product (earthworms) which can have other uses is produced (Lorimor et al. 2001). Therefore, vermicomposting seems to be more appropriate and an efficient technology to convert industrial waste to a valuable community resources at low input basis. However, the composting efficiency and biology of only a few epigeic earthworm species has been studied, for example, *Eisenia foetida* (Maboeta and van Resenburg 2003; Kaushik and Garg 2004; Gupta et al. 2005), *Eisenia andrei* (Elvira et al. 1997, 1998; Nogales et al. 2005; Benitez et al. 1999), and *E. eugeniae* (Kale 1998).

Compost is an excellent product; being homogenous and retaining most of the original nutrients and reduced levels of organic contaminants with respect to the starting material (Ndegwa et al. 2000), it can be applied to soil to increase soil organic matter content and content of nutrients, which can be released upon decomposition, improve soil structure, and increase cation exchange capacity. Composting has been updated to process organic wastes of different origin, such as sewage sludge, animal manure, and agro-industrial wastes (Paredes et al. 1996; Bernal et al. 1998). However, composting is a time-consuming process taking at least 6 months and requiring frequent mixing with possible losses of nutrients, that is, NH_3. Additionally, earthworms reduce numbers of pathogens and the same effect is obtained in traditional composting by the increase in temperature. Vermicomposting has been successfully used for composting different types of wastes, such as municipal and industrial sludges (Edwards and Bohlen 1996; Elvira et al. 1998), though optimal moisture and the best proportions of organic waste are required for an efficient vermicomposting.

Vermicomposting technology involves harnessing earthworms as versatile natural bioreactors, which play a vital role in decomposition of organic matter, maintaining soil fertility, and bringing out efficient nutrient recycling and enhanced plants' growth. A variety of organic solid wastes, domestic, animal, agro-industrial, human wastes, etc. can be vermicomposted. The value of vermicompost is further enhanced as it has simultaneously other benefits: excess worms can be used in medicines and as protein rich animal feed provided they are not growing on polluted wastes and can be used as an antisoil pollutant.

Earthworm can be used as bioindicators for the monitoring of ecosystem state and changes. Various workers identified the earthworms for evaluating the effect of soil contamination with heavy metals and pesticides, agricultural practices, and acid rain, etc. (Paoletti et al. 1991). There are numerous studies about the heavy metal influence on the growth, reproduction, and mortality of earthworms. Marcel et al. (1997) reported that earthworms increase the water infiltrations rate of the soil and observed a mean rate of 150 mm h^{-1} per 100 g m^{-2} of earthworms, however the anecic species shows maximum infiltration (282 mm h^{-1} per g m^{-2}).

Heavy metals are perhaps of greatest concern, and it may be possible to exploit some aspect of earthworm behavior for their removal. Processing by the earthworms may alter the solubility or stability of some heavy metals or perhaps, enhance other physical, chemical, or microbial means of removal (e.g., Tyagi and Couillard 1991). Accumulation of pesticide may be less of a problem as these chemicals and their metabolites often have known rates and products of decay. Earthworms may be used in combination with conventional composting techniques to reduce pathogens, although the temperatures involved are incompatible for earthworm survival (Blakemore 1995).

Vermicomposting of municipal wastes may be particularly suitable option for production of useable products. Composition and consistency of these products would largely depend on the composition of the initial waste materials and of any materials with which they are combined. As for sludge treatment, there would be a requirement to constantly monitor nutrients, contaminants and to prevent pathogen regrowth, in both the raw materials and the final products.

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Chapter 14
Earthworm Innate Immune System

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14.1 Introduction

The immune system is an essential component for survival against a wide range of pathogens. Rapid deployment of innate immune cells and molecules in vertebrates including humans is the first line of defense against pathogens until interaction with the slower adaptive system becomes activated. This is essential since these cells correspond to the necessary antigen-presenting cells for lymphocytes that then proceed to complex effector activity. Recently, more invertebrate animals have become candidates for analyzing innate immunity to reveal the strategies and complexities of vertebrate adaptive immunity. Additional reasons for using invertebrate models while analyzing in parallel vertebrate organisms to study innate immunity concern vitality and ethical questions. First, these consider the facts that invertebrate animals have short life-spans, their laboratory maintenance is easy, and no ethical problems are derived from their use. Second, invertebrates have developed a variety of active immune mechanisms including production of antimicrobial peptides, coagulation, phagocytosis, and encapsulation reactions. These mechanisms depend on innate immune receptors, the so-called "pattern recognition receptors" (PRRs), that can discriminate "self" from "non-self" membrane components. Recognition of "pathogen associated molecular patterns" (PAMPs) by germ-line

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encoded receptors initiates essential signaling pathways, which then lead to activation of various genes that encode inflammatory mediators, antimicrobial peptides, and regulators of phagocytosis. Third, invertebrate recognition receptors have unique pathways unknown in vertebrates, but that are also more universal mechanisms found throughout the animal kingdom. For example in *Drosophila* two distinctive signaling pathways, the *toll* and immune deficiency pathway (*imd*), share high similarity to mammalian counterparts of signaling pathways (Lemaitre and Hoffmann 2007). Essential knowledge of the seemingly less complex invertebrate immune strategies can help understand the more sophisticated vertebrate immune system, the evolution of the immune response and identify new biomolecules with possible therapeutic use (Salzet 2002).

14.2 Innate Immune Recognition in Earthworms

Oligochaeta annelids (earthworms) have become a model for comparative immunologists through several approaches that involved innate immunity. Some of the earliest experimental results focused on transplantation experiments that proved the existence of self/nonself recognition, immune mechanisms in earthworms, before more extensive studies on other forms of earthworm immunity were even initiated (Cooper 1968, 1969). Numerous works have focused on proteolytic, hemolytic, antibacterial, and cytolytic properties of coelomic fluid (Bilej et al. 2000; Cooper et al. 2002; Kauschke and Mohrig 1987). Recently Coelomic Cytolytic Factor (CCF) has been identified and characterized as an immune molecule of the coelomic fluid and coelomocytes (immune cells of body cavity) from *Eisenia fetida* recognizing lipopolysaccharide (LPS)-, β-1,3-glucans-, muramic acid-, and *N,N′*-diacetylchitobiose microbial molecules. Binding to microbial antigens CCF triggers the prophenoloxidase cascade, an important invertebrate immune mechanism (Beschin et al. 1998, 1999), and also exerts opsonizing properties helping phagocytosis (Bilej et al. 1995). The prophenoloxidase (proPO) cascade as an immune mechanism is well known since it exists in other invertebrates, especially well studied in arthropod species (insects, crustaceans). These multiple biological activities point to a crucial role of CCF in innate immune reactions in earthworms, and evidence accumulates that lectin-like interactions serve as the initial recognition events. So far, it is known that CCF has unique antigen recognition characteristics (Bilej et al. 2001); although it is possible that CCF-mediated nonself recognition may accelerate various signal transduction pathways still unknown in annelids.

14.3 Coelomocyte Proliferation After Depletion and Mitogen Stimulation

Coelomocytes are circulating leukocytes in the earthworm's coelomic cavity (hyaline amoebocytes, granular amoebocytes, and chloragocytes; SC and LS), which possess various immune functions (phagocytosis, encapsulation, production of

cytotoxic/antimicrobial molecules). We have characterized subgroups of earthworm coelomocytes using cytochemical, immunological [using specific monoclonal antibodies (mAbs)] and functional approaches (Engelmann et al. 2004, 2005a). Several studies have analyzed cellular and humoral immune compartments, but the precise molecular data of immune molecules are limited (Cooper et al. 2002; Engelmann et al. 2005b). Restricted data are available concerning activation of coelomocyte subgroups. Crucial results are contradictory concerning whether earthworm coelomocytes are terminally differentiated cells or do they proliferate (Liebmann 1942; Hostetter and Cooper 1972; Lemmi and Cooper 1981; Roch and Valembois 1978; Tuckova et al. 1988).

A recent report reveals that isolated coelomocytes proliferate after cell depletion as analyzed by flow cytometry (Homa et al. 2008). In addition, several lines of evidence indicate mitotic activity of coelomocytes under stress conditions (Bilej et al. 1992) that could be triggered by lectins and transplantation antigens; however, cellular activation has not been analyzed at the molecular level. In our recent experiments, we observed in vivo proliferative ability of free coelomocytes from *E. fetida* by flow cytometry. Freshly isolated coelomocytes were stained with propidium iodide for cell cycle analysis. We did repeated isolations from the same earthworms and allowed various resting times. Results revealed an increase in proliferating cell numbers that indicate mitotic division of coelomocytes (Fig. 14.1a). We also measured coelomocyte proliferation after using various stimuli. Our results clearly show effects of different mitogens (lipopolysaccharide, pokeweed mitogen, concanavalin A). Lectin stimulations caused increased activation and proliferation (Fig. 14.1b).

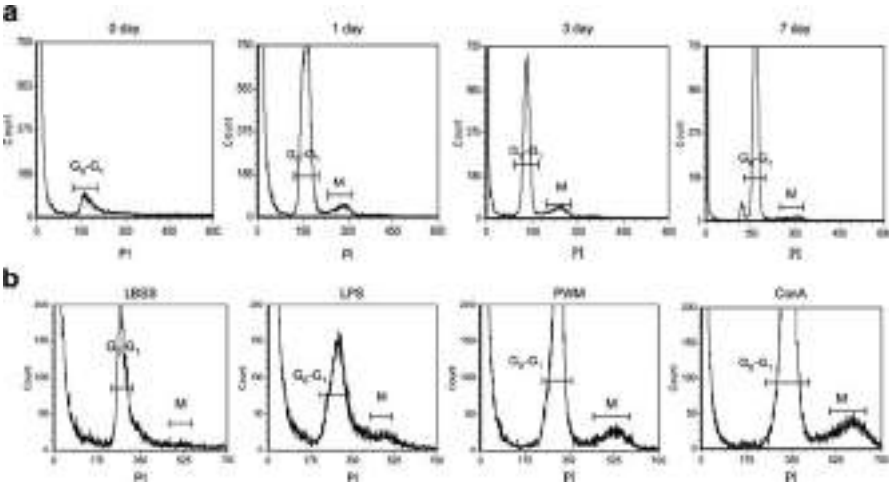

Fig. 14.1 Mitotic activity of coelomocytes

14.4 Earthworm Coelomocytes Are Positive for Different Mammalian Antigen-Specific Monoclonal Antibodies

14.4.1 Molecules with Cytokine-Like Activity

In recent years, more attention has been focused on the evolution of immune systems in certain invertebrate species (Cooper 2008). Comparative studies for detecting analogous and perhaps homologous cellular and molecular mechanisms concerning immune-active components have become increasingly important. Molecules with cytokine-like activity are of particular interest as they may add a parallel dimension that could help to better understand immune reactions in vertebrates as well. There is evidence for the existence of cytokines during early phases of evolution, leading to the conclusion that these molecules were highly conserved during phylogenesis (Beck and Habicht 1991a; Ottaviani and Franceschi 1997). Analogs of inflammatory cytokine molecules (IL-1, IL-6, and TNF-α) have been described in invertebrates including sponges, mollusks, insects, annelids, and tunicates (Beck and Habicht 1991b; Clatworthy 1996; Cooper 1996; Hoffmann 1995; Hoffmann and Reichhart 1997; Hughes et al. 1990; Müller et al. 1999; Ottaviani et al. 1993; Parrinello et al. 2008; Zhang et al. 2009).

Annelid coelomocytes form rapidly immune mechanisms against bacteria, parasites, and yeast (Cooper and Stein 1981; Metchnikoff 1891; Porchet-Henneré and Vernet 1992; Valembois et al. 1985) through cellular reactions, for example, phagocytosis and encapsulation. This rapid response is mediated through soluble antibacterial molecules like lysozyme (Hirigoyenberry et al. 1990; Josková et al. 2009), fetidin (Lassegues et al. 1997). We found host defense related cytokine molecules (TNF-α, TGF-α) and enzyme (Cu/Zn SOD) in earthworm coelomocytes. Anti-TNF-α antibody showed positive reaction both in coelomocyte cytoplasms and on cell surfaces (Engelmann et al. 2002).

CCF-1 shows functional similarity with TNF-α (Bilej et al. 1995, 1998). In addition to CCF-1, several other humoral lytic components have been identified with respect to molecular sequence and function (fetidins, lysenin); these characteristics must be elucidated for still others: eiseniapore, H1 H2 H3, and perforin. Lumbricin I is a small antimicrobial peptide that appears to be unrelated to the lytic molecules as viewed by sequence analysis (for a review see Cooper et al. 2002; Kauschke et al. 2001). These results offer strong support for the in vivo existence of other bioactive molecules produced by earthworm coelomocytes (Hanušová et al. 1999).

14.4.2 FACS Reveals Distinct Coelomocyte Subtypes Based on CD Marker Properties

Self and nonself tissue recognition is an important component of earthworm immune responses, which suggests the presence of recognition molecules and cell

adhesion molecules on coelomocyte surfaces (Cooper et al. 1999; Shalev et al. 1983; Toupin and Lamoureux 1976).

A nongranulated coelomocyte population, which can neutralize and recognize foreign antigens, has been described from an electron microscopic study (Toupin and Lamoureux 1976). Other reports have described the presence of Thy-1 and β_2 microglobulin molecules on coelomocyte surfaces (Roch et al. 1983; Shalev et al. 1981). These molecules, present in other invertebrates as well, belong to the immunoglobulin (Ig) superfamily (Mansour and Cooper 1984; Mansour et al. 1985; Negm et al. 1991; Shalev et al. 1983). Surprisingly, serological evidence of homology with the J-chain of immunoglobulins has also been revealed in association with different invertebrate cells including earthworm's (Takahashi et al. 1996). According to some reports, coelomocytes can be divided into more subpopulations by light- and electron-microscopic examinations. By flow cytometric analysis, two different subpopulations have been found in earthworm immune-competent cells that reacted with mammalian epitope-specific antibodies. Small coelomocytes (SC) had surface positivity with anti-CD11a, CD45RA, CD45RO, CDw49b, CD54, β_2-m, and Thy-1 antibodies. These cells were negative using other markers. Large coelomocytes (LC) proved to be negative for each marker but were active in phagocytosis (Cooper et al. 1999; Cossarizza et al. 1996).

By flow cytometric analysis we found three different cell populations. Although these populations have been well characterized by means of morphological methods, the third population has not been identified by flow cytometry. We were able to identify highly conserved molecules, including cell surface markers. Consistent with literature, we found cross-reactivity with anti-Thy-1 mAb and we described reactions with anti-CD24 and anti-TNF-α antibodies on coelomocyte surfaces. Other surface markers, for example, human CD45, CD4, CD8, and MHC molecules were negative in our experiments (Engelmann et al. 2002). However, as we mentioned earlier, other authors (Cossarizza et al. 1996) have found anti-CD45RA, CD45RO positivity on SC. When cocultured in vitro with tumor cell targets (e.g., K562) these small lymphocyte-like cells may release perforin-like molecules that seem to complete a contact-mediated cytolytic destruction process (Kauschke et al. 2001) (Fig. 14.2a, b). At this level of evolution this suggests the first evidence that supports the dissociation of phagocytosis from NK-like functions.

14.5 Earthworm Leukocytes Deploy Lysosomal Enzymes That Respond to Bacterial Challenge

Coelomocytes recognize nonself tissues that consequently trigger a rejection process that may be partially enzyme mediated (Cooper 1968, 1969; Linthicum et al. 1977). The released lysosomal acid phosphatase in the coelom may exert an effect during this response (Marks et al. 1981). In the various subpopulations of earthworm coelomocytes several enzyme activities with different patterns have been

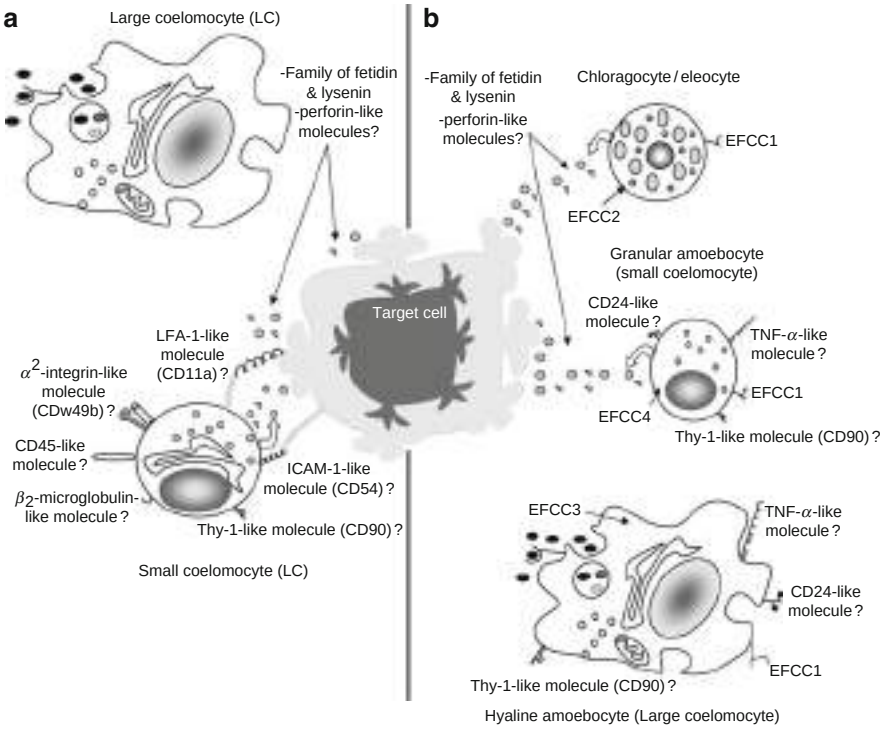

Fig. 14.2 Hypothetical drawings show coelomocyte subtypes from *Eisenia fetida*

defined. In our study, we have shown that bacterial infection of earthworms stimulated an increased number of lysosomes in coelomocytes (Engelmann et al. 2004).

Cytochemical methods have been used to characterize earthworm coelomocytes including their enzymes. In our cytochemical experiments, granular and hyaline amoebocytes but not chloragocytes release acid hydrolase activity, in accordance with recent reports (Hamed et al. 2002). Enzyme reactivity was bound to discrete granules (e.g., lysosomes) in the cytoplasms of coelomocytes as observed by electron microscopy; however, the staining pattern was different. Variable staining patterns may indicate different cell activation and differentiation stages. Coelomocytes released the contents of the granules (including enzymes) into the coelomic cavity to inactivate pathogens. With Western blotting we found a 39-kDa peptide that reacts with anti-AcP antibody. Subsequently, intracellular acid phosphatase decreases compared to the controls in the samples which contain bacterial strains. In contrast, ELISA results show that the samples had an increased level of extracellular acid phosphatase after phagocytosis (Engelmann et al. 2004).

One research group has isolated an acid phosphatase from another earthworm species, *Eisenia veneta*, which has two isoenzymes of acid phosphatase: one is 113 kDa composed of identical peptide chains of 36 kDa (Stubberud et al. 2000).

As bacteria are bound to leukocytes they are removed from circulation and may be destroyed by the release of degradative enzymes, reactive oxygen metabolites, or any antimicrobial molecules secreted from blood cells of the mussel *Mytilus edulis* (Pipe et al. 1997). This release of digestive enzymes may play a role in autophagocytosis. Several reports have concluded that other coelomocyte types clear degraded chloragogen cells. Examinations have revealed chloragosomes inside "brown bodies" (encapsulated particles in earthworms). These bodies contain lipofuscin and melanin, which render them capable of killing parasites, microbes and of clearing altered cells (Valembois et al. 1994). In this process different coelomocytes may have specialized tasks. Granular coelomocytes may play a role in encapsulation, while hyaline amoebocytes affect primarily phagocytic activity. The functional distribution of different leukocyte types has been characterized in other annelid species, notably marine polychaetes (Porchet-Henneré 1990).

Some reports indicate that chloragogen cells (which constitute the chloragogue tissue) have high acid phosphatase activities (Cancio et al. 1995). In contrast free chloragogen cells exhibited low enzymatic activities in our experiments. This coelomocyte type is found in all lumbricid worms, and chloragocytes change during the expulsion of granules and biochemical alterations of cytoplasmic inclusions (Valembois 1971; Valembois and Roch 1977; Valembois et al. 1985). Chloragocytes in the coelomic fluid have low acid phosphatase content as measured by flow cytometry, while immunocytochemical, enzyme cytochemical examinations could not detect any acid phosphatase in this coelomocyte subpopulation (Engelmann et al. 2004). This may refer to a developmental stage of chloragocytes that may occur causing loss of these hydrolytic enzymes, thus giving these cells a status of secondary importance in immune mechanisms.

14.6 Specific Monoclonal Antibodies Identify Four Distinct Earthworm Coelomocyte Markers

14.6.1 Establishing a CD Marker Library

Modern biology has benefited enormously from the development of hybridomas for producing mAbs. mAbs are highly specific molecular probes that are widely used in analyzing cells of vertebrate immune systems (Caruso et al. 1997; Koumans-van Diepen et al. 1995). They are also potentially suitable for mapping of invertebrate immunity in broad terms as well, but specific antibodies against certain invertebrate immune cells are not always available. mAbs have been developed against some disparate invertebrate taxa (Dyrynda et al. 1997; Porchet-Henneré 1990; Porchet-Henneré and Vernet 1992; Reinisch et al. 1983; Yoshino and Granath 1983). However, most mAbs have been raised against insect hemocytes in order to characterize them during different developmental stages and according to certain functional differences (Gardiner and Strand 1999; Kurucz et al. 2003; Pech and

Strand 1996; Strand and Johnson 1996; Willott et al. 1995). To extend our understanding of the earthworm immune system, focusing on coelomocytes, we have developed for the first time a family of mAbs against their leukocytes using hybridoma technology.

We aimed to establish an earthworm cluster of differentiation (CD) library so that later we could then move further towards a more specific analysis of how leukocytes are organized functionally. Up to now we know that the cellular components consist of different coelomocyte populations; they are neither homogenous in morphology nor in certain functions. Although we used mAbs for further characterization of earthworm cell surface and intracellular markers, these were developed initially against different mammalian antigens and are apparently specific for conserved molecules (cytokines, cell surface molecules, and enzymes) (Engelmann et al. 2002). Thus, a more detailed analysis of cell differentiation markers (CD markers) using specific mAbs against earthworm coelomocytes is essential to further analyze and refine aspects of immunity. We raised selective antibodies to characterize earthworm coelomocyte subpopulations (EFCC). Different clones of anti-EFCC mAbs were selected for comparative analyses, especially with respect to origins and development. In this study we were able to: (1) define coelomocyte clusters using specific mAbs (anti-EFCC clones); (2) assess whether anticoelomocyte mAbs bind to other cells or tissues; (3) determine functional heterogeneity between coelomocyte subgroups; (4) compare the EFCC clusters determined by immunological markers to classical microscopic classifications and to flow cytometric classification; (5) investigate the possible origin of coelomocyte subgroups (Engelmann et al. 2005a).

14.6.2 Characterization of Coelomocyte Differentiation Clusters

This was the first report that outlined the production and characterization of earthworm-specific mAbs against earthworm coelomocyte differentiation clusters (EFCC), as well as their use for comparative analysis (Fig. 14.2b). The anti-EFCC1 clones recognize common antigens present on earthworm cells and tissues, and certain distribution patterns in snails but localized only in well-circumscribed organs. In contrast, anti-EFCC mAbs were positive neither for *Drosophila melanogaster* hemocytes nor for different mammalian cells or tissues. These results suggest a close evolutionary relationship between cellular components of the immunodefense system in earthworms and snails but not with insects or vertebrates. Similar observations reveal reactivity patterns of antibodies specifically associated with immune cells of shrimp and the moth *Manduca sexta* compared to cells and tissues of other test animals from different evolutionary groups (Van de Braak et al. 2001; Willott et al. 1994).

The anti-EFCC2, EFCC3, and EFCC4 antibodies are positive only for earthworm cells and tissues. This selective class/species specificity supports the view

that differentiation of certain cellular functions occurred during species evolution. The anti-EFCC2, a-EFCC3, and a-EFCC4 antibodies can discriminate between coelomocyte clusters by selectively labeling different populations with characteristic morphological appearance and specific functions. The anti-EFCC2 mAb recognizes exclusively the chloragocyte population: the EFCC2 cluster seems to be equivalent with classical chloragocyte/eleocyte population. Chloragocytes play a role in various functions: in earthworms the chloragogenous tissue is often considered as a functional analog of the vertebrate liver and the hepatopancreas of mollusks and arthropods. They also have hemopoetic functions, producing the extracellular respiratory pigment in oligochaetas and leeches (Fischer et al. 1975, 1976; Fischer 1993). Both sessile and free wandering chloragocytes express the same marker suggesting common tissue origin but with different maturation stages.

The EFCC3 cluster seems to be equivalent to hyaline coelomocyte populations. These leukocytes seem to be responsible for phagocytosis. The EFCC4 is a relatively small cell population in the coelomic fluid. They have been previously identified as granular amoebocytes and are present mainly in smaller numbers using flow cytometry. After labeling earthworm tissues with different anti-EFCC clones, especially with three and four, a characteristic staining pattern was found in the mesodermal area. The anti-EFCC3 clone labels epithelial cells of the somato- and splanchnopleure, while the anti-EFCC4 clone labels cells with splanchnopleural origin in the gut wall beneath the inner epithelium (Engelmann et al. 2005a, b).

14.7 Signaling Mechanisms by Mitogen-Activated Protein Kinases in Invertebrate Immunocytes

14.7.1 Signaling a Common and Important Mechanism

Signaling by receptor tyrosine kinases (RTKs) has a highly conserved role in all metazoan organisms controlling cell fate, proliferation, migration, and differentiation (Friedman and Perrimon 2006). Sequencing of genomes shows early appearance of RTK in all metazoans and higher diversification in several subfamilies (Kurz and Tan 2004). One of the conserved signal transduction pathways is the mitogen-activated protein kinase (MAPK) pathway, which is crucial for stress response and immunity in plants and animals (Asai et al. 2002; Kyriakis and Avruch 2001; Troemel et al. 2006; You et al. 2007) (Figs. 14.3 and 14.4). MAPK system corresponds to immune response in invertebrates and vertebrates (Dong et al. 2002). Members of MAPK pathway are key players in pro- and anti-inflammatory processes (Han et al. 1994; Salojin and Oravecz 2007). This signaling pathway is activated by the invader (inducer) in mammalian phagocytes. These have been analyzed extensively and include among others tyrosine kinases, serine kinase (MAPKs), small GTPases, and lipid signaling pathways (Nahas et al. 1996).

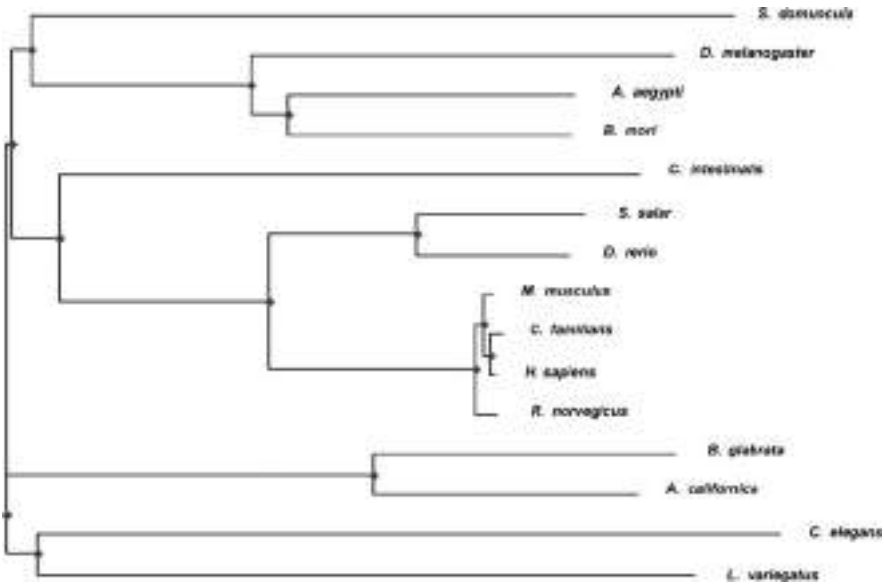

Fig. 14.3 Phylogenetic tree of p38 MAPK enzyme

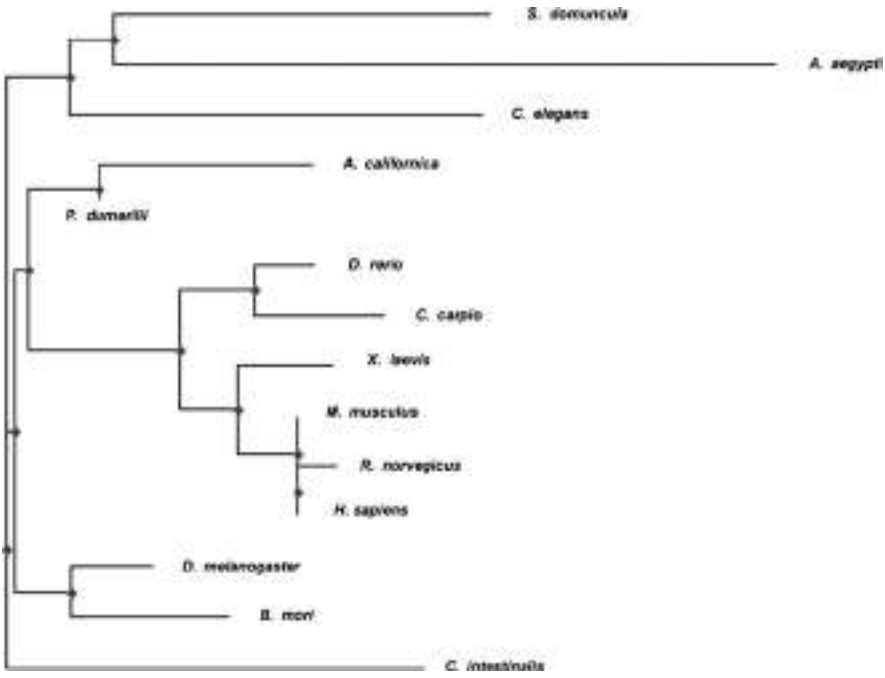

Fig. 14.4 Phylogenetic tree of JNK protein

14.7.2 The Essential Role of Phosphorylation to Activate MAPKs

Several lines of evidence demonstrate that the phosphorylation and activation status of MAP kinases exert a crucial impact on the outcome of downstream events that regulate cytokine production. For example, med-fly hemocytes, key mediators of cell-mediated immunity in insects, respond to *Escherichia coli* LPS by activating the three major MAPK subfamilies: extracellular signal-related kinase (ERK), c-jun *N*-terminal kinase (JNK), and p38 in a Ras/Rho-dependent manner. The functional association of *E. coli* phagocytosis with the activation of MAPKs appears to be the secretion of proPO-activating enzymes. Activation of hemocyte surface proPO via MAPKs activation is an important component of innate immunity and hence the process of phagocytosis (Lamprou et al. 2005, 2007). Information on MAPK signaling in invertebrate immunity is limited; most data have been derived from insects and mollusks (Canesi et al. 2002; Gaitanaki et al. 2004; Lacchini et al. 2006; Larade and Storey 2006; Zelck et al. 2007). No information is available from earthworms concerning MAP kinases or any other signaling mechanisms.

14.8 Is MAPK Pathway Involved in Earthworm Immune Response?

Mitogen-activated protein kinase (MAPK) signaling pathways likely exist in all eukaryotic organisms. Regarding their pivotal function and ubiquitous appearance of MAPK enzymes in eukaryotic cells, we propose to study MAPKs in earthworms. MAP kinases (p38, JNK-c jun terminal kinase) are conserved as in all living organisms. Although there are no available data from earthworms, our recent results clearly show evidence for the presence of MAPK enzymes in earthworm coelomocytes. Total coelomocyte lysates along with lysates from Jurkat human leukemia cell line were separated on SDS PAGE gels then blotted to nitrocellulose sheets. The nitrocellulose membranes were probed with commercially available p38 and JNK-specific antibodies for Western blot. The results revealed distinct bands from coelomocytes for both molecules and their molecular weights were similar compared to the bands from the Jurkat positive control (data not shown).

In vivo Gram-positive and Gram-negative bacteria challenge will affect the expression of CCF in *E. fetida*. CCF expression is upregulated upon bacteria challenge within a few hours along with demonstrable lysozyme activity (Köhlerová et al. 2004). In a separate experiment, we used phospho p38 and phospho-JNK-specific antibodies; both antibody showed reaction pattern on earthworm coelomocyte lysates (data not shown). Another problem concerns the specificity of commercially available phospho-p38 and phospho-JNK antibodies. Commercial antibodies may not be phospho specific in earthworms in contrast to clearer specificity for mammalian proteins. In polychaete annelids and leeches MAPK pathway members have been detected. JNK was cloned and p38 detected

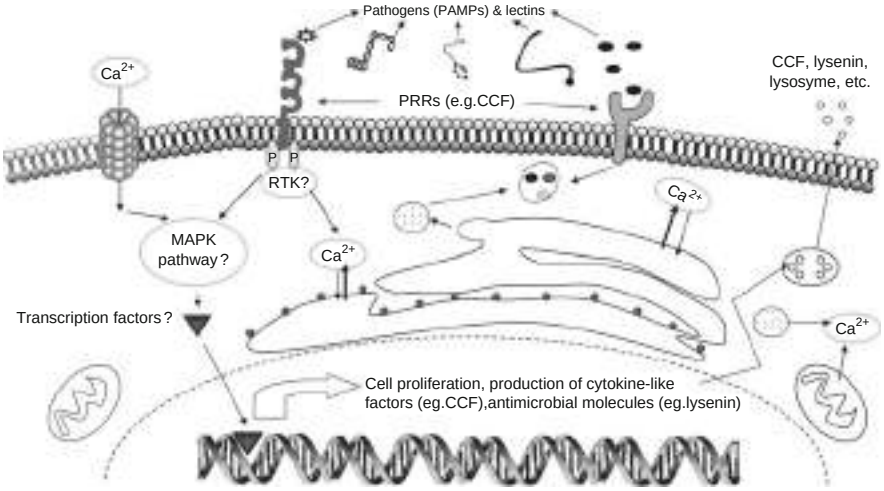

Fig. 14.5 Hypothetical model that show proposed intracellular signaling events after activations of *Eisenia fetida* coelomocytes

at the protein level (Gonsalves and Weisblat 2007; Steinmetz et al. 2007). Thus, there is compelling evidence to propose that earthworm coelomocytes would possess a signaling system (Fig. 14.5). The challenge is to find the appropriate assay system.

14.9 Conclusions and Future Prospects

Indeed, earthworms possess a highly effective innate immune system against environmental pathogens. Enormous data are collected concerning humoral and cellular immune components. However, many important questions are still unanswered. One of them is related to signaling, whose conserved RTKs and/or MAP kinases are involved in the activation events.

MAPK pathways likely exist in all eukaryotic cells as suggested by the recent identification of p38 and JNK orthologs in the phylogenetically oldest extant metazoans, the sponges (Müller et al. 2002). Interestingly, there are no experimental data available from earthworms about MAP kinases or even other signaling mechanisms. Why would there be interest in analyzing invertebrate signaling mechanisms in model organisms? Most studies concern the identification of PRRs and the subsequent signaling cascades in limited model organisms. MAPK cascade is induced by stress-signals; different organisms in various environments must face variable challenges.

We must consider that earthworms are adapted to a special environment with somewhat different mechanisms and strategies for survival from those of other

organisms. Uncovering major components of MAPK pathway would allow analysis of receptors, target molecules (transcription factors), and regulators of earthworm signaling mechanisms. Second, messengers such as intracellular calcium may also have an important role during coelomocyte activation and immune response.

Further work should concentrate on cooperating partners of p38 and JNK kinases such as MAPK kinase (MEK) and MAPK phosphatase molecules. Another interesting question concerns a definition of the target/docking molecules for MAP kinases, and which transcription factors are affected by this signaling system in earthworms. The broad importance and implication of MAPK pathways draw attention to human diseases, primarily to cancer. Discovery of novel components of this pathway can show regulatory network that are required to control this system and also to reveal new implications for human diseases. Finally, novel regulators of MAPK cascades found by comparative analysis of signal transduction systems may even be attractive targets useful for drug discovery.

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Chapter 15
Earthworms: A Potent Herbal Target for TCM (CAM) Research

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15.1 Introduction

Being a vital component of traditional Chinese medicine (TCM), earthworms have attracted scientists' interest based on their therapeutic effects on human disease and their impact on and responses to the environment. This chapter begins by illustrating how earthworms regenerate their injured or lost body parts. The unique ability of earthworm regeneration stimulates researchers' focus on studying the potential therapeutic effects of earthworm extract (EE) on peripheral nerve regeneration. Peripheral nerve regeneration requires a permissive environment around neurons. Multiple factors including neurotrophic factors, extracellular matrix (ECM) proteins, and hormones participate in Schwann cell dedifferentiation, proliferation, and remyelination. Next, we will discuss several factors required for nerve regeneration

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and activation of the intrinsic growth capacity. The mitogen-activated protein kinase (MAPK) family is crucial for Schwann cells proliferation and migration. Insulin-like growth factor-I (IGF-I) also plays a crucial role on proliferating Schwann cells. IGF-1 stimulates growth and differentiation of fetal neurons and increases neurite sprouting and outgrowth. In addition, pituitary adenylate cyclase-activating polypeptide (PACAP) is now reported to promote and increase the possibility of functional recovery following nerve injury. The glycolipoprotein tissue homogenate extract from *Eisenia foetida* (G-90) is another essential factor that can activate signal transduction pathways and cause an increased concentration of EGF and FGF. These growth factors (GDNF, BDNF, FGF, and NGF) can activate MAPK pathway to stimulate Schwann cell migration. The remainder of this chapter details evidence regarding the fibrinolytic, anticoagulative, and anti-oxidative effects of earthworms and new trends of clinical application. Finally, the chapter will end with aspects concerning roles of earthworms on our ecological systems. The digestive process of the earthworm converts nutrients from plant matter forms that can be absorbed by plant root systems. Adversely, the balance of ecological systems could be broken by invasive earthworm populations. Therefore, further studies are needed to clarify the real "earthworm power."

15.2 Peripheral Nerve Regeneration

The capacity for regenerating injured or lost parts of earthworm bodies has been well studied. Some species have less ability to replace lost segments than others, for example, *Eisenia fetida* regenerates more segments than does *Lumbricus terrestris* (Gates 1974). In addition, earthworms can regenerate either the anterior or posterior portions of their bodies but the posterior region grows again more readily than the anterior. The other essential result indicates that earthworms can only regrow amputated parts of their body if the nervous system remains intact (Hasegawa and Kobayashi 1991). Attracted by the unique ability of regeneration of earthworms, recently researchers have studied the potential therapeutic effects of EE on peripheral nerve regeneration.

In mammals, central neurons without a myelin sheath are difficult to regenerate. In contrast, peripheral nerves with a myelin sheath exhibit easier spontaneous regrowth after injury. Bunge (1993) investigated the effect of buyang huanwu decoction on regeneration of rat sciatic nerve (Cheng et al. 2001). Results showed that the buyang huanwu decoction had remarkable growth-promoting effects on regenerated nerves, including significantly higher numbers of myelinated axons, larger endoneurial areas, higher axon densities, and a larger percentage of axon area per total nerve area in the buyang huanwu group than in control groups. Buyang huanwu decoction, which is composed of earthworm, milkvetch root, Chinese angelica, red peony root, peach seed, safflower, and Szechwan lovage rhizome, has been used as a potent treatment agent on spinal

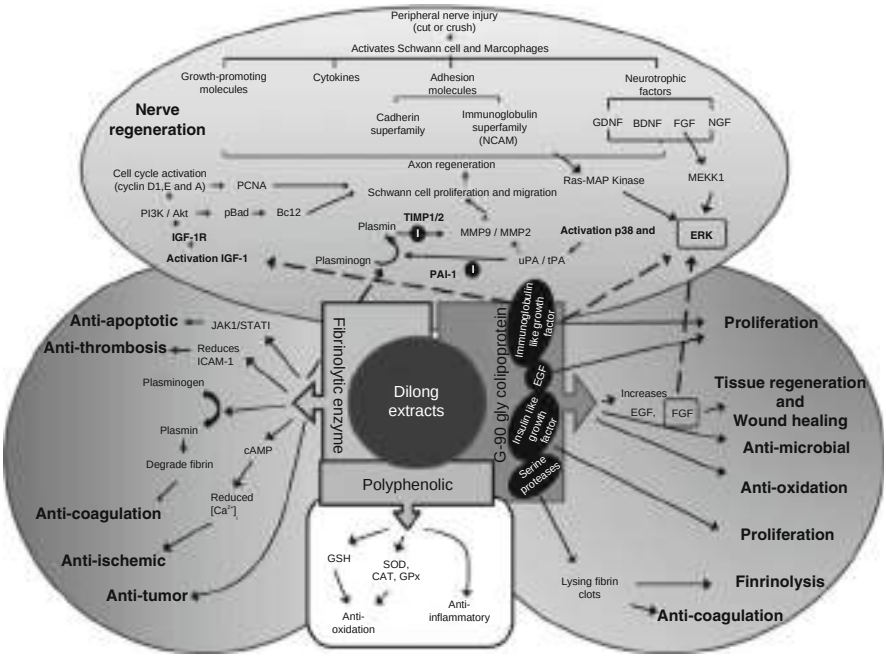

Fig. 15.1 Schematic model of peripheral nerve regeneration of RSC96 cells. This figure represents that the multiple neurotrophic factors likely involved in RSC96 Schwann cell migration, survival, and proliferation. *MAPK* mitogen-activated protein kinase, *IGF-I* insulin-like growth factor 1, *FGF* fibroblast growth factors, *PACAP* pituitary adenylate cyclase-activating polypeptide, *AC* adenylyl cyclase

ischemia/reperfusion injury (Wang and Jiang 2009), myocardial ischemia (Yang et al. 2009), and neurite outgrowth and differentiation of neuroepithelial stem cells (NEPs) (Sun et al. 2007). The EEs appear to enhance nerve regeneration and functional recovery following injury, suggesting the clinical potential of *Lumbricus* extract for treating peripheral nerve injury in humans (Wei et al. 2009). Further experimental works are needed to focus on investigating the molecules and potential mechanisms of EE and how it is involved in peripheral nerve remyelination.

Peripheral nerve regeneration requires a permissive environment and activation of the intrinsic growth capacity of neurons. Axon regrowth and remyelination of the regenerated axons by Schwann cells are both essential. Multiple factors including neurotrophic factors, ECM proteins, and hormones participate in Schwann cell dedifferentiation, proliferation, and remyelination. Recent studies reveal that locally applied neurotrophins can enhance survival of damaged neurons and regrowth of injured axons in the central and peripheral nervous systems in rats (Chen et al. 2001; Jessen and Mirsky 2008; Svaren and Meijer 2008). Next, we will discuss several factors required for nerve regeneration (Fig. 15.1).

15.2.1 MAPK Pathway: Stimulating Schwann Cells Migration

The MAPK family is a crucial regulator of pathways involved in cell proliferation (Pearson et al. 2001) and migration (Meintanis et al. 2001). Extracellular signal regulated protein kinase (ERK), a component of MAPK family, has been implicated in the migration of fibroblasts (Huang et al. 2004) and carcinoma cells (Reddy et al. 2003). The MAPK (ERK1/2, JNK, and p38) pathways for earthworm-induced matrix-degrading proteolytic enzyme (PAs and MMP2/9) production in Schwann cell play a critical role on stimulating Schwann cells migration. The signaling migration pathway in earthworm-stimulated Schwann cells, inducing the activation (Chang et al. 2009a) of uPA and tPA, mediated through the ERK1/2 and p38. To promote migration, cells secrete proteases (PA and uPA) that are thought to degrade matrix molecules and cell adhesion. The ERK1/2 and p38 phosphorylation leads to the expression of uPA and tPA occurred in a time-dependent manner, during the elevation of MMP9 and MMP2 levels and activity.

15.2.2 IGF-I Pathway: Stimulating Schwann Cells Proliferation

IGF-I is a polypeptide hormone synthesized by proliferating Schwann cells (Schumacher et al. 1993). IGF-I level elevation increases sympathetic neuron proliferation in vivo (Zackenfels et al. 1995), stimulates the growth and differentiation of fetal neurons (Cicco-Bloom and Black 1988), and increases neurite sprouting and outgrowth in vitro (Aizenman and de Vellis 1987; Caroni and Grandes 1990). Interestingly, IGF-I not only stimulates proliferation but also promotes survival in several cell types (Gong et al. 1993). Moreover, IGF-I seems to act as a therapeutic target for treating peripheral nerve injury and motor neuron diseases (Sullivan et al. 2008). Therefore, the molecular mechanisms by which EE promotes neuron regeneration have been recently investigated. (Chang et al. 2009b) show that treatment with dilong extract induces phosphorylation of the IGF-I-mediated phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/Akt) pathway and activates protein expression of cell nuclear antigen (PCNA) in a time-dependent manner. Cell cycle analysis showed that G_1 transits into the S phase in 12–16 h, and S transits into the G_2 phase 20 h after exposure to EE. Strong expression of cyclin D1, cyclin E, and cyclin A occurs in a time-dependent manner. Small interfering RNA (siRNA)-mediated knockdown of PI3K significantly reduced P13K protein expression levels, resulting in Bcl_2 survival factor reduction and a marked blockage of G_1 to S transition in proliferating cells. These results demonstrate that EE promotes the proliferation and survival of RSC96 cells via IGF-I signaling. The mechanism is dependent primarily on the PI3K protein (Chang et al. 2009b).

15.2.3 PACAP-Like Compound

It is now well established that PACAP plays important roles in development of the nervous system in vertebrate animals. A human subject research indicated that PACAP promotes regeneration factors and increases the possibility of functional recovery following the facial nerve injury (Kimura et al. 2004). PACAP and its receptors are present in many regions of the developing brain. It stimulates different signaling cascades in neurons, involving cAMP, MAP kinase, and calcium. These characteristics suggest that PACAP may influence neuronal development (Vaudry et al. 2000). Recent extensive research is in progress on earthworm PACAP-like compounds found in the body wall, alimentary canal, and coelomocytes. The results show that PACAP-like peptides accumulate in earthworm regenerating tissues, suggesting similar trophic functions of these compounds in earthworm and vertebrate tissues (Somogyi et al. 2009; Varhalmi et al. 2008).

15.2.4 G-90

A biologically active glycolipoprotein extract from whole earthworm tissue homogenates (*E. foetida*, *Lumbricus rubellus*) was first isolated and named G-90 in 1992 (Hrzenjak et al. 1992). G-90 possesses several growth factors including an insulin-like growth factor (IGF-like), an immunoglobulin-like growth factor (IgFG-like), and epidermal growth factor (EGF) (Cooper et al. 2004). The results revealed that G-90 also participates in tissue regeneration and wound healing. G-90 can promote the synthesis of EGF and FGF (fibroblast growth factor) during wound healing of mouse skin (Grdisa et al. 2004). The glycolipoprotein tissue homogenate extract from *E. foetida* (G-90) can activate signal transduction pathways and cause an increased concentration of EGF and FGF as observed 6 h after wounding. Stimulation of cell proliferation on nerve regeneration usually involves initiation and progressive activity of growth factors. As mentioned previously, these growth factors (GDNF, BDNF, FGF, and NGF) can activate MAPK pathway to stimulate Schwann cell migration. Highly expressed uPA in the epidermis of damaged tissue is regulated by the FGF-2 which activates MAPK kinase (MEKK-1) and its downstream ERK1/2 (Witowsky et al. 2003). Therefore, the possible beneficial effect of G-90 extracted from earthworm on peripheral nerve regeneration remains unclear and requires further study to investigate and confirm.

15.2.5 Optimal Dosage of Earthworm Extract

Although the treatment benefits of earthworms have been strongly supported, more precise analyses are still required to even suggest accurate doses of medication. In Boyd's study, results suggested a dose-dependent facilitation and inhibition of

peripheral nerve regeneration by brain-derived neurotrophic factor (BDNF). In contrast to low-dose group, the high doses of BDNF significantly inhibited motor axonal regeneration (Boyd and Gordon 2002). Chang's study suggested similar results (Chang et al. 2009a, b). As a word of caution, an excessive EE load in the medium could provoke an adverse response to recovery of neuron regeneration.

15.3 Pharmacological and Clinical Application

The complementary and alternative medicine (CAM)-related natural products can be used as therapeutic agents in the treatment of human diseases (Cooper 2005a, b). Commonly used in TCM, earthworms (Dilong) benefit in the acquisition of novel molecules that may contribute to human therapeutic needs. To understand the underlying pharmacological mechanisms, substantial research has investigated the potential therapeutic effects of earthworms. EEs exhibit various biological activities, such as the mitogenic effect of insulin-like proteins (Hrzenjak et al. 1993), fibrinolytic and anticoagulative activities (Hrzenjak et al. 1998), and potential antioxidative effects (Popovic et al. 2001). In the next sections, a variety of compounds purified from earthworms will be discussed. (Balamurugana et al. 2009; Chang et al. 2009; Chen et al. 2007; Cooper et al. 2004, 2009; Grdisa et al. 2001, 2004; Hrzenjak et al. 1998; Hu and Fu 1997; Popovic et al. 2001, 2005; Schumacher et al. 1993; Sullivan et al. 2008; Tang et al. 2002; Zackenfels et al. 1995; Zhao et al. 2006; Zhao et al. 2007 (Table 15.1)).

15.3.1 Fibrinolytic Enzyme

In 1997, a novel fibrinolytic enzyme was isolated from *Pheretima aspergillum*. It can dissolve human thrombi and fibrin directly and strongly and also activate human plasminogen to plasmin (Hu and Fu 1997). Advances in the development of molecular and cellular biology techniques have greatly assisted biologists to analyze underlying molecular basis of extracts derived from earthworms. The crude EE has a thrombolytic effect that could significantly promote blood circulation that then removes stasis (Zhang and Wang 1992). Lumbrokinase (LK), a group of proteolytic enzymes extracted from the earthworm *L. rubellus*, has been used as the therapeutic agent in treating stroke and cardiovascular diseases (Jin et al. 2000). In Wang's study, those mechanisms involved in antiischemic actions of LK in brain have been examined (Ji et al. 2008). Results indicated that the antiischemic activity of LK was due to its antiplatelet effect that occurs by elevating cAMP levels and attenuating calcium release from calcium stores. The antithrombosis action was due to inhibiting ICAM-1 expression, and the antiapoptotic effect is due to the activation of JAK1/STAT1 pathway. LK can dissolve fibrin directly and also act as a plasminogen activator (Hu and Fu 1997; Tang et al. 2002). Purified from another

Table 15.1 Analysis of purified components derived from earthworms. Earthworm extracts exhibit various biological activities, such as the fibrinolytic, anticoagulative, and potential antioxidative effects. This table represents a variety of compounds purified from earthworms and their applications

Purified compounds	Earthworm specie	Effects	Mechanism
A. Fibrinolytic enzyme			
Lumbrokinase(LK)	*Lumbricus rubellas*	• Antiplatelet activity: elevate cAMP level attenuate the calcium release • Antithrombosis action: inhibit ICAM-1 expression • Antiapoptotic effect: activate JAK1/STAT1 pathway	• Potentiate adenylate cyclase (AC) • Increaset cAMP level • Inhibit rat platelet intracellular Ca(2+) • Attenuate glycoprotein IIB/IIIA (GPIIB/IIIA) and P-selectin • Dissolve fibrin directly • Act as a plasminogen activator
Earthworm fibrinolytic enzymes (EfP-0-2, EfP-I-1 and EfP-I-2)	*Eisenia fetida*	• Only EfP-I-1 exhibited distinct thrombolytic activity	Unknown
Earthworm protease-III-1 (*Ef* P-III-1)	*Eisenia fetida*	• Play a role in the balance between procoagulation and anticoagulation	• Fibrinogenolysis: cleave α, β, and γ chains of fibrinogen • Fibrogenesis: activate plasminogen and released active plasmin suggest a tPA-like function show factor Xa-like function on prothrombi produce α-thrombin
Earthworm-derived Factor Xa inhibitor (eisenstasin)	*Eisenia andrei*	• Bidirectional alternation between coagulation and inflammation	• Inhibit proteinase-activated receptor 2-mediated FXa activation during the propagation step of coagulation • Reduce endothelial nitric oxide (NO) • Inactivate protease-activated receptor-2 (PAR-2) • Reduce the expressions of proinflammatory cytokines (IL-1α, IL-1β, IL-8, IL-16, MCP-1, MIP-1α, and MIP-1β)
Earthworm fibronectinase (EFNase)	*Eisenia fetida*	• Antitumor activity	• Cleave fibronectin and inhibit HBV infection through its suppressing the level of HBeAg
B. G-90 glycolipoprotein	*Eisenia foetida*, *Lumbricus rubelus*	• Proliferation	• Immunoglobulin-like growth factor • Pidermal growth factor (EGF) • Increase EGF and FGF

(*continued*)

Table 15.1 (continued)

Purified compounds	Earthworm specie	Effects	Mechanism
		• Tissue regeneration and wound healing	
		• Fibrinolysis and anticoagulation	• Lysing fibrin clots
C. Polyphenolic	Indigenous earthworm powder (*Perionyx excavatus*)	• Hepatoprotective and antioxidant effect	• Decrease the activities of enzymatic antioxidant enzymes (SOD, CAT, GPx) • Decrease nonenzymatic antioxidant (vit C, vit E) • Reduce GSH
	Lampito mauritii	• Antiinflammatory and antioxidant property	• Restore antioxidants-reduced glutathione, glutathione Peroxidase, superoxide dismutase, catalase, and thiobarbituric acid reactive substances • Normalize the values of erythrocyte, leukocyte, differential levels of neutrophils, lymphocytes, eosinophils, hemoglobin, and serum biochemical contents

earthworm, *E. fetida*, protease (EfP) is able to hydrolyze fibrin and other protein, and it also activates proenzymes such as plasminogen and prothrombin, suggesting a tPA-like function (Zhao et al. 2006, 2007).

Recently, a novel Factor Xa inhibitor synthesized from the earthworm *Eisenia andrei* was named eisenstasin (Joo et al. 2009). Results suggested that eisenstasin-derived small peptide (ESP) could be an effective anticoagulant that targets earthworm-derived Factor Xa during the propagation step of coagulation. ESP can inhibit proteinase-activated receptor 2-mediated FXa activation, which may then trigger endothelial inflammation. ESP also can inactivate protease-activated receptor-2 (PAR-2) and reduce expressions of proinflammatory cytokines. Therefore, ESP may effectively control the bidirectional alternation between coagulation and inflammation. Recent studies reveal that the fibrinolytic enzymes could dissolve blood fibrin clots and inhibit platelet activation and aggregation (Ji et al. 2008). Earthworm fibrinolytic enzyme showed significant antitumor activity in human hepatoma cells both in vitro and in vivo, an effect since earthworm fibrinolytic enzyme induces apoptosis of hepatoma cells and inhibits expression of MMP-2 (Hrzenjak et al. 1992). ELISA results showed that secretion of HBeAg from HepG2.2.15 cells was significantly inhibited in the presence of earthworm fibronectinase (EFNase) (Wang et al. 2008). It cleaves fibronectin rapidly and shows a potential to inhibit HBV infection by suppressing levels of HBeAg. Above all, therapeutic and preventive effects of fibrinolytic enzymes for thrombosis-related

disease have been clinically confirmed (Chen et al. 2007), but for other major diseases such as malignant tumors there is substantial need for further analysis.

15.3.2 G-90 Glycolipoprotein

G-90 promotes anticoagulative and fibrinolytic activities by shortening the physiological time of fibrin clot lysis and completely inhibited blood clotting (Hrzenjak et al. 1998). Recently, tG-90 has been shown to be neither allergic nor toxic, and possesses antibacterial activity and antioxidative effects. G-90 also plays a key role in cell proliferation (Hrzenjak et al. 1993) and adhesion (Popovic et al. 1998). There is substantial confirmation that the G-90 mixture contains various growth factors, such as insulin-like growth factors, immunoglobulin-like growth factor, serine proteases, and EGF (Cicco-Bloom and Black 1988; Aizenman and de Vellis 1987; Hrzenjak et al. 1993, 1998; Popovic et al. 2001). Furthermore, in vivo, experiments reveal that it also contains molecules that can activate signal transduction pathways and increase EGF and FGF synthesis; in vivo, this would be essential to facilitate the tissue regeneration process, that is, wound healing (Kimura et al. 2004). In cell cultures, after treatment with H_2O_2 for 4 h, G-90 allows cells to recover and stimulates their growth. Thus, G-90 could be a useful wound-healing agent based on its apparent protective effect against the toxicity of H_2O_2 (Grdisa et al. 2001). G-90 exhibits an inhibitory effect on in vitro growth of nonpathogenic and facultative-pathogenic bacteria (Popovic et al. 2005). In a recent comparative study, biologists investigated the antipyretic and antiinflammatory activities of whole tissue extract from indigenous earthworm species, *Lampito mauritii* (Balamurugana et al. 2009). The EE was prepared and revealed properties similar to G-90. Both standard experimental groups and EE groups showed significant inhibition of paw oedema and granuloma and reduction in hyperpyrexia in rats. But administration of EE exhibits better results in a dose-dependent manner. Therefore, data revealed that the effects of antiinflammatory and antipyretic properties of EE were similar to glycoprotein complex (G-90). These may be of interest for further clinical investigations aimed at human medicine.

15.3.3 Polyphenolic

The antiinflammatory activity together with antioxidant properties seems to be due to the high polyphenolic content [e.g., antioxidants-reduced glutathione, glutathione peroxidase (GPx), superoxide dismutase (SOD)] in earthworm tissues (Balamurugan et al. 2007). The indigenous earthworm *Perionyx excavatus* could afford a significant hepatoprotective and antioxidant effect against alcohol-induced toxic rats by increasing the activities of enzymatic antioxidant enzymes: SOD, catalase (CAT), GPx,

and nonenzymatic antioxidant vitamin C, vitamin E, and reduced glutathione (GSH) (Prakash et al. 2008).

15.4 The Impacts on Ecological Systems

By their activity in the soil, earthworms offer many benefits important for increasing nutrient availability, improving better drainage and building soil fertility, all of which help improve farm productivity. Because of the earthworm's soil-based origin, the application of chemical fertilizers, sprays, and dusts on soil can have a disastrous effect on earthworm populations. Therefore, soil enzyme activity and metal bioaccumulation by earthworms can be used as ecological indicators of metal availability and environmental monitoring (Lee et al. 2009; Cikutovic et al. 1999; Cooper and Roch 1992; Fitzpatrick et al. 1990; Rodriguez et al. 1989). Research in New Zealand and Tasmania found earthworms introduced to worm-free perennial pastures produced an initial increase of 70–80% in pasture growth, with a long-term 25% increase. Researchers also found that the most productive pastures in the worm trials produced to 7 million worms per hectare, weighing 2.4 tons. There was a close correlation between pasture productivity and total worm weight, with some 170 kg of worms for every ton of annual dry matter production.

15.5 The Challenges After Breaking the Balance of Ecological Systems

The problem of invasive earthworms has become a concern that has increased recognition from the public. Its impacts are especially serious in the tropical and subtropical regions that are invaded by *Pontoscolex corethrurus* and in temperate regions that were previously devoid of earthworms due to glaciation (Hendrix et al. 2008). In addition, exotic earthworms altered the emergence of plant seedlings from the seed bank and the functional composition of the established plant seedlings (Eisenhauer et al. 2009). Further studies are essential to clarify these results and their influence.

15.6 Conclusion and Perspectives

Recently, evidence-based research has examined the therapeutic effects of earthworms, the current state of knowledge, and their impact on and responses to their environment. Here we have discussed observations: neurobiological mechanisms, especially nerve regeneration induced by earthworms, pharmacological functions, and impact on brief conclusion of ecological systems. Peripheral nerve regeneration requires a special environment and activation of intrinsic growth capacities of

neurons. Axon regrowth and remyelination of regenerated axons by Schwann cells are both essential. Multiple factors include those that are neurotrophic, ECM proteins, and hormones that participate in Schwann cell dedifferentiation, proliferation, and remyelination. Locally applied neurotrophins enhance survival of damaged neurons and regrowth of injured axons in the central and peripheral nervous systems in rats revealing that EE promotes proliferation and survival of RSC96 cells via IGF-I signaling. The mechanism is dependent primarily on the PI3K protein. The PACAP-like peptides accumulate in earthworm regenerating tissues, and those similar trophic functions of these compounds in earthworm and vertebrate tissues. However, a possible beneficial effect of G-90 extracted from earthworms on peripheral nerve regeneration remains unclear and requires further investigation and confirmation.

Earthworms exhibit various biological activities, for example, mitogenic effect of insulin-like proteins, fibrinolytic and anticoagulative activities, and antioxidative effects.(Cooper 2009) Purified from another earthworm specie, *E. fetida*, protease (EfP) not only hydrolyzes fibrin and other proteins but also activates proenzymes such as plasminogen and prothrombin, suggesting a tPA-like function. Results of ELISA showed that secretions of HBeAg from HepG2.2.15 cells were significantly inhibited in the presence of the EFNase. It cleaves fibronectin rapidly and shows a potential to inhibit HBV infection by suppressing the level of HBeAg. To go further, therapeutic and preventive effects of fibrinolytic enzymes for thrombosis-related disease have been clinically confirmed, but for other major diseases such as malignant tumors there is substantial need for further analysis. An EE has been prepared with properties similar to G-90. Both standard drug groups and EE groups showed significant inhibition of edema and granuloma and reduction in hyperpyrexia in rats. But administration of EE exhibits better results in a dose-dependent manner. Therefore, results revealed that the effects of anti-inflammatory and antipyretic properties of EE were similar to the glycoprotein complex (G-90). These may be of interest for further clinical investigations aimed at human medicine.

Humans have been aware of earthworms for many centuries. Usually, the first impression in our minds is that these small, reddish creatures reflect a healthy earthworm population that usually correlates with fertile soil to farmers; or they are mutilated by fisherman! However, the real “earthworm power” may be underestimated. Spread all over the earth’s international boundaries, earthworms not only form the basis of many food chains but also provide a basic biological model that may reflect similar conditions in other animal models in similar environmental niches. Recently, in a newly released book *What on Earth Evolved?*, the author ranked the top 100 dominated species according to their influences on Mother Earth and all life forms. Results reveal that the specie that has the greatest impact on the planet is not intelligent humans or giant dinosaurs, but the humble earthworm! In this year of Darwin, his book on earthworms is all the more pertinent to this book. Despite this remarkable revelation the world remains largely ignorant of “earthworm power” except their power to dangle in front of a fish hook!

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Chapter 16
Earthworms as Bioindicators of Soil Quality

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16.1 Introduction: Soil Quality and Soil Health

A soil quality assessment is characterized by an assessment of various parameters (soil quality indicators) in order to get an integrated view of the ecological soil functions at the place of investigation (Doran et al. 1994).

The Soil Science Society of America (SSSA) has defined soil quality as "the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health" (SSSA 1997). In particular, a soil of good quality (1) mediates water flow through the environment, (2) buffers and mineralizes organic wastes and xenobiotics, (3) is toxicologically safe, and (4) sustains biodiversity.

Although some authors use the term soil health interchangeably with soil quality (e.g., Harris and Bezdicek 1994), it may be useful to make a distinction. Soil quality as defined by the SSSA pertains to the functional properties of a soil not making a difference if these properties have developed naturally or if they are the result of anthropogenic degradation or amelioration. Soil health on the other hand is monitored to detect illness, that is, a deviation of the soil conditions from an ideal "healthy" state. In this view an untouched peat bog is a healthy soil of poor quality. The peat bog is loosing health and is gaining quality if it becomes drained and fertilized.

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Motivations for the assessment of soil quality may be as follows:

- Raise public awareness for soils as a valuable resource (Karlen et al. 2008).
- Indicate soil functionality: living space, biodiversity resource, biomass production; water uptake, distribution, storage; decomposition and degradation.
- Monitor global trends of change in ecosystems and in soil in particular.

Motivations for the monitoring of soil health may be as follows:

- Monitor the sustainability of (agricultural) land use (Palojärvi and Nuutinen 2002).
- Evaluate the need for and the success of soil remediation (e.g., against acidification, or contamination).

Bioindication is a constitutive element in the assessment of soil quality. Earthworms are often suggested as bioindicators of soil quality because they are an important part of the soil system and also because they are frequent, easy to collect, and rather simple to identify (Buckerfield et al. 1997; Paoletti 1999; USDA-NRCS 2009). The occurrence and the effects of earthworms are generally associated with good soil quality. Markert et al. (2003) defined bioindication as qualitative indication of environmental properties, and biomonitoring as quantified bioindication in order to detect trends in time and space.

Earthworms can indicate soil quality by (1) the abundance and species composition of the earthworm fauna at a particular site, (2) the behavior of individual earthworms in contact with a soil substrate (preference/avoidance/activity), (3) the accumulation of chemicals from the soil into the body, and (4) the biochemical/cytological stress-biomarkers in the earthworm.

This chapter will give an overview of the use of earthworms as bioindicators and biomonitors. At first, some examples are presented for the role of earthworms in long-term soil monitoring in Europe. This is followed by a brief account of laboratory tests with earthworms for the detection of toxic contaminants and for the assessment of general soil properties. Finally, the chapter discusses the use of earthworms as accumulation indicators for the bioindication of soil contaminants. Stress-marker bioindication with earthworms is not covered in this chapter. This topic is reviewed in Didden (2003).

16.2 Monitoring of Earthworm Communities

16.2.1 Monitoring Programs

The monitoring of earthworms in the field can be done on different levels. On-farm assessment schemes provide for a rough estimate of earthworm occurrence in general by counting “worms per shovel” (Table 16.1) or just looking at the soil surface for visible signs of activity like casts or middens (Evans et al. 1947;

Table 16.1 Representation of earthworms in soil quality cards for farmers in some US federal states (compiled from http://soils.usda.gov/sqi/assessment/sq_card.html)

State	Mentioning	Rating
Connecticut	33. Worms and other bugs in the soil	None/a few/many
Georgia	8. Biological activity	Little or no sign of animal life in the soil/some living organisms or signs of animal activity in the soil/ numerous signs of animal life in the soil
Illinois	Earthworms per shovel	0–1/2–10/>10
Maryland	Earthworms	0–1 worms in shovelful of top foot of soil. No casts or holes/2–10 in shovelful. Few casts, holes, or worms/ 10+ in top foot of soil. Lots of casts and holes in tilled clods. Birds behind tillage
Montana, North Dakota	Soil organisms (spring)	Few insects, worms or fungi/some insects, worms and fungi/many insects, worms and fungi
	Porosity (spring before tillage)	Very few worm and/or root channels, solid mass, hard plow pan/some new and old worm and/or root channels, weak plow pan/many worm and/or root channels
Nebraska	2. Biological activity	Very old residue that does not decompose; no sign of soil life (insects, worms, etc.)/moderate decomposition of residue; few soil organisms (insects or worms)/rapid decomposition of residue; many soil organism; and diverse population
Ohio	Soil life – earthworms	No visible signs of earthworm activity/some earthworms, few holes and casts/lots of earthworms, many holes and casts
Oregon	5. Are earthworms abundant in the soil?	No earthworms/few earthworms, holes or casts/many earthworms, earthworm holes and casts
Pennsylvania	Soil biodiversity	No or little evidence of earthworm activity; no nightcrawler mounds; spiders and ground beetles absent/some evidence of earthworm activity; some nightcrawler mounds; spiders and ground beetles scarce/much evidence of earthworm activity; many nightcrawler mounds; spiders and ground beetles visible under residue
Colorado	Biological activity	Little or no signs of insects, worms, etc./some living insects, worms, etc./large amounts of insects, worms, etc.

Hauser et al. 1998). A higher level of information is reached in programs with standardized sampling of earthworms at monitoring sites where details about species abundances and age structures are assessed.

In Germany, the federal states are running long-term soil monitoring programs with a total of about 800 permanent soil monitoring sites (BDF) focusing mainly on the chemical and biological soil conditions and covering arable land, grassland, and forest. The first BDF went into operation in 1985. The mandatory soil zoological parameters according to Barth et al. (2000) are earthworms and enchytraeids, but

this is not yet realized in all federal states. Investigations on earthworms are performed in eight German federal states so far, at intervals of 5–10 years (UBA 2007).

In the Netherlands, the Biological Indicator system for Soil Quality (BISQ) is a program that has been incorporated into the Netherlands Soil Monitoring Network (NSMN) with 300 locations selected in a random stratified design comprising stringent combinations of land use and soil type, and running since 1997. Investigated biological parameters are earthworms, enchytraeids, springtails, mites, nematodes, and microorganisms, sampled in a 6-year cycle (Rutgers et al. 2009).

While Germany and the Netherlands are the only EU countries with working long-term monitoring programs that include earthworms, few other EU-countries have made soil biology inventories with earthworms as key soil zoological bioindicators. One example is the French RMQS Biodiv, a regional pilot research program running from 2006 to 2008 in Britanny at 109 sites (16 × 16 km grid) of the French Soil Quality Monitoring Network (Peres et al. 2008). So far, RMQS Biodiv is a one-time investigation of earthworms, macroinvertebrates, springtails, mites, nematodes, and microflora. Parts of it have served as pilot study within the EU FP6 project ENVASSO (Environmental Assessment of Soil for Monitoring) aiming to devise a set of indicators in relation to the eight threats to soil identified by the European Commission (2002). For assessing the threat "decline in soil biodiversity" ENVASSO resulted in proposing three first-level indicators: earthworm species (enchytraeid species if no earthworms), Collembola species, and soil respiration (Bispo et al. 2009).

Table 16.2 lists the soil zoological parameters investigated at soil monitoring sites in northern Germany (Schleswig-Holstein, Hamburg, Nordhein-Westfalen) and the kind of information they indicate (Graefe et al. 2001).

Table 16.2 Soil zoological parameters investigated at soil monitoring sites in northern Germany

Parameter	Indicator function
Total abundance of earthworms (ind./m^2)	Zoological indicators of the biological activity in the soil
Total biomass of earthworms (g/m^2)	
Total abundance of microannelids (ind./m^2)	
Community structure: species composition, species number, abundance, dominance, and frequency of species	Zoological indicators of soil biodiversity
Vertical distribution of enchytraeids: total and species level	Indicator of the vertical extent and strength of the biological activity
Biomass and biomass dominance of earthworm species	Ecological significance of the species
Aggregated community parameters: Trait types spectra and functional indices Average indicator values Decomposer community type	Biological indicators of soil quality – meaning both the biological condition of the soil and the impact of environmental factors on the soil biota

16.2.2 Monitoring Results

Twenty years of earthworm monitoring in Bavaria (1985–2005) showed a significant increase of the earthworm abundance averaged over roughly 100 arable sites. Climatic reasons can be ruled out since there was no significant trend at the grassland sites (Bauchhenß 2005). The average increase of earthworm populations can be seen as an indication of improving soil management practices in plant production in Bavaria.

16.2.3 Indicator Values of Earthworm Species Derived from Their Habitat Requirements

Inventories and monitoring of the earthworm fauna in relation to soil properties and site characteristics have revealed a body of experience about the habitat preferences and requirements of different earthworm species (e.g., Irmler 1999; Mascato et al. 1987; Bouché 1972; Nordström and Rundgren 1974). Based on this, Römbke et al. (2005) set up expectation values for the 15 most frequently observed earthworm species in Central Europe with respect to 5 soil properties (soil texture, pH value, moisture, C/N ratio, and organic matter). From this, they assigned "main species" and "possible species" to 28 different site types. Tischer (2008) modified this classification based on the assessment of earthworms at 84 soil monitoring plots in Central Germany. Krück et al. (2006) proposed a classification scheme for earthworms in arable land in the German federal state Brandenburg which is characterized by sandy soils and rather dry climate. The classification used principal components analysis (PCA) and revealed soil texture and soil organic matter content as key variables (Krück et al. 2006). Graefe (1997, 2005) investigated the annelids (earthworms and enchytraeids) at 60 soil monitoring sites in northern Germany including arable land, grassland, and forest (Beylich and Graefe 2009). He adopted the system of plant indicator values (Ellenberg 1979) and assigned reaction values (for soil pH) and soil moisture values to the species (Graefe 1993; Graefe and Schmelz 1999). The bioindication could be improved considerably by integrating the enchytraeids into the assessment procedure. Indicator values allow to calculate mean values of the annelid community per site and sampling occasion, indicating changes of the soil condition over time. In the evaluation of earthworm data, the functional traits of species (anecic, endogeic, epigeic) were given special emphasis.

16.2.4 Constraints on the Interpretation of Earthworm Abundance as an Indicator of Soil Quality

The abundance of an earthworm population is not only determined by the soil quality (Curry 2004, Fig. 16.1).

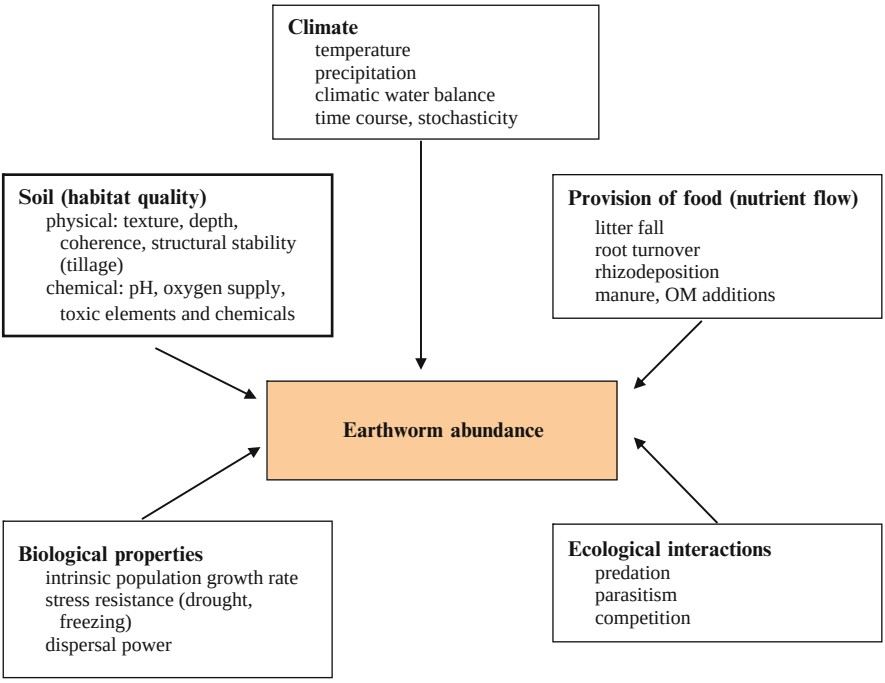

Fig. 16.1 Factors determining the abundance of earthworms in the field

Soil, climate, and food, the three main factor complexes depicted in Fig. 16.1, may be characterized as follows: The soil quality is determining the physical and chemical habitat quality in relation to the species-specific preference and tolerance. The provision of food is determining the carrying capacity/maximum population size. Weather is decimating populations and initiates phases of recovery. Catastrophic breakdowns of the earthworm population after events of exceptional drought or freezing have been observed by Graff (1964) and Ehrmann et al. (2007). Timmermann et al. (2006) observed that earthworm numbers in wet grassland in the Netherlands fluctuated by a factor of 5.8 during a 10 years observation period. The fluctuations were correlated with the sum of daily average temperature values below 0°C in the preceding winter. Other authors found soil moisture to be the key limiting factor for earthworm populations (Auerswald et al. 1996; Eggleton et al. 2009). When earthworm abundance is monitored it has to be considered that the population may be in a phase of recovery and not be typical for the soil component of its environment. The maximum speed of recovery is determined by the intrinsic growth rate of the population. Estimates for European endogeic species are $\times 2$ to $\times 10$ per year (Fründ et al. 2004). Species number and community composition of earthworms have been found to be more reliable monitoring parameters than abundance and biomass (Joschko et al. 2006).

16.3 Bioindication with Earthworms in Laboratory Assays

While the results of field assessments are influenced by weather and other nonsoil factors laboratory experiments have the advantage of controlled conditions. Their results should be better reproducible than field results, and the experiments can be carried out at any time in the year. Earthworms have been used in a multitude of microcosm experiments either investigating their response to environmental conditions, or looking for the impact of earthworms on their environment (Fründ et al. 2010). Microcosm experiments with earthworms can also be used for the assessment of soil quality. For practical reasons laboratory assays with earthworms should be of short duration. Therefore, behavioral responses are tested. Life cycle parameters are usually beyond the scope of bioindication assays with earthworms in the laboratory.

Two experimental systems are applicable for the testing of soil quality with earthworms as bioindicators: the earthworm avoidance test (ISO 2008) and the observation of burrowing and casting activity in 2D (two-dimensional) terraria (Evans 1947; Topoliantz and Ponge 2003; Fründ et al. 2009b).

16.3.1 Avoidance Test

The avoidance test with *Eisenia fetida* has been proposed by Yeardley et al. (1996). Subsequently, the test was further elaborated by Hund-Rinke and Wiechering (2001) and Hund-Rinke et al. (2003). Eventually the test was described as an ISO Standard (ISO 2008). In the test, a test soil and a reference soil are put separately in either half of a box. Ten worms (usually clitellate *E. fetida*) are put on the surface of the substrate at the line where both soil substrates are touching. The box is left undisturbed in the dark for 24–48 h. After this time a dividing sheet is inserted into the box to separate the two soils and the number of worms in either half is counted. A significant uneven distribution of worms between the two substrates in the box (exceeding 20:80%) is taken as indicating toxicity (adversity) of the avoided substrate. A reference soil is necessary for the execution of the test. This may be field soil from a somehow comparable site (van Zwieten et al. 2004; Fründ et al. 2005), artificial soil (OECD-soil), or a certified reference soil (Refesol http://www.refesol.de). The avoidance test has been used for the assessment of contaminated floodplain soils (Fig. 16.2) and corresponded well with the field abundance of endogeic earthworms and with the concentration of the main contaminant copper in the soil at the sampling points (Fründ et al. 2005).

Figure 16.2 shows that the avoidance test clearly indicated soil contaminations in the floodplain exceeding 100 mg Cu in the arable fields and the set-aside land. In the meadows, the contamination effect was probably less pronounced. The strong avoidance effect in the set-aside land on the other hand raises the question if there are other contaminants in addition to the copper analyzed. Similar results

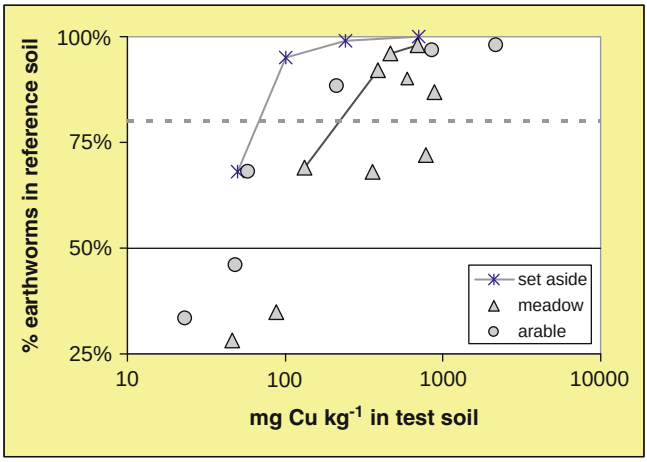

Fig. 16.2 Summary of *E. fetida* avoidance tests with soils from a contaminated floodplain. *Connecting lines* indicate dilution of soils from the same sampling point with reference soil. The *dotted line* at 80% indicates the toxicity criterion according to ISO 17512-1 (Fründ et al. 2005 and Prjanikov unpublished)

were obtained by van Zwieten et al. (2004) in soils of an Australian avocado farm with high copper concentrations from fungicide spraying.

The avoidance test has also been done with *Aporrectodea* species, which are more natural to field soils than the laboratory-bred compost worm *E. fetida* (Lukkari and Haimi 2005; Capowiez and Bérard 2006).

16.3.1.1 Two-dimensional terraria

Soil-filled cuvettes [also referred to as Evans' boxes or two-dimensional (2D) terraria] (Evans 1947) easily allow for the visual assessment of earthworm activity. These observation cages are an established tool for the study of the burrowing, feeding, and egestion behavior of earthworms (Evans 1947; Schrader and Joschko 1991; Schrader 1993). Observation cages have been used mainly to investigate aspects of earthworm biology. The studied aspects include burrowing and feeding activity, mucus and cast production, and the reaction of earthworms to specific soil properties and soil amendments. Observation cages also offer the possibility to test the quality of a soil substrate and use the earthworm as a bioindicator. Tests can be set up with only one soil substrate in the cuvette ("mono-design"). It is also possible to fill the left and right half of the cuvette with different soil substrates so that the earthworms can chose between two soil substrates (choice design). For the choice design a sheet dividing the cuvette during the filling up with soil is removed before the earthworms are introduced.

An example of soil testing with the mono-design is the study of Capowiez et al. (2003). In a series of 2D terraria with increasing concentrations of the insecticide

Imidacloprid, they found that sublethal concentrations of the insecticide significantly reduced the burrow length of *Aporrectodea nocturna* and *Aporrectodea icterica*. Effects of sublethal concentrations of Imidacloprid on these earthworm species were not detected in an avoidance test (Capowiez and Bérard 2006). The choice design was used by Topoliantz and Ponge (2003) to investigate the effect of charcoal on *Pontoscolex corethrurus*. After 2 weeks, the visible area of burrows and casts was significantly smaller in the part of the cuvette containing a mixture of soil and charcoal compared to the other half filled with soil only.

These examples show that 2D terraria are suited for the bioindication of soil quality by earthworms. When a soil quality test is executed with 2D terraria the general rules for the experimentation with earthworms should be followed (Fründ et al. 2010). The visible traces of burrowing and casting activity at the cuvette sides have often been calculated as burrow length or with more sophisticated geometric parameters. In a standardized test design, this can be simplified to a simple measurement of burrow and cast area. The interpretation of earthworm activity in 2D terraria is not straightforward because (1) the burrowing and casting activity in the first 3 days (settlement of substrate) may differ from the following period when feeding from soil predominates (Bolton and Phillipson 1976), (2) burrowing and casting activity may be higher in a soil containing less food (Lee 1985), (3) humidity differences between substrates compared in the choice design may determine the earthworm preference overlaying a difference in substrate quality (Fründ et al. 2009b).

16.4 Monitoring of Earthworms as Accumulators of Metals and Xenobiotica

16.4.1 Suitability of Earthworms as Accumulation Indicators

For a number of reasons earthworms are well suited to serve as accumulation indicators for the presence of bioavailable chemicals in the soil:

1. “Earthworms reside in soil and are more or less in constant contact with some portion of the soil.
2. Earthworms reside in contaminated sites, allowing field validation of chemical bioavailability.
3. Earthworms are found in a wide variety of soil types and horizons.
4. The exterior epidermal surface of the earthworm is vascularized with no cuticle, allowing the uptake of contaminants directly from the soil.
5. Earthworms ingest soil or specific fractions of soil, providing a means for the dietary uptake of contaminants.
6. Earthworms have a large mass, so contaminant concentrations can be determined in individual organisms.

7. There is a low level of mixed-function oxidase (MFO) activity, allowing greater potential for the accumulation of organic compounds that would normally be metabolized in other organisms.
8. We have an understanding of their physiology and metabolism of metals." [cited from Lanno et al. (2004)].

Environmental chemicals in earthworms may also be a relevant issue for wildlife protection since earthworms are an important food of many vertebrate and invertebrate species (Beyer and Stafford 1993). There are two important parameters when earthworms are used as accumulation indicators: The body concentration in the earthworm indicates the risk of secondary poisoning for predators feeding on earthworms. The bioaccumulation factor (BAF = concentration of chemical in worm/concentration of chemical in soil) indicates the bioavailability of the contaminant in the soil.

16.4.2 What Chemicals/Elements Have Been Found to Accumulate?

There is an extensive body of literature dealing with the uptake of metals by earthworms. In general, it has been found that Cd, Hg, and Zn have a tendency of becoming bioconcentrated in the earthworm with a bioaccumulation factor (BAF: ratio concentration in worm/concentration in soil) > 1 (Neuhauser et al. 1995; Rahtkens and von der Trenck 2006; Ernst et al. 2008; Tischer 2009; Nahmani et al. 2009). The distribution of heavy metals in earthworm tissues has been reviewed by Hopkin (1989) and Peijnenburg and Vrijver (2009). The alimentary tract of earthworms is a relatively straight tube from the mouth to the anus divided into a foregut, midgut, and hindgut. The midgut is where much of the metabolic activity takes place and therefore is involved intimately in the uptake, transport, storage, and excretion of metals (Hopkin 1989). Surplus essential and nonessential metals are stored as metal-bound proteins and/or metal-containing granules. The granules are stored in the chloragogenous tissue surrounding the lumen of the gut. The earthworm species have different ways to deal with heavy metals. *Lumbricus terrestris* has active calciferous glands and as a result accumulates less Pb, whereas *Aporrectodea longa* relies more on production of waste nodules for excretion of Pb and hence accumulates relatively more (Hopkin 1989). Cd is mainly handled by the formation of metallothioneines (metal-binding proteins). Formation of these proteins results in highest Cd levels in the gut wall (Andersen and Laurensen 1982) and nephridia (Prinsloo et al. 1990).

The equilibrium partition theory provides a framework for the estimation of the accumulation of organic chemicals in earthworms (Belfroid et al. 1995; Jager 1998). According to this theory, only substances in soil water could be accumulated by earthworms. A pharmacokinetic model to estimate bioconcentration in earthworms was developed recently by Henson-Ramsey et al. (2009). BAF for persistent

organic pollutants (POP's) were reported for DDT (BAF = 5), Dieldrin (BAF = 8), and heptachlor epoxide (BAF = 10) (Beyer and Gish 1980). PCBs are accumulated less (BAF = 1.8) (Beyer and Stafford 1993). Ma et al. (1998) found an average BAF of 0.1 (range 0.03–0.26) for the uptake of PAHs into *Lumbricus rubellus* at contaminated floodplain sites.

16.4.3 What Influences the Uptake of Chemicals by Earthworms?

There is high variation in the BAF of metals in earthworms collected from different sites. Table 16.3 shows BAF in earthworms at 84 soil-monitoring plots in Germany with various land use types (Tischer 2009; no gut clearance). Table 16.4 presents BAF in earthworms collected from 27 forest soils in Switzerland (Ernst et al. 2008; with gut clearance).

The bioaccumulation of an environmental chemical into the earthworm is a complex process which is determined by several abiotic and biotic factors (Fig. 16.3).

In the soil, the bioaccumulation depends on the concentration, on the chemical speciation (chemical bioavailability), and on the spatial distribution (physical bioavailability) of the chemical.

- *Concentration*: Neuhauser et al. (1995) investigated the bioconcentration of Cd, Cu, Ni, Zn, and Pb from sewage sludge contaminated soils in *Aporrectodea tuberculata* and *L. rubellus* and compiled data from another 20 published studies. A significant correlation between the metal concentration in the soil and the metal concentration in the worm turned out for Cd, Zn, Pb, Cu but not for Ni. The BAF declines at high concentrations in soil. This holds in particular for Cd and the essential metals Zn and Cu. Tischer (2009), analyzing earthworms without gut-clearance from 84 soil-monitoring sites, found significant positive correlations between metal contents in earthworms and metal contents in soil for Cd ($R^2 = 0.72$**), Cu ($R^2 = 0.65$**), Cr ($R^2 = 0.54$*), Pb ($R^2 = 0.51$*), Zn ($R^2 = 0.47$*), and Ni ($R^2 = 0.45$*).

Table 16.3 Bioaccumulation factors (ratio of metal content in soil to metal content in worm) according to Tischer (2009) for lumbricid species (n = number of plots)

Species	n	Cd	Zn	Pb	Ni	Cr	Cu
Dendrobaena octaedra	7	7···85	3···19	0.1···0.7	0.2···0.9	0.2···0.5	0.3···0.9
Lumbricus castaneus	8	2···23	2···10	0.1···0.3	0.2···0.4	0.1···0.4	0.3···1.6
Lumbricus rubellus	22	3···60	2···9	0.1···0.3	0.1···0.4	0.1···0.5	0.2···1.0
Aporrectodea caliginosa	41	2···58	1···16	0.1···0.9	0.1···0.9	0.1···1.3	0.3···1.3
Allolobophora chlorotica	18	5···21	1···7	0.3···0.9	0.3···0.8	0.3···1.3	0.4···1.9
Aporrectodea rosea	30	3···52	1···10	0.3···0.9	0.3···0.9	0.3···1.0	0.5···1.8
Octolasion cyaneum	17	4···29	2···8	0.4···0.7	0.4···0.9	0.3···1.2	0.4···1.3
Octolasion tyrtaeum	8	2···36	1···9	0.1···0.8	0.2···0.7	0.2···0.8	0.4···1.8
Lumbricus terrestris	52	3···45	1···14	0.1···0.8	0.1···0.8	0.1···0.9	0.3···1.2
Aporrectodea longa	7	4···34	2···8	0.2···0.7	0.3···0.7	0.1···1.4	0.5···2.8

Table 16.4 Bioaccumulation factors (ratio metal content in soil to metal content in worm tissue) for earthworm species in Swiss forest soils (Ernst et al. 2008)

Ecophysiological species group		BAF soil earthworms		
		Pb	Cd	Hg
Epigeic	*Lumbricus rubellus* ($n = 16$)	0.2 ± 0.1	26.9 ± 26.6	1.1 ± 0.6
	Dendrodrilus rubidus ($n = 8$)	4.7 ± 3.5	37.8 ± 43.4	4.1 ± 3.3
Endogeic	*Aporrectodea caliginosa* ($n = 5$)	0.8 ± 1.5	11.2 ± 19.6	2.9 ± 2.5
	Aporrectodea rosea ($n = 5$)	4.5 ± 9.8	120.6 ± 179.9	15.2 ± 15.3
	Octolasion tyrtaeum ($n = 7$)	0.3 ± 1.5	28.1 ± 16.9	7.6 ± 4.5
	Octolasion cyaneum ($n = 9$)	1.2 ± 2.4	47.9 ± 70.1	14.7 ± 14.3
Anecic	*Lumbricus terrestris* ($n = 4$)	0.3 ± 0.2	26.4 ± 15.8	1.5 ± 0.8
	Aporrectodea longa ($n = 6$)	0.7 ± 0.5	57.4 ± 86.8	10.7 ± 11.3

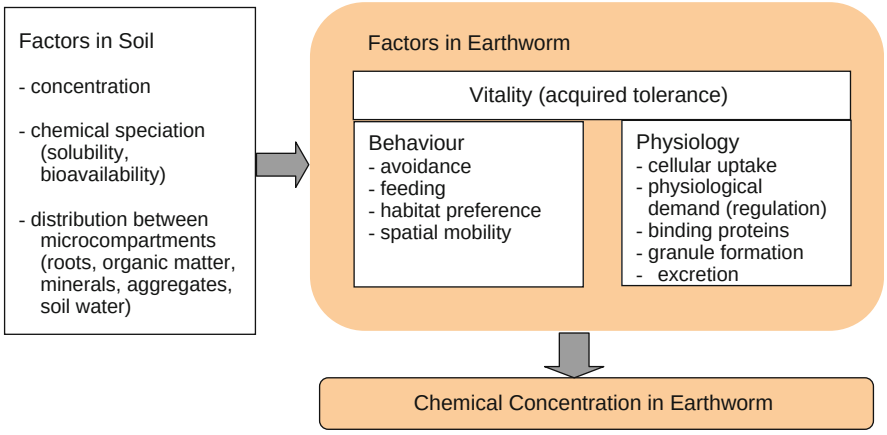

Fig. 16.3 Factors determining the concentration of environmental chemicals in earthworms from field populations

- *Chemical speciation*: Alberti et al. (1996) showed that lead that has been added to the soil as a salt is accumulated more than lead from an aged soil contamination. It is a general experience that aging reduces the bioavailability of contaminants. Soil properties like pH and redox potential are important determinants for the speciation/solubility of metals in the soil.
- *Distribution between microcompartments*: Morgan and Morgan (1999) showed experimentally that the Pb distribution in the soil profile affected the pattern of tissue concentration between the epigeic *L. rubellus* and the endogeic *Aporrectodea caliginosa*. Plant roots are microcompartments in the soil with enriched concentrations of heavy metals (Ernst et al. 2008).

Among the factors in the worm vitality is a prerequisite for the indication of bioaccumulation. Only worms can be analyzed from a contaminated site that have been able to survive the contamination. Often these worms have adapted to the contamination and therefore are not representative for the average population.

Consequently, accumulation monitoring with earthworms will probably be biased at highly contaminated sites. The concentration of a substance in the worm is regulated by the interplay of physiological mechanisms such as uptake, internal demand, internal sequestration, and excretion. These mechanisms are subject to adaptations as well as to the regulatory influence of environmental factors such as the pH or the Ca-ion concentration (Nahmani et al. 2009).

Behavioral aspects include the feeding and habitat choice of the earthworm (ecological group). Ernst et al. (2008) showed in a PCA analysis that there were specific patterns in the Cd, Pb, and Hg content in earthworms of ecological groups in relation to the metal content in different compartments of the soil-litter system.

16.4.4 What is the Use of Taking Earthworms as Accumulation Indicators of Environmental Chemicals?

As mentioned above, the concentrations of chemicals in earthworms from severely contaminated sites are likely to be influenced by site-specific adaptations. This leads to the conclusion that analyzing chemical concentrations in earthworms is better for the monitoring of background contaminations than for the assessment of highly contaminated sites.

In regional inventories of soil contamination the concentration in the earthworm indicates the bioavailable part of the contamination (e.g., Ernst et al. 2008; Tischer 2009). Monitoring of trends can be done by the repeated assessment of chemical concentrations in a bioindicator species. A fine example is *L. rubellus* in the soil monitoring program of the State Institute for Environment, Measurements and Nature Conservation Baden-Württemberg (LUBW) in Germany (Fig. 16.4).

The environmental specimen bank ("Umweltprobenbank") of the German Umweltbundesamt contains specimen of *L. terrestris* and *A. longa* from a set of annually sampled sites that are stored in liquid air for retrospective monitoring purposes (Gies et al. 2007).

In the laboratory, chemical analysis of experimentally exposed earthworms is also used as a bioassay for the validation of chemical extraction methods (Yu et al. 2005).

16.5 Conclusion

The ecological importance of earthworms and their rather easy handling in collection, species determination, and chemical analysis have made them the best studied group of soil fauna. Earthworms can serve as reaction indicators and as accumulation indicators. In both cases, it has to be kept in mind that earthworms and their populations are regulated by various influences responding not only to soil

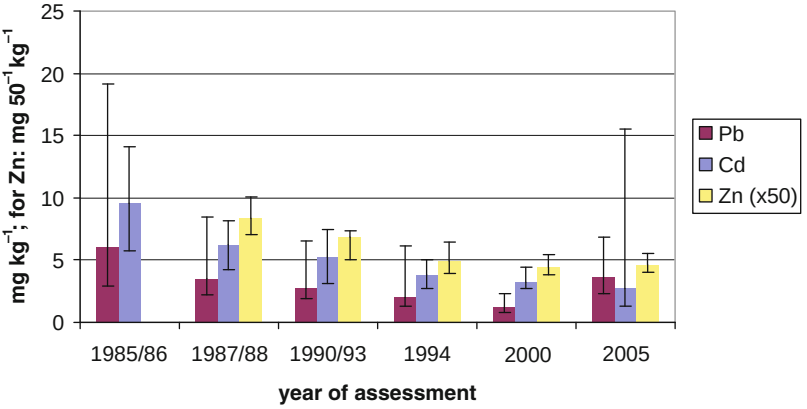

Fig. 16.4 Median values with 1. and 3. quartile of Pb-, Cd-, and Zn-concentration in the earthworm *Lumbricus rubellus* sampled from 1985 to 2005 at 60 permanent forest monitoring sites in Baden-Württemberg, Germany. (Rahtkens and von der Trenck 2006 and Rahtkens pers. comm.)

conditions but also to climate and to agricultural management. A combination of field assessments and controlled laboratory tests may help in differentiating the influence of climatic effects from that of soil chemical effects in the interpretation of monitoring data.

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Chapter 17
Application of Molecular Genetics to Earthworm Ecology: Current Research and Promising Future Directions

F. Lazrek, T.P. Velavan, J. Mathieu, and L. Dupont

17.1 Introduction

Understanding the movement of individuals among populations is fundamental in ecological studies because gene flow can profoundly influence population size and dynamics, genetic diversity and local adaptation processes. Only little information is available concerning earthworm dispersal and gene flow (Edwards and Bohlen 1996), in part because dispersal is highly difficult to measure in such species hidden in the soil. Molecular tools provide a great variety of potential solutions for measuring gene flow. The detailed analysis of the genetic structure within and among populations can indeed be used to depict the nature of dispersal and its consequences on gene flow regimes among a set of wild populations (Bohonak 1999; Broquet and Petit 2009). Although molecular markers were increasingly used in earthworm ecological research during the last decades, the use of molecular tools was, however, restricted to certain areas of research such as ecotoxicology (review in Dupont 2009; Sturzenbaum et al. 2009). Significant changes in genotype/allele frequencies and overall genetic variation may indeed occur in contaminated environments as a result of population bottlenecks and/or selection at certain loci. Gene flow is another key factor affecting the amount and kind of genetic variation in populations that have been rarely investigated in earthworms with molecular markers.

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A well understanding of the reproductive behaviour is a pre-requisite to infer gene flow patterns from population genetic structure. Indeed, mating system, especially self-fertilization, correlates with the distribution of genetic diversity within and among populations (Charlesworth et al. 1993). Earthworms are exclusively simultaneously hermaphroditic animals with reciprocal insemination, which present a great diversity of reproductive modes. It is generally accepted that diploid (2n) earthworms are amphimictic (i.e. sexually reproducing by cross-fertilization) while orthoploids (4n, 6n, etc.) reproduce both parthenogenetically and sexually and anorthoploids (3n, 5n, etc.) are only parthenogenetic (Viktorov 1997). In parthenogenetic species, population genetic study might be difficult to carry on because of low clonal variability (but see Terhivuo and Saura 2008) while in amphimitic species, a higher genetic diversity is expected, although inbreeding may also alter the genetic diversity within a population. Many other aspects of the mating behaviour of earthworms remain poorly understood. For instance, issues such as occurrence of self-fertilization or mate choice are still unresolved. Analyses of relatedness and parentage using polymorphic molecular markers could be highly informative for mating behaviour inferences in amphimitic earthworms.

In this chapter, current molecular markers appropriate to address various issues of earthworm ecology are first presented, putting the emphasis on microsatellites. Because the problematic taxonomy of earthworms is a critical drawback for intra-species population genetic studies, we briefly summarize the recent advances in molecular taxonomy of earthworms. Finally, the interest of using molecular markers in studies of mating behaviour and dispersal mode is discussed.

17.2 Current Molecular Markers for Earthworm Genetics

Genetic markers are inherited variations that can be used to study genetic structure within and among populations. Markers allow one to determine which alleles are present in populations, and constitute therefore an effectual toolbox for modern ecologists, for inference in a great number of situations (Avise 1994; DeYoung and Honeycutt 2005). In particular, quantifying patterns of genetic variation allows insight into population processes (e.g. dispersal, demographic history, hybridization). Moreover, genetic markers can identify individuals and populations, permitting for instance to define units for management, to infer source populations and to investigate parentage.

Different classes of markers exist and provide different levels of resolution and statistical power, as well as various advantageous and disadvantageous properties (Avise 2004; Freeland 2005). A population genetic survey must start with a decision regarding appropriate genetic markers. The selection of a marker is based on its mode of inheritance, sensitivity for the question, comparability among studies, reproducibility and mode of revelation (i.e. assayable by polymerase chain reaction, PCR). Molecular markers may be classified as dominant or co-dominant markers. Co-dominant markers allow one to identify all of the alleles that

are present at a particular locus, whereas dominant markers will reveal only a single dominant allele but at several loci simultaneously. Co-dominant data are generally more precise than dominant data but dominant markers usually require less development time and may therefore be a more convenient way to obtain data (Freeland 2005). Some markers commonly used in animal population genetics are mtDNA markers, single nucleotide polymorphisms (SNPs), allozymes, restriction fragment length polymorphisms (RFLPs), microsatellites or simple sequence repeats (SSRs), random amplification of polymorphic DNA (RAPDs) and amplified fragment length polymorphisms (AFLPs). Their principal characteristics are described in Table 17.1.

Until 1998, molecular markers used for earthworms were exclusively proteins (i.e. isozymes and allozymes) but were found to have substantial limitations in population studies. In particular, it was realized that protein electrophoresis reveals only a small fraction of the genetic variation. RAPD is another type of marker that has been widely used in earthworms (see Dyer et al. 1998; Kautenburger 2006; Lentzsch and Golldack 2006). For instance, RAPDs revealed that *Lumbricus terrestris L.* individuals possess a relatively similar genetic structure within sampling sites as well as among adjacent sites in western Germany (Kautenburger 2006). The popularity of RAPDs has, nevertheless, decreased in recent years because of their general lack of reproducibility, combined with their dominant nature (Freeland 2005). More promising molecular markers, such as mitochondrial DNA (mtDNA) markers, microsatellites and AFLPs, are increasingly used in earthworms and are described later.

17.2.1 mtDNA Markers

mtDNA represents rapidly evolving DNA sequences that are informative for answering population-level issues. Thus, studies of intraspecific phylogeography, which focus on patterns of variation resulting from either historical or recent barriers to gene flow among populations, were initiated using mtDNA (Avise 2000). Inferences drawn from mtDNA sequences are, however, limited by the fact that the mtDNA genome comprises a single uniparentally inherited locus. The widely used mtDNA markers are ribosomal RNA markers (i.e. 12S rDNA and 16S rDNA), protein-coding genes markers (e.g. cytochrome b, cytochrome oxidase subunits I and II, NADH dehydrogenase subunits) and control region markers (i.e. D-loop).

So far, phylogeographical studies have been rarely undertaken in earthworms (but see Cameron et al. 2008) although mtDNA sequences have been used for several years to discriminate morphologically similar earthworm species (i.e. cryptic species, see references in Chang et al. 2009; Dupont 2009 and Table 17.2). Indeed, morphological identification of lumbricid species is difficult because of the lack of stable and readily scorable diagnostic characters (Pop et al. 2003), and identification of juveniles is currently impossible in most cases. Recently, DNA

Table 17.1 Current molecular markers for earthworm genetics

Marker type	PCR-based?	Single-locus	Codominant	Comparability among taxa	Level of polymorphism	Already employed for earthworm research?	Suitability for exploring[a]				
							Phylogeography	Genetic diversity	Gene flow at fine/medium scales	Inbreeding	Relatedness/parentage
MtDNA markers	Yes	Yes	N/A[b]	Direct	Low-High	Yes[c]	+++	++	+	−	−
SNPs	Yes	Yes	Yes	Direct/indirect[d]	Low-High	No	+++	++	+	−	−
Allozymes	No	Yes	Yes	Direct	Low-moderate	Yes[c]	+	++	+	+	+
RFLPs	No	Yes	Yes	Direct	Low-moderate	No	+	++	+	+	+
Microsatellites	Yes	Yes	Yes	Indirect	High	Yes[c]	++	+++	+++	+++	+++
RAPDs[e]	Yes	No	No	Limited	High	Yes[c]	+	+	+	−	−
AFLPs	Yes	No	No	Limited	High	Yes[f]	++	+++	+++	−	+

(+++) excellent; (++) good; (+) moderate; (−) unlikely to be used or useless

[a]In the context of earthworm research. Adapted from Sunnucks (2000), DeYoung and Honeycutt (2005), Freeland (2005), Jarne and David (2008)

[b]mtDNA is haploid

[c]See references in Dupont (2009)

[d]SNPs can be amplified using either universal primers (direct comparability) or specific primers (indirect comparability).

[e]RAPDs suffer from reproducibility problems

[f]King et al. (2008)

Table 17.2 Recent data of intraspecific genetic divergence in earthworms

Family	Species	Mitochondrial markers[a]	Nuclear markers	N_i	Intraspecific genetic divergence	Cryptic species?	Reference
Hormogastridae	*Hormogaster elisae*	COI		82	5.75–20.20%	Yes	Novo et al. (2009)
Lumbricidae	*Allolobophora chlorotica*	COI		10	11.55%	Yes	King et al. (2008)
	Aporrectodea caliginosa	16S, 12S, ND1, COII, tRNA-$S_{Asn-\ Asp-Val-Leu-Ala-Ser-Leu}$	28S	11	Two subclades	Yes	Perez-Losada et al. (2009)
		COI		13	0.89%	No	King et al. (2008)
	Aporrectodea longa	16S, 12S, ND1, COII, tRNA-$S_{Asn-\ Asp-Val-Leu-Ala-Ser-Leu}$	28S	6	Two subclades	Yes	Perez-Losada et al. (2009)
		COI		12	6.8%	Yes	King et al. (2008)
	Aporrectodea rosea	COI		10	12.35%	Yes	King et al. (2008)
	Aporrectodea trapezoides	16S, 12S, ND1, COII, tRNA-$S_{Asn-\ Asp-Val-Leu-Ala-Ser-Leu}$	28S	12	Two subclades	Yes	Perez-Losada et al. (2009)
	Aporrectodea tuberculata	16S, 12S, ND1, COII, tRNA-$S_{Asn-\ Asp-Val-Leu-Ala-Ser-Leu}$	28S	5	N/A	No	Perez-Losada et al. (2009)
	Lumbricus castaneus	COI		4	3.02%	No	King et al. (2008)
	Lumbricus rubellus	COI		10	8.28%	Yes	King et al. (2008)
	Lumbricus terrestris	COI		9	3.66%	No	King et al. (2008)
		ND2, ND4		199	Up to 6.59%	No	Field et al. (2007)
	Satchellius mammalis	COI		2	0.35%	No	King et al. (2008)
Megascolecidae	*Amynthas formosae*	COI		9	0.2–10%	No	Chang and Chen (2005)
	Amynthas wulinensis	COI		22	> 14.6%	Yes	Chang et al. (2007)
	Amynthas yuhsii	COI		17	2.9–10.3%	No	Chang and Chen (2005)
	Metaphire sieboldi	COI, 16S		68	COI: 0.1–18.1%, 16S: 0–6.2%	No	Minamiya et al. (in press)

For each species, the markers used, the number of individuals analyzed (N_i), intraspecific genetic divergence as well as the possible occurrence of cryptic species, as described in the cited paper, are presented

[a]*COI* cytochrome oxidase subunit I; *12S* 12S rDNA; *16S* 16S rDNA, *ND1-4* NADH dehydrogenase subunits 1–4 genes

barcoding (i.e. a technique that uses a short DNA sequence from a standardized and agreed-upon position in the genome), generally the gene of the cytochrome oxidase I (COI), as a molecular diagnostic for species-level identification (Hebert et al. 2004), has been increasingly used in earthworms, leading to moderate reactions. While Huang et al. (2007) were enthusiastic about this tool, Chang et al. (2009) highlighted the caveats of this approach and concluded that they 'have identified several issues regarding the evolution of the COI gene in these organisms which remain to be further elucidated' (Chang et al. 2009; their summary). Describing the promise and pitfalls of DNA Barcoding, Moritz and Cicero (2004) also emphasized that the real challenge of DNA barcoding research concerns taxa with limited dispersal, and thus substantial phylogeographic structure, such as that expected in earthworms. Zhang et al. (2010) demonstrated in their recent study that sample sizes used in DNA barcoding (i.e. 5–10 individuals of one species) are generally insufficient to assess the genetic diversity of a species. In particular, they highlighted that genetic population structure affects the estimation of population parameters and hence species identification. They conclude that sample sizes for DNA barcoding projects must take into consideration the evolutionary history of the studied species. Table 17.2 shows recent data of intra-species genetic divergence in earthworms as well as sampling sizes, which are highly variable. It is worth noting that cryptic species have often been discovered in earthworms. However, estimated values of genetic divergence depend on the species, and there is no clear consensus regarding the establishment of the species limit. Novo et al. (2009) stated that authors generally suspect the existence of new species when divergence values around or greater than 10 or 11% are found.

Although the DNA barcoding technique needs to be improved in earthworms, barcode results can be of high value in aiding the selection of species for more detailed analysis (Hajibabaei et al. 2007). Moreover, in population genetics investigations, DNA barcodes can provide a first signal of the extent and nature of population divergences and may facilitate comparative studies of population diversity in many species since universal primers may be used (Hajibabaei et al. 2007).

17.2.2 Microsatellites

Of particular interest in the field of earthworm population genetics are microsatellites, also known as SSRs. They are stretches of nuclear DNA that consist of tandem repeats of 1–6 bp. Microsatellites represent powerful co-dominant markers in population genetics (Jarne and Lagoda 1996) primarily because they mutate much more rapidly than most other types of sequences. Moreover, they are suitable for automation by PCR with high reproducibility and resolution. Until now, the major hurdle in the development of SSRs is that they have to be developed de novo from either enriched or non-enriched genomic libraries (Fig. 17.1). There is now an alternative approach – in silico mining (i.e. retrieving microsatellites from sequence databases using microsatellite search tools; Sharma et al. 2007). The screening of

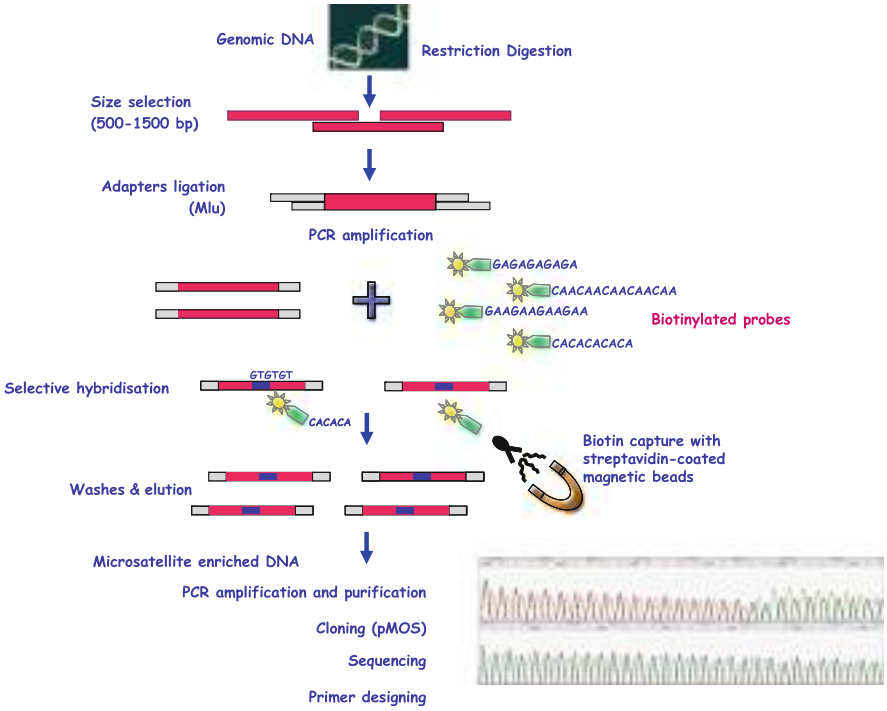

Fig. 17.1 The strategy employed to obtain microsatellite markers from *Lumbricus terrestris* and *Hormogaster elisae* (Velavan et al. 2007; Novo et al. 2008)

large amounts of sequence data with microsatellite-finding algorithms can yield many new candidate markers. The next-generation sequencing technology (Margulies et al. 2005; Wheeler et al. 2008) will further encourage large-scale sequencing and the development of database derived microsatellite markers (Sharma et al. 2007).

So far, microsatellites have been developed for three earthworm species. Velavan et al. (2007) had developed and characterized ten highly polymorphic microsatellite loci from an SSR-enriched genomic DNA library of the common earthworm *Lumbricus terrestris* L. Characterization of these loci in a sample of 32 individuals revealed high levels of genetic diversity (i.e. 5–18 alleles per locus and a high degree of heterozygosity; H_e ranged from 0.43 to 0.97). Three of these markers have been utilized to characterize the population genetic structure and genetic diversity at a micro geographic scale across 26 German sites. The results were related to the level of parasitic infections of earthworms by *Monocystis sp*. A lack of genetic structuring among German populations and an absence of any relationship between parasite load and genetic diversity was revealed. Interestingly, Velavan et al. (2009) showed substantial population differentiation between the German populations and a Canadian population, a result suggesting that these markers are quite reliable to differentiate populations of different origins.

Using the same approach than Velavan et al. (2007), Novo et al. (2008) have developed and characterized ten highly polymorphic microsatellite loci for the earthworm *Hormogaster elisae* (26 individuals, 8–15 alleles per locus and H_e ranging from 0.61 to 0.98). The overall schematic illustration of the approach is depicted in Fig. 17.1. Harper et al. (2006) also used a microsatellite enrichment protocol as described by Ciofi et al. (1998) and characterized eight microsatellite markers for the earthworm species *L. rubellus* (34 individuals, 2–15 alleles per locus and H_e ranging from 0.06 to 0.92). All three studies failed to standardize a cross species amplification of these markers across related species. This ensures that these motifs fished from microsatellite enriched library remained quite species specific.

17.2.3 AFLPs

AFLPs are highly reproducible dominant markers. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA; 50–100 bands are usually produced per assay. With this technique, the presence or absence of a fragment is used as a molecular marker. Such markers restrict many standard analyses because only one allele can generally be identified at each locus, and therefore heterozygotes cannot be differentiated from homozygotes. Thus, banding patterns represent individual phenotypes rather than genotypes. AFLPs are, however, popular because they are relatively easy to use and do not require knowledge of targeted sequences in the genome. Thus, similar primers can be tested on any species. Until now, AFLPs were used only in one earthworm species. King et al. (2008) conducted an AFLP analysis using two primer combinations, which produced 665 scorable bands in *Allolobophora chlorotica*. Their AFLPs results were highly congruent with mtDNA data showing the presence of five highly divergent lineages in this species.

17.3 Inferring Mating Strategies from Molecular Data in Amphimictic Earthworms

Molecular markers can reveal and quantify the occurrence of mating behaviours that otherwise may remain hidden from field naturalists. Moreover, the use of PCR-based markers in molecular analysis of mating system can extend to case in which a small amount of tissue is available, as with cocoons. Many aspects of the mating behaviour of earthworms remain poorly understood, and molecular methods have not been used yet to study earthworms breeding strategies. In this paragraph, we will highlight which issues of earthworm reproductive strategies could benefit from molecular data.

17.3.1 Selfing and Inbreeding

Self-fertilization (or selfing) is the fusion of gametes from a single genetic individual. It is common in hermaphroditic individuals such as molluscs and trematodes (Jarne and Auld 2006). Estimating selfing rate is an important issue in population and evolutionary biology. Indeed, selfing substantially affects the distribution of genetic variation within and among populations, and therefore the response of populations to selection (review in Charlesworth 2003). Selfing has a deleterious effect known as inbreeding depression (i.e. decrease in the fitness of offspring, Charlesworth and Charlesworth 1987). Inbreeding increases homozygosity and inbreeding depression is thought to result mainly from the unmasking of deleterious alleles (Charlesworth and Charlesworth 1999). In species where inbreeding is common, it is hypothesized that selection would already have eliminated recessive deleterious alleles; the cost of inbreeding is thus likely to be low or nonexistent (e.g. Shields 1993; Waser 1993; Jarne and Städler 1995; Keller and Waller 2002). Thus, several mechanisms, including mating between relatives, may serve to reduce inbreeding depression over time and lead to a greater tolerance of self-fertilization. Selfing is therefore predicted to be more common in species with limited mobility, such as earthworms.

Although several authors reported that some earthworms, reared in isolation since birth, were capable of producing cocoons (review in Dominguez et al. 2003), there is a considerable controversy about self-fertilization in the Lumbricidae, the major family of earthworms. Uniparental reproduction in earthworms may be achieved by either self-fertilization or parthenogenesis. Parthenogenesis is well known in several earthworm species: most parthenogenetic species retain meiosis with a premeiotic doubling of the chromosome set but some species produce eggs through mitosis instead of meiosis. Since parthenogenetic species are generally characterized by high ploidy (review in Beukeboom et al. 1998), self-fertilization is often assumed in diploid species producing cocoons without mating. A study of Dominguez et al. (2003) confirmed that uniparental reproduction in the diploid species *Eisenia fetida* may be due to self-insemination. To our knowledge, the level of inbreeding in natural population of amphimictic earthworms has only been investigated in a recent study that revealed a low level of inbreeding in a large population of the earthworm *L. terrestris* (Velavan et al. 2009).

Molecular methods for estimating selfing rates and inbreeding level in populations are reviewed in Jarne and David (2008). Co-dominant markers are preferable because it is possible to unambiguously identify heterozygous individuals. Moreover, highly polymorphic markers (such as microsatellites) are preferred since the precision of all estimates increases with genetic diversity.

17.3.2 Multiple Mating

Genetic studies of parentage have played a major role in the study of evolution and behavioural ecology and have become one of the central themes in the field of

molecular ecology (Avise et al. 2002; Freeland 2005). In particular, molecular techniques provide the opportunity to test for multiple paternity and to genetically quantify reproductive success in the wild. Multiple mating seems to be common in earthworms. For instance, copulation with different partners during a mating season was observed in *E. fetida* (Monroy et al. 2003) and *L. terrestris* (Butt and Nuutinen 1998; Michiels et al. 2001). However, little is known of the actual fate of sperm after it is transferred to a mate. Copulation may not always result in insemination and subsequent fertilization of eggs. Mating outcomes can only be deduced from molecular information. Methods and techniques of parentage analysis in natural populations are reviewed in Jones and Ardren (2003) and Blouin (2003). So far, no paternity analysis has been achieved in earthworms, although microsatellite markers have been defined in two species for this purpose (Velavan et al. 2007; Novo et al. 2008).

17.3.3 Post-Copulatory Sexual Selection

Multiple mating has ramification for sexual selection, providing scope for sexual conflict (i.e. conflict that exists as a result of the divergent evolutionary interests of males and females Parker 1979), sperm competition (i.e. competition among sperms of different males to fertilize the available ova Parker 1970; Birkhead and Möller 1998) and cryptic female choice (i.e. female behaviour, physiology or morphology that favours certain males against others after copulation has already begun, Eberhard 1996). Sexual selection may be an important force shaping simultaneous hermaphroditic mating strategy (Charnov 1979; Michiels and Newman 1998; Angeloni et al. 2003). Having both sexual functions at the same time leads to a dilemma: how to allocate resources between the production of eggs and sperms (van Velzen et al. 2009)? It is expected that individuals show a preference for adopting the sex role that tends to offer the higher potential fitness gain per mating (Anthes et al. 2006). In the earthworm *E. fetida*, Meyer and Bouwman (1997) showed differences in the number of cocoons and hatchlings produced between the individuals of pairs suggesting that there are individuals that are sperm donor (i.e. low producer of cocoons) and others that are rather sperm receiver (i.e. high producer of cocoons) in this species. The preferred sex role may depend on factors such as the body size, the quality of the partner, the sperm precedence pattern and the mating history of mates (see Janicke and Scharer 2009; Scharer 2009; van Velzen et al. 2009 for review). The partner selection may be either pre-copulatory or post-copulatory.

Pre-copulatory partner evaluation has been well studied in several species of earthworm. For instance, a prolonged courtship that involves short and repeated touches between partners before mating attachment was observed in *Eisenia andrei* (Velando et al. 2008). Tato et al. (2006) confirmed that these earthworms (*E. andrei*) are able to discriminate their partners and adjust their breeding effort accordingly but they could not identify the criterion of choice. In *E. fetida*, Monroy

et al. (2005) showed that individuals preferred same-sized mates to differently sized one, suggesting the existence of mate selection in this species. In *L. terrestris*, Michiels et al. (2001) assessed that relative partner size and also the distance between the putative partners, the risk of being pulled onto the surface and size-related fecundity all appear to play key roles in mating behaviour.

On the contrary, post-copulatory sexual selection has been rarely investigated in earthworms, although multiple mating and sperm storage organs provide opportunities for sperm competition and/or cryptic female choice. The study of Velando et al. (2008) suggests that sperm competition is a powerful evolutionary force that influenced the mating behaviour in earthworms. They found that *L. terrestris* individuals responded to the mating status of their partners and triplicated the donated sperm when they mate with a non-virgin mate. Sperm receivers, on their part, can potentially choose sperm from different donors if, for instance, the sperm carry information on male quality (i.e. cryptic female choice).

Parentage analyses allow investigating the effects of post-copulatory sexual selection on the patterns of reproductive success. For instance, in a cross-breeding experiment with a recipient individual that mated with two sperm donors, a paternity analysis on the eggs showed that post-copulatory events influenced the patterns of sperm precedence (i.e. non-random differential fertilization success among mating males) in the simultaneous hermaphrodite snail *Helix aspersa* (Evanno et al. 2005).

17.3.4 Genetic Compatibility and Mate Choice

In gonochoric species (i.e. species with separate sex), several studies suggested that pre-copulatory or post-copulatory female choice may allow to drive paternity towards more genetically compatible males (see Tregenza and Wedell 2000 for review). One source of genetic incompatibility is the degree of genetic relatedness among mates: unrelated males are expected to be genetically more compatible with a female than her relatives. Inbreeding avoidance through female mate choice based on relatedness recognition has therefore been suggested in several mating systems; selection pressure being particularly important in species where indirect inbreeding avoidance through dispersal is poorly developed (Pusey and Wolf 1996).

A few studies investigated the effect of inbreeding on earthworm mating behaviour. Examining the number of cocoons, the number of viable cocoons and the number of hatchlings per viable cocoons in sibling and non-sibling pairs, Nakagawa et al. (2002) asserted that relatedness does not affect the mating effort of the earthworm *E. fetida*. On the contrary, undertaking laboratory experiments in which earthworms were mated with their sibs and with non-sibs from the same population and with no-sibs from a geographically isolated population, Velando et al. (2006) showed that *E. andrei* adjusted its reproductive effort, measured as the number of cocoon produced, according with the degree of relatedness of its partner: both inbreeding mating (mating of full siblings) and outbreeding mating (mating of

a sibling with a virgin earthworm from a distant population) caused a strong reduction of cocoon production, suggesting an optimal outbreeding degree. However, no study has considered mate choice in relation to relatedness patterns in earthworms. Using molecular markers, inbreeding avoidance through post-copulatory mate choice may, for instance, be investigated by examining patterns of paternity in an experiment where each recipient individual mates with both a related and an unrelated sperm donor, in either order (e.g. Bretman et al. 2004).

17.4 Determining Patterns of Earthworm's Dispersal Using Molecular Data

Dispersal (i.e. the movement of organisms away from their parent source) is a fundamental biological process that operates at multiple temporal and spatial scales (Nathan 2001). Understanding why and how animals and plants are moving has become of prime importance in a context of global changes and habitat fragmentation. Although dispersal has traditionally been measured using field methods such as capture-mark-recapture protocols, more recent methods use molecular data to infer dispersal from gene flow patterns. Initially, genetic approaches for estimating dispersal rate focused on the consequences of dispersal upon the apportionment of genetic variation among populations (e.g. methods based on *F*-statistic, Wright 1951). However, these approaches do not distinguish between contemporary and historical gene flow and dispersal. Contemporary patterns of dispersal may now be determined using assignment tests (review in Berry et al. 2004). Although this approach can provide more detail than pairwise F_{ST} values (Berry et al. 2004), the distances that individuals disperse cannot be directly measured. Alternatively, parentage assignment can quantify the distances offspring disperse as well as the frequencies of those distances (Cullingham et al. 2008). The primary challenge of parentage analysis is, however, ensuring that an adequate proportion of the population was sampled for optimizing the probability of including parent–offspring pairs. Co-dominant genetic markers are needed for assessment of *F*-statistics and determination of the reproductive scheme within a population. Moreover, assignment tests perform better when detailed genetic profiles of populations are obtained from a relatively large number of individuals, using multiple highly polymorphic loci. Microsatellites, which fulfil both demands, are thus the marker of choice to investigate gene flow in diploid species. In polyploid species, however, data obtained with co-dominant markers may be ambiguous (impracticality to decipher between heterozygote and homozygote individuals). Moreover their mode of reproduction (i.e. parthenogenesis) may originate in extremely low genetic diversity even with microsatellite markers; a higher number of loci are thus required. AFLPs offer great potential for polyploid species, since they are highly reproducible dominant markers and typically 100–300 loci can be surveyed at once.

Because the concept of 'population' is central in any study of population genetics, we will first discuss what a 'population' is for earthworms. We will then emphasize which information on earthworm dispersal patterns can be gained from molecular data.

17.4.1 What Is a 'Population' for Earthworms?

A 'population' may be defined as a group of individuals of the same species that occupy a more or less well-defined geographical region and exhibit reproductive continuity from generation to generation (Futuyma 1998). The boundaries of earthworm populations are very difficult to determine because individuals are hidden in the soil. Geostatistics studies showed that earthworms are often distributed in patches which are 10–50 m wide (Rossi et al. 1997; Cannavacciuolo et al. 1998). However, these patches may disappear temporarily (Rossi 2003), and thus they do not necessarily correspond to different populations.

Most population genetic studies have used models that assume populations to be mutually isolated and internally panmictic (i.e. at Hardy–Weinberg equilibrium). Thus, these population genetics studies have been traditionally designed to collect samples from a minimum of 24–30 individuals per a priori delineated 'population'. However, a priori delineation is no longer necessary because of the development of genetic clustering algorithms. Instead, models such as STRUCTURE (Pritchard et al. 2000; Evanno et al. 2005) that cluster individuals by minimizing Hardy–Weinberg and gametic disequilibrium might be used to delineate limits of earthworm populations. It is thus possible to test to which extent the patches of earthworms in the field are related to specific genetic entities or conversely if the earthworms are forming continuous populations from a genetic perspective.

17.4.2 Modes and Pathways of Earthworms Dispersal

Dispersal of earthworms can be active (i.e. earthworm movements in soil or on the soil surface) or passive (i.e. via anthropochory, hydrochory or zoochory, Terhivuo and Saura 2006). Low rates of active dispersal are generally recorded in the literature. Thus, Terhivuo and Saura (2006) asserted that earthworms do not disperse more than 10–15 m/year. Surface dispersal distance has been, however, reported to reach 19 m in one night from field observations of *L. terrestris* (Mather and Christensen 1988). Clobert et al. (2004) proposed to classify the factors suggested to promote the evolution of active dispersal in three categories: habitat specific factors, factors related to mate choice (i.e. inbreeding, mating system) and social factors (e.g. density-dependence dispersal). These three hypotheses are not necessarily mutually exclusive. For numerous species, dispersal patterns are influenced by more than one evolutionary constraint at the same time (review in

Nutt 2008). A recent mesocosm experiment (Mathieu et al. 2010) showed that the dispersal of endogeic (*Aporrectodea icterica*) and epigeic (*Dendrobaena veneta*) earthworms can be affected by habitat quality and conspecific density. Similarly, field experiments (Grigoropoulou and Butt 2010) revealed that dispersal of the anecic species *L. terrestris* is dependent on population density and resource availability. So far, no study has investigated the factors related to mate choice (e.g. inbreeding avoidance, see Sect. 22.3.4).

Both traditional (i.e. F_{ST}-based) and newly developed (i.e. assignment tests) methods may be used to assess the respective roles of active and passive dispersal in earthworms. If passive dispersal is predominant, a pattern of jump dispersal is expected (i.e. long-distance and stochastic dispersal) while a pattern of gradual dispersal (e.g. a pattern of isolation by distance; Slatkin 1993) is expected in the other case. Connectivity among individuals and populations may be investigated using landscape genetic approaches, where individuals are sampled across landscapes, genetic relatedness between individuals assessed, and these relationships correlated with landscape features (Manel et al. 2003; Storfer et al. 2007; Holderegger and Wagner 2008). Such approaches allow identifying both barriers to movement and corridors that facilitate dispersal in heterogeneous landscape. The extent to which such features determine population connectivity will depend on the vectors and modes of dispersal of a given species. For instance, Wilmer et al. (2008) investigated the interaction between landscape structure and dispersal in the endemic aquatic snail, *Fonscochlea accepta*, using a spatial genetic clustering programme (GENELAND, Guillot et al. 2005) and two other Bayesian assignment methods (implemented in STRUCTURE, Pritchard et al. 2000 and BAYEASS, Wilson and Rannala 2003), and showed that short-range dispersal occurs via active movement while long-range dispersal is facilitated by animal vector in this species.

17.4.3 Invasion by Earthworms

Human-aided dispersal may have a great impact in terms of biological invasions. There is growing evidence that lumbricid invasions are increasing worldwide, sometimes with significant effects on soil processes and plant communities (Hendrix 2006). For instance, invasion of northern USA and Canada by European Lumbricidae has been the matter of several recent reviews (e.g. James and Hendrix 2004; Parkinson et al. 2004; Hendrix 2006). Characteristics of some earthworm species (e.g. parthenogenesis, environmental plasticity, ability to aestivate) appear to make them particularly successful as invaders (James and Hendrix 2004).

Analyses of genetic and evolutionary processes are key features in studies of biological invasions. Through the modification of genetic characteristics and architecture in invasive populations, evolutionary forces such as selection or genetic drift play a major role in determining the spread and the long-term establishment of alien species (Sakai et al. 2001; Lee 2002). In addition of providing new insights into evolutionary processes that act during the invasion process, population genetic

techniques can offer powerful tools for determining specific and practical aspects critical to successful management of invading species, namely: (1) identification of source population(s), (2) enumeration of successful introductions, (3) detection of selective tensions at the points of introduction that directly limit the spread of the species and (4) potential for hybridization with indigenous species (Sakai et al. 2001). Thus, investigating the spread of the parthenogenetic earthworm *Dendrobaena octaedra* in the boreal forest of Alberta using a mitochondrial marker, Cameron et al. (2008) showed that multiple introductions had occurred although individual populations might have been established by either single or multiple invaders introduced on one or more occasions.

17.5 Conclusion

Polymorphic DNA markers, such as microsatellites, provide a powerful tool for modern ecologists especially when they are used in combination with behavioural, demographic or spatial information (DeYoung and Honeycutt 2005). In general, multidisciplinary approaches are needed to move forward researches on the ecology of ecosystem engineers, such as earthworms (Lavelle 2000). Thus, the new field of landscape genetics, which combines landscape ecology and population genetics (Manel et al. 2003), promises to facilitate our understanding of how geographical and environmental features act on earthworm movements. In addition, the joint analysis of realized dispersal, as measured by genetic tools, and potential earthworm dispersal based on simulations (e.g. Vorpahl et al. 2009) may be an efficient way to investigate the modes and pathways of earthworms' dispersal.

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Chapter 18
Population Dynamics of Earthworms in Organic Farming Systems

James B. Kotcon

18.1 Introduction

The dynamics of earthworm populations in agricultural soils have been studied extensively, in large part because the effects of earthworms are generally dependent on the species diversity and population density at each site. Earthworms can influence organic matter decomposition, carbon and nitrogen content of soils, soil aggregate structure, porosity and water infiltration, and abundance of various soil flora and fauna. In native ecosystems, invasive earthworms can alter the soil habitat and adversely affect populations of native flora and fauna (Bohlen et al. 2004). But in agricultural soils, earthworms provide well-known benefits through cycling of organic matter and improving soil porosity and aeration, and are generally considered to be important indicators of soil quality (Brown 1995; Edwards 1998; Syers and Springett 1984).

Earthworms are known to be sensitive to many synthetic pesticides and respond favorably to additions of organic matter, thus transition from conventional to organic practices is believed to enhance earthworm activity in soil, which in turn, enhances the soil quality on which organic agriculture depends. Enhanced soil quality may result in greater plant growth, particularly below ground, thereby providing a larger food source to support earthworm populations (Syers and Springett 1984).

But these ecological relationships may be complex. For example, Stinner et al. (1997) demonstrated that higher weed biomass was present in plots with augmented populations of earthworms and the higher weed biomass resulted in lower corn yields. Suppression of a variety of soilborne plant diseases was associated with increased plant growth, increases in population densities of filamentous actinomycetes and fluorescent Pseudomonads, and reductions in the densities of total soil

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bacteria (Elmer 2009). Chapman (2006) demonstrated that the microbial populations in soil were altered during passage through the earthworm intestine. He found that the microbial community in earthworm casts could be distinguished from communities in both the food source (cattle manure) and the bulk soil based on bacterial numbers, community-level substrate utilization, and denitrifying activity.

Benefits to soil quality come from a variety of mechanisms, including improved soil porosity from earthworm burrows, enhanced organic matter decomposition and nutrient cycling, and from increased soil aggregate stability and microbial activity associated with earthworm casts. Syers and Springett (1984) suggest that all of these mechanisms may be operating simultaneously, and all depend on the population density, species diversity, and activity level of the earthworm community present.

Many practitioners of organic farming value these benefits to soil quality and depend on soil quality to sustain crop yields. Thus, earthworm population dynamics, and the influence of farming practices on earthworms, are of particular interest to organic growers. In this chapter, some key literature on earthworm population dynamics generally, and the influences of organic farming systems in temperate climates specifically, is reviewed. Additional results from long-term cropping systems studies at the West Virginia University Organic Research Farm are presented.

18.2 Earthworm Life Cycle

An in-depth study of the life history of earthworms has been completed for only a few of the hundreds of earthworm species (Curry 1998), but certain aspects are common to most species. Earthworms are hermaphroditic, having both male and female pores on the ventral body surface. While parthenogenesis may occur in some species, mating is required for many lumbricids. After copulation, the clitellum secretes a cocoon into which ova and sperm are deposited as the cocoon slips off the worm. The embryos develop inside the cocoon, with the incubation period ranging from 5 to 20 weeks, depending on species and temperature (Edwards and Lofty 1972).

In temperate climates, cocoons are produced in spring and fall, hatching mostly in mid-summer. The number of cocoons ranges from 3 to 79 per individual, depending on species (Edwards and Lofty 1972). While as many as 20 eggs may be laid in a cocoon, generally only one or two juveniles survive to hatch. Maturation requires 3 months to a year, and while individuals have survived 4–10 years in culture, their life span in the field may be only a few months to a few years. Depending on the species, a facultative or obligatory diapause occurs during hot dry weather in mid-summer, during which worms generally migrate to lower soil depths, coil, and become inactive until environmental conditions improve. Some authors have divided the life cycle into stages of young immature, well-grown immature (adolescent), mature, and senescent; however, growth appears to be more

or less continuous with no distinct stages or morphological differences separating the categories until maturation of the sex organs and clitellum of the adults.

Reproductive rates vary among species and growing conditions. Holmstrup (1999) estimated a production of about 15 cocoons per adult per year for *Aporrectodea longa* under field conditions, while *Aporrectodea rosea* adults produced 30–40 cocoons per year. These rates were much higher than reported in laboratory studies, suggesting that earthworm species may be much more differentiated to microsites in the field than can easily be replicated in the laboratory.

While numerous studies have described earthworm population dynamics in a variety of settings (Edwards 1998), more quantitative approaches are needed to develop a conceptual understanding of the factors that drive responses to the environment. For example, Klok et al. (1997) modeled the life cycle of *Lumbricus rubellus* in four distinct stages: cocoon, juvenile, subadult, and adult. The model assumes that maturation is correlated with reaching a certain weight, and progression through the various stages depends on food availability and the energy budget for individual animals. Food energy is partitioned first to maintenance, then growth and, in adults, reproduction. Cocoons develop into juveniles in approximately 42 days, and the maximum lifespan was 710 days. Responses to an environmental contaminant (copper) were modeled as an increase in maintenance energy needed to detoxify the contaminant, and resulted in lower growth rates, which reduced reproduction. The model results predicted population extinction at soil copper concentrations above 300 mg/kg, a result that was in good agreement with laboratory toxicity tests.

Jager et al. (2006) used a Dynamic Energy Balance model to describe the physiological processes that govern growth and reproduction of an epigeic species, *Eisenia veneta*. Reproduction begins when worms reach a certain size. They measured body size and cocoon production over 200 days at two temperatures (15 and 25°C) and two densities (5 and 10 worms per container) but with the same worm-to-food ratio at both densities. The model used five parameters and produced a good fit between the observed and predicted growth rate and reproduction rate. When food was not limiting, an increase in temperature increased both growth and reproduction rates, as expected. They observed a great deal of interindividual variability in growth and reproduction rates, with a few worms never reaching reproductive size. Increasing the population density had little effect on growth curves, but delayed cocoon production until worms reached a larger body size and lowered the reproduction rate. These results suggest that the worms at higher densities have an altered energy allocation, or that other phenomena influence the model parameters.

Behavioral characters have been used to assign earthworm species to functional guilds by Bouche, Lavelle, and others (Brown 1995). Epigeic species are litter-dwellers, rarely make burrows in soil, and are important in early stages of litter decomposition. Endogeic species are typically soil inhabitants, generally producing shallow, horizontal burrows, and ingest large amounts of soil. Anecic species produce deep, vertically oriented burrows, and feed on surface litter or bury it deep within their burrows. Not all species fall neatly into mutually exclusive

categories, as some species can exhibit a range of burrowing and feeding behavior. For example, *L. rubellus* has been described as both epigeic and endogeic.

These differences in behavior produce differences in soil characteristics among earthworm species. Burrows of *L. terrestris* are larger and tend to be more vertically oriented than those containing *Aporrectodea caligenosa* (Pitkanen and Nuutinen 1997; Jegou et al. 1998). This can influence the growth of roots through the burrows, as well as water infiltration, leaching of nutrients and soil chemicals, and gas exchange. In field soils, Pitkanen and Nuutinen (1997) found that burrows near the surface tended to be smaller diameter, and were generally occupied by *A. caligenosa*, whereas those below 60 cm generally contained *L. terrestris* and were larger diameter, suggesting that both tillage and species may influence the burrow frequency and diameter.

18.3 Long-Term Effects of Farming Systems on Population Dynamics

Major abiotic stresses influencing earthworm population dynamics include soil moisture, temperature, and food availability (Edwards 1998; Edwards and Lofty 1972). Earthworms are also sensitive to a variety of environmental pollutants such as pesticides, heavy metals, and organochemicals, and numerous studies have evaluated earthworms as bioindicators for pollution stress (Curry 1998). In agricultural soils, tillage operations can kill or damage earthworms directly, or severely disturb earthworm burrows, and shift community composition toward species more tolerant of disturbance.

However, relatively few studies have examined earthworm population dynamics over more than a few years. After 21 years of a long-term farming systems comparison of organic and conventional systems in Switzerland, Mader et al. (2002) found that organic and biodynamically farmed soils contained 3.2 times more earthworms and 1.3 times more earthworm biomass than conventionally managed soils. These values are comparable to those reported from Denmark (Hansen et al. 2001) where earthworm densities increased by a factor of 2.88 compared to conventional cultivated land farming. Earthworm densities in conventional livestock farming were higher than in cultivated crops systems, with earthworm populations in conventional dairy farms comparable to those on organic farms. Riley et al. (2008) compared six farming systems over 14 years, and found that conventional cultivated crops consistently had the lowest earthworm densities, but that conventionally produced forage crops did not differ greatly from organic systems. These data suggest that livestock production systems, with the emphasis on manure applications and pasturelands, provide suitable conditions for earthworms.

Pfiffner and Luka (2007) compared low-input integrated conventional systems versus organic and biodynamic systems, and found higher earthworm abundance, species diversity, and biomass in organic systems. The low-input conventional

systems used no insecticides or fungicides but did include herbicides and mineral fertilizers. They found that late fall plowing, when earthworms were active, significantly reduced populations, regardless of farming system, but that summer plowing, when anecic species were in deeper soil layers, had less severe effects on worm populations.

Hole et al. (2005) reviewed 13 studies comparing earthworm abundance and activity in organic versus conventional farming. While most studies found higher earthworm abundance in organic systems, a few found no difference, and one study observed lower earthworm populations in the organic system. They concluded that excessive tillage in some organic systems may suppress earthworm populations, in spite of the higher levels of organic matter input.

Hutcheon et al. (2001) compared farming systems using conventional tillage (plowing) versus integrated systems using noninversion tillage equipment. They found that few differences in earthworm populations were observed in the first 3 years, but that the integrated systems consistently had higher earthworm populations, biomass, and diversity in later years, indicating that long-term experiments with multiple seasons are needed to detect the influence of farming systems on changes in earthworm populations. They also showed that earthworm populations were variable among seasons, with a collapse of the populations following the winter of 1992–1993 leading to low populations until 1998, regardless of tillage system. These results highlight the need for long-term studies, to distinguish the effects of specific farming systems from the short- to intermediate-term fluctuations that are exhibited in naturally occurring populations.

18.4 Methodological Limitations

Field studies of population dynamics are inherently constrained by the methods used to monitor earthworm parameters. Three general approaches have been used, and each has limitations that influence the quality of the data obtained.

Hand-sorting, which involves collecting a volume of soil and picking the earthworms from it, is generally considered the most accurate approach for collecting and quantifying earthworm populations. Unfortunately, it is also one of the most time-consuming, particularly if a standardized soil volume of adequate size is to be sampled. Hand-sorting can collect immobile stagers such as aestivating worms or cocoons, but a fairly deep sample is needed to collect anecic species, especially during hot dry weather when the worms migrate to deeper depths. Most studies limit hand-sorting to the upper 15–30 cm, which results in an underestimation of worms from deeper layers.

Various types of irritants can be applied to induce earthworms to come to the surface. Dilute formalin, mustard or pepper solutions, and even shocking with electric currents have been used with great success. However, these methods only sample those worms that are actively mobile. Some studies suggest that toxins such as formaldehyde may kill some worms before they reach the surface, and that this

effect is more pronounced with certain epigeic species, and thus underestimates their population. In addition, worms may respond by moving deeper, rather than toward the surface, and this behavioral response also varies among species.

A third approach is to quantify the number and location of earthworm burrows (Pitkanen and Nuutinen 1997). This gives an indication of the density of the worm population, as well as the relative activity with regard to soil penetration and organic matter cycling, key parameters in measuring the impact of earthworms on soil quality. Some specificity in burrows has been reported, for example, larger species such as *Lumbricus terrestris* produce burrows with wider diameters. Anecic species produce burrows that tend to orient vertically and go deeper than those of epigeic species. While this gives an indication of earthworm activity, Kretzschmar (1998) reported that the tunneling behavior of some species is variable. For example, *A. caligenosa*, generally considered a strictly endogeic species, makes typical short, disconnected burrows in the Northern Hemisphere, but produces long vertical burrows typical of anecic species in temperate areas of the Southern Hemisphere. Many species create burrows that tend to follow cracks and zones of less-compacted soils (Jegou et al. 1998). Some worms use burrows produced by other species. Because of this behavioral plasticity, assigning burrows to species or life stages is inherently dubious. Furthermore, it is not always clear how long burrows persist after worms die.

The number, size, and distribution of samples to be collected are important considerations, particularly since earthworm populations often have a patchy distribution in the field. Dickey and Kladivko (1989) found that the sample size and frequency affected sampling efficiency. Because motile stages oriented along a crop row, hand-sorting of soil from a pit 10 cm along the row by one-half the row width was found to be most efficient for juveniles and adults, whereas a square pit was needed for cocoon sampling.

Some workers have suggested that a combination of methods produces the most consistent results (Pfiffner and Luka 2007). Because of the effort involved, and the spatial variability even within a single field, the monitoring objectives of the study and the limitations of each method should be carefully considered in designing sampling methods.

18.5 Earthworm Population Dynamics at the WVU Organic Research Farm

The West Virginia University Organic Research Farm project was initiated in 1999 as a long-term evaluation of organic farming systems. Prior to this time, the Farm had been in conventional horticultural production, primarily tree fruits and vegetables. Soils are silt-loam and slopes range from 0 to 24%, typical of Appalachian hill top farms. The initial 3 years of the project involved a transition from conventional to organic management, with organic certification granted in 2003. Since that

time, farming practices in this trial adhere to USDA organic certification requirements.

The project was designed to evaluate the impact of several organic farming systems on crop and livestock productivity, soil quality, populations of pests and beneficial organisms, and overall farm profitability. One of the many objectives of this study was to assess the long-term changes in earthworm populations, biomass, and age structure in response to several distinct farming systems.

Two replicated farming systems experiments, market garden and field crop/livestock, were conducted. Each compared two treatments for managing soil quality during the transition from conventional to organic practices: a low-input transition using cover crops only, and a high-input treatment using off-farm compost amendments with cover crops. The field crop system also included two additional treatments, with and without livestock, arranged in a factorial design with the two transition (high versus low input) treatments. Prior to initiation of the experiment, all plots had been managed as permanent grassland or as a conventional apple orchard with grass sod as ground cover for at least 10 years.

18.5.1 Low-Input Treatment

Plots were cover cropped intensively beginning in fall 1999 and throughout the 2000 growing season. Rye, sown in fall, 1999, was followed by clover in spring, 2000, and by rye and vetches in the fall of 2000. All cover crops were plowed in as green manure. This treatment was used to build soil quality and yielded no saleable product in 2000. Market garden plots were cropped, starting in 2001, with a 4-year rotation sequence of legumes (beans and peas), leafy vegetables (spinach and lettuce), solanaceous crops (tomato and pepper), and cucurbits (zucchini and pumpkin). Field crop plots in the without-livestock systems were cropped to wheat, potato, forage soybean, or Brussels sprouts. A rye-vetch winter cover crop was planted each year on all plots, except those with an established overwintering crop. Beginning in 2003, forage rape was substituted for Brussels sprouts, and a summer cover crop of cowpea was inserted in the rotation between wheat and forage rape crops. Field crop plots in the with-livestock systems followed a 7-year rotation, with the same cultivated crops as in the low-input system for 4 years, followed by an additional 3 years with orchard grass and red clover, which was either harvested as hay or grazed by sheep.

18.5.2 High-Input Treatment

Following the rye cover crop planted in fall 1999, plots were amended with a dairy manure compost at 10 T/acre in the spring of 2000 (equivalent to approximately 100 lbs N/acre). Crops in the field crop and market garden plots were the same

rotation as for the Low-Input system. Thus, the High-Input treatment used off-farm compost to improve soil quality and produced saleable crops in the first year of transition. Compost was applied at 10 T/acre each year to High-Input plots in the Market Garden and through 2003 in the Field Crop plots. Beginning in 2004, compost was applied at 20 T/acre in the potato and wheat crops, with no compost applied in the high-input soybean, cowpea, or forage rape crops.

The market garden had four replications of the two treatments and four crop families in all combinations (32 plots total). The field crop system had three replications of the low- and high-input systems with and without livestock, in all combinations (66 plots total). Sheep grazed the plots, with stocking density assigned at a level to minimize purchases of off-farm feed.

Soil samples were collected to monitor soil earthworm fauna. Three soil cores (10-cm-diam by 15-cm-deep) were collected from each plot and earthworms were collected in the field by hand-sorting. Worms were placed in vials on ice and returned to the laboratory where they were sorted by species and age class, and then oven-dried at 45°C to determine biomass. Worm fragments were counted as one-half of a worm, and fragments without an identifiable head were designated as unknown species. Worm populations were monitored in the Market Garden systems in spring of each year from 2000 to 2007 (except 2005), generally before the first tillage operations. Field crop systems and summer and fall populations in the Market Garden were also monitored through 2004.

18.5.3 Observations

The dominant species in both field crop and market garden plots was *Aporrectodea caliginosa*, with *L. rubellus* also occurring frequently. *L. terrestris* was rare, and largely disappeared from the plots after a few years of cultivation. Population density was significantly greater in market garden plots with high compost inputs on 6 of the 15 sample dates (Fig. 18.1a, b). Similar trends in field crop plots were observed, but differences were statistically significant only for biomass at one date (Fig. 18.1c, d).

The effect of including livestock and 3 years of orchard grass/red clover into the crop rotation was generally not significant (data not shown). Although plots with livestock tended to have higher earthworm numbers and biomass at some dates than plots without livestock, the trend was not consistent, and was largely a result of greater earthworm numbers in the orchard grass/red clover plots, rather than an effect of the farming system that carried over into the cultivated years of the rotation. The compost-by-livestock interaction was not significant, indicating that effects of compost amendment and livestock treatments were additive.

The population age structure of earthworm species in our plots was also affected. Although 57% of identified individuals collected in 2000 and 2001 were adults, the populations became increasingly dominated by juveniles, with adults constituting only 26% of the population by 2007. The declines observed in mid-summer may

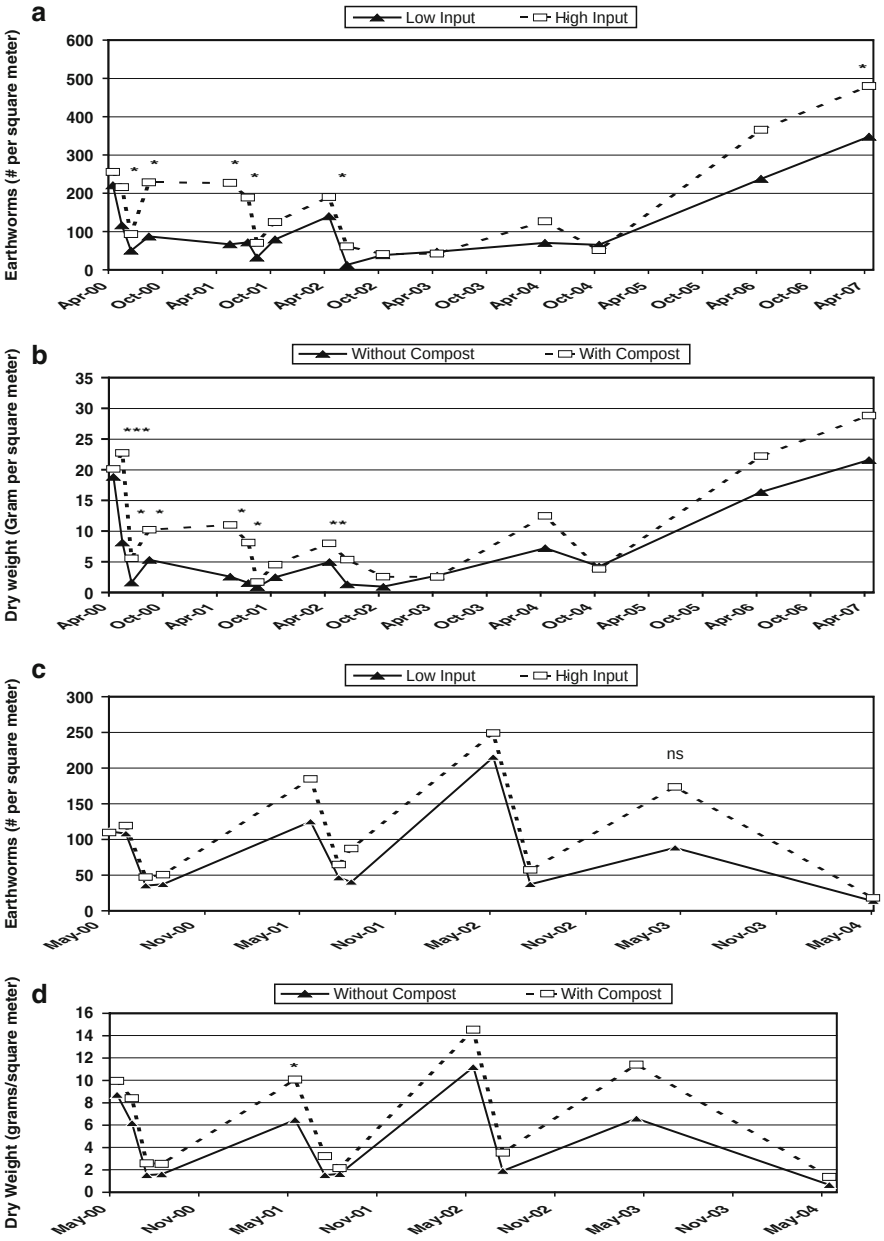

Fig. 18.1 Earthworm population density (**a**) and biomass (**b**) in vegetable market garden systems and in Field Crop Systems (**c** and **d**) amended with 10 tons dairy manure compost per acre (High Input) or unamended (Low Input). [From Kotcon (2008)]

have been a direct result of spring tillage operations, or simply a sampling artifact due to earthworms moving during hot dry weather to soil layers deeper than the 15-cm depth of the soil samples.

18.6 Conclusion

Earthworm populations respond to agroecosystem management practices. Adverse effects of tillage on earthworm populations are well known (Berry and Karlen 1993; Edwards and Lofty 1982; Rovira et al. 1987). Anecic species such as *L. terrestris* were particularly sensitive to tillage, and largely disappeared from plots in this study after continuous cultivation began.

Other studies show that addition of organic substrates that serve as food sources can stimulate earthworm populations (Berry and Karlen 1993; Curry 1976). In our study, population density and biomass were consistently greater in both field crop and market garden plots receiving dairy manure compost than in plots without compost, although differences were not always statistically significant. The host crop planted rarely had a significant or consistent effect, although there was a trend toward higher populations in market garden plots with tomato and pepper, and in field crop plots planted to orchard grass-red clover grasslands. The higher earthworm populations in tomato and pepper plots may have been due to the use of hay mulch for weed suppression, rather than a specific effect of these crops.

Additions of organic matter provide an important determinant of earthworm population dynamics. Several studies have found that adding animal manures increases the population density of earthworms (Estevez et al. 1995; Berry and Karlen 1993). While acidifying fertilizers may be directly toxic to earthworms, nonacidifying fertilizers, by increasing plant growth, indirectly result in more food available for earthworms (Syers and Springett 1984).

Few studies have examined the effects of livestock on earthworms. Hutchinson and King (1980) indicated that earthworm populations were greatest when the stocking rate of sheep was kept at levels associated with maximum productivity, however few other studies have found comparable effects. While the absence of cultivation may tend to promote earthworm populations, trampling has been shown to adversely affect earthworms living near the soil surface. In our plots, sheep grazed only for short periods, and no effects were discernible.

Whether the adoption of organic practices per se is inherently superior to conventional management for increasing earthworm population densities is still undetermined, but the absence of toxic pesticides, the addition of organic matter in the form of compost and manures, and the longer crop rotations appear to compensate for the increased tillage practiced in organic farming.

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